PHYLOGENIC AND BIOCHEMICAL STUDIES ON CUPROZINC, MANGANIC AND FERRIC SUPEROXIDE DISMUTASES

SUMIO KANEMATSU

(1986)

PHYLOGENIC AND BIOCHEMICAL STUDIES ON CUPROZINC, MANGANIC AND FERRIC SUPEROXIDE DISMUTASES

SUMIO KANEMATSU

(1986)

CONTENTS

CHAPTER I

GENERAL INTRODUCTION

Oxygen is indispensable to most life forms. Aerobic respiration using molecular oxygen as the terminal electron acceptor provides much energy than does fermentation. Oxygen is utilized as an oxidant in oxidase reaction and used as a substrate in oxygenase reactions producing many important metabolites. It should be noted, however, that unique properties of oxygen are responsible for its ubiquitous requirement by organisms. Molecular oxygen, though apparently simple two atom molecule, is characterized by its spin state at ground state (1). While the ground state of most molecules are singlet, molecular oxygen has a spin multiplicity of three at ground state, and it is therefore triplet. This spin restriction renders significant kinetic barriers to molecular oxygen in reactions with organic compounds, although these processes are thermodynamically favored. Furthermore, molecular oxygen has a potentiality to be excited states or to be oxidized or reduced states (1). In biochemical reaction, oxygen is utilized through activated intermediates under strict control, since these intermediates have a potent reactivity toward cell components. However in some cases these bring about non-specific oxidation of cell components without control. Therefore oxygen has a two-sidedness when it is used, in other words, oxygen is potentially toxic to all organisms depending on molecular oxygen.

One cannot recognize the harmful action derived from oxygen in organisms under normal conditions, but, it can be easily understood when organisms are exposed to hyperbolic oxygen. Strict anaerobes cannot survive under low concentration of oxygen and even aerobes suffer oxygen damage in a hyperbolic oxygen (2). This indicates the presence of defense system against deleterious action of oxygen in aerobes and lack of this system in anaerobes. The defense system by aerobes should have acquired during the evolution of organisms with a close relation to the

ability of oxygen evolution of plants. Further it is most likely that organisms have evolved to derive greater benefit and lesser disadvantage from oxygen upon utilization of molecular oxygen during the course of evolution of organisms.

The oxygen concentration in the atmosphere is currently 21% and is maintained by the balance of respiration and photosynthesis of organisms. However, at the begining of the evolution of organisms, approximately 3.4×10^9 years ago, it is presumed that only trace amount of free oxygen existed in the atmosphere. Oxygen at this time was mainly derived from the decomposition of water by ultraviolet irradiation (Urey reaction) and its concentration has been estimated to be less than 0.002% (3,4). Obviously, there was no need to require the protection system against oxygen for the earliest organisms on the earth such as fermenting bacteria capable of utilizing organic compounds accumulated in environment as the result of chemical evolution.

The most important event in the evolution of organisms was the development of oxygen-evolving photosynthesis by blue-green algae. Using water as electron donor and carbon dioxide as electron acceptor in photosynthesis, they became to be independent of limiting energy sources and could multiply enormously. The accumulation of free oxygen, by-product of photosynthesis, first by blue-green algae followed by eukaryotic algae and then land plants changed the atmosphere from a reducing environment to an oxidizing one, brought aerobic respiration to anaerobes, and formed ozone layer which eliminate ultraviolet irradiation on the surface of the earth. Thus, the free oxygen made possible the appearance of all higher forms life as seen at present day. Behind this, the defense system against oxygen toxicity should have been prerequested to ensure the usage of oxygen by organisms.

Molecular oxygen itself has a low toxicity, but its partial reduction intermediates such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH\cdot$) and its excited species, singlet molecular oxygen ($^1O_2$) are very reactive toward cell components and are responsible for oxygen toxicity (5-8). In sequential one electron reduction from O_2 to H_2O, or oxidation from H_2O to O_2, the superoxide radical, hydrogen peroxide and the hydroxyl radical as the

intermediate can be present (Fig. 1). In addition to these intermediates, excited singlet oxygen ($^1O_2$) is formed from ground state triplet oxygen ($^3O_2$). These species are termed as "active oxygen" because of their high reactivity in biology.

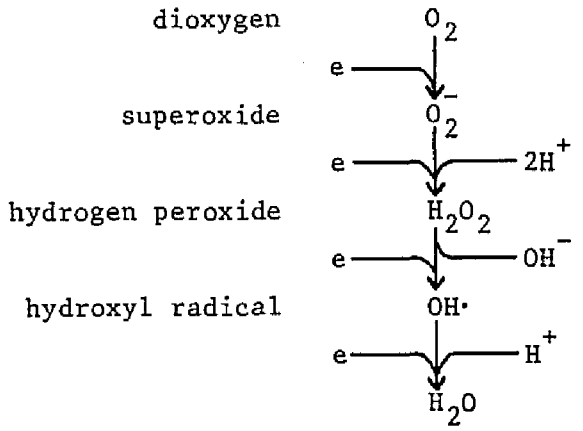

Fig. 1. The univalent pathway of oxygen reduction.

The active oxygens are produced in various biological and non-biological processes (5-8). The superoxide radical, which is most important since from which other active oxygens are derived, is produced by oxidizing enzymes, metaloproteins, and autooxidations of electron carrier proteins and of low molecular weight compounds. Furthermore it has been demonstrated that the superoxide is produced in subcellular organelles such as chloroplasts, chromatophors, mitochondria, microsomes and cell nucleus, and intact cells such as leucocyte, erythrocyte, macrophage and Escherichia coli. In addition to above, the superoxide is produced physically from water by ultraviolet or X-ray radiation (9,10).

Hydrogen peroxide is generated through two electron reduction of oxygen by oxidases (glucose oxidase, glycolate oxidase, etc.) (11,12) and through the enzymatic or non-enzymatic disproportionation of superoxide (13). The latter is a major path of hydrogen peroxide production in chloroplasts (14), which is known as the Mehler reaction.

There is no evidence indicating the production of the hydroxyl radical in enzymatic reaction. It is reasonable that oxygen-utilizing

enzymes do not release the hydroxyl radical, since the hydroxyl radical is a strong oxidant having a high redox potential and oxidizes biomolecules unspecifically. In the presence of both the superoxide and hydrogen peroxide, however, the hydroxyl radical is formed through the following reactions:

$$Fe^{3+} + O_2^- \longrightarrow Fe^{2+} + O_2$$

$$H_2O_2 + Fe^{2+} \longrightarrow HO\cdot + OH^- + Fe^{3+}$$

The overall reaction corresponds to the Haber-Weiss reaction in which O_2^- instead of Fe^{2+} is a reductant for H_2O_2 (15). However the reaction between H_2O_2 and O_2^- is very slow. The requirement of iron as indicated in above scheme has been demonstrated (16,17).

Beside the superoxide, hydrogen peroxide and the hydroxyl radical, electronically excited oxygen constitues active oxygen (5-8). Several kind of excited species are possible, of which $^1\Delta_g$ is most important in biological system, since half life of this speices in water is comparatively longer to react biomolecules than other speices (18). Due to spin restriction the ground state molecular oxygen has low reactivity toward organic compounds which are in general singlet at ground state, however, singlet oxygen reacts very rapidly with compounds having double bond and oxidizes sulfides to sulfoxides, phenols to free radical products. Singlet oxygen is produced through photosensitized reaction in the presence of sensitizer (18) according to the following reaction:

$$^1D + h\nu \longrightarrow {}^1D^* \longrightarrow {}^3D^*$$

$$^3D^* + {}^3O_2 \longrightarrow {}^1O_2 + {}^1D$$

where ^1D, $^1D^*$, and $^3D^*$ are photosensitizer in ground state and excited singlet and triplet states, respectively. Naturally occurring pigments including chlorophyll and flavine play a role as a snsitizer (19). In addition to above, several routes by which singlet oxygen is formed has been proposed. The oxidation of superoxide generates singlet oxygen (20,21).

Organisms are equipped with several kinds of defense system against active oxygen which has been prerequested upon the utilization of molecular oxygen as mention above. The most efficient and sophisticated measure is prevention of active oxygen production during use of oxygen or water. One of those example can be seen in the case of respiration and photosynthesis. In respiratory chain cytochrome oxidase as a terminal oxidase undergoes four-electron reduction of oxygen to water during which no liberation of intermediate is observed (22). This indicates that the reduction of oxygen proceeds in active site successively without liberation of intermediates. Oxygen evolution process in photosynthesis which is apparently the reversed reaction of respiration withdraws four electron from water to evolve molecular oxygen (23,24). Again there is no evidence indicating the liberation of intermediate.

Several compounds including thiols and SH-enzymes are susceptible to autooxidation in the presence of molecular oxygen (25-28). Thus, the concentration of oxygen inside the cells should be low enough to prevent non-specific oxidation of their constituents. This strategy can be considered as the defense system against oxygen toxicity. In fact cytochrome c oxidase has a very low K_m values of 10^{-9} M for oxygen and is able to use oxygen as final oxidant under very low oxygen concentrations (29). Aerobic diazotrophs, for example in the case of Azotobacter, vigorous consumption of oxygen by respiratory activity results in the decrease in oxygen concentration inside cells and prevent their nitrogenase system from inactivation by molecular oxygen (30).

Since the generation of active oxygen is inevitable, these should be removed as soon as possible. The following enzymes participate in the scavenging of active oxygen. Superoxide dismutase catalyzes the disproportionation of the superoxide and reduces its stady-state concentration (13). Hydrogen peroxide is removed by catalase and/or peroxidase. Recently ascorbate-specific peroxidase responsible for scavenging H_2O_2 in cytoplasm or chloroplasts have been isolated in Euglena (31) and spinach (32). Glutathione peroxidase may participate in the degradation of lipid hydroperoxide (33).

The hydroxyl radical is the strongest oxidant among active oxygen and non-specifically oxidizes carbohydrates, amino acids, proteins,

lipids and neucleic acids. Although it has not been established whether there is specific scavenger or enzyme for the hydroxyl radical, it is no doubt that many compounds play a role in scavenging the radical, otherwise the oxidations due to the hydroxyl radical give rise to deleterious effect.

Singlet oxygen has a short life-time of 10^{-5} sec in aqueous solution. Thus, most singlet oxygen are quenched by water molecules (34). In hydrophobic environment, i.e. membrane, α-tocopherol and β-carotine quench singlet oxygen at a diffusion-controlled rate (35-37).

Among active oxygen the superoxide radical is most important since from which the other active oxygen are derived (5-8). The interconversion of active speices of oxygen are as follows:

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$O_2^- + H_2O_2 \xrightarrow{Fe} HO\cdot + HO^- + O_2$$

$$O_2^- + A \longrightarrow {}^1O_2 + A^-$$

Thus, the stady state concentration of the superoxide determines the production of hydrogen peroxide, the hydroxyl radical and singlet oxygen. Fig. 2 represents the mechanisms for production, scavenging and biological effect of superoxide radical.

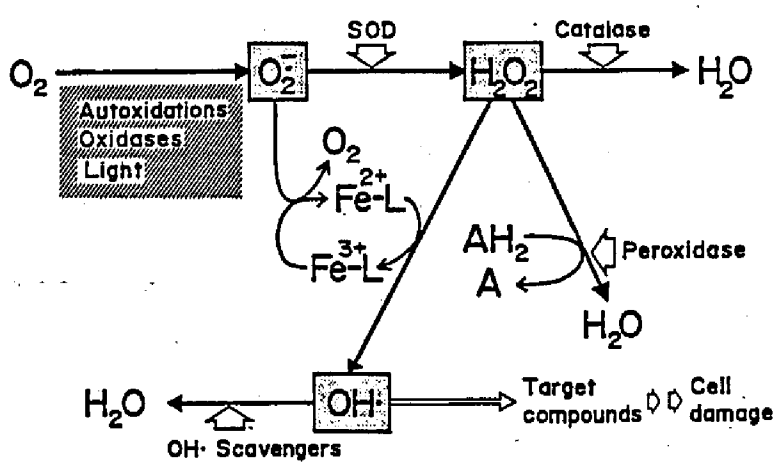

Fig. 2. Mechanisms for generation, scavenging and biological effects of superoxide radical. L, ligand; AH_2, electron donor.

Superoxide dismutase (superoxide: superoxide oxidoreductase, EC 1.15.1.1, SOD) is a key enzyme in defense system for active oxygen which catalyzes the disproportionation of the superoxide anion radical according to the following reaction (13):

$$O_2^- + O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2 \qquad [1]$$

The reaction mechanism of the enzyme has been elucidated to be a simple oxidation-reduction cycle (38) as

$$E\text{-}M^{n+} + O_2^- \longrightarrow E\text{-}M^{(n-1)+} + O_2 \qquad [2]$$

$$E\text{-}M^{(n-1)+} + O_2^- + 2H^+ \longrightarrow E\text{-}M^{n+} + H_2O_2 \qquad [3]$$

where $E\text{-}M^{n+}$ is oxidized enzyme and $E\text{-}M^{(n-1)+}$ is reduced counterpart. The second-order rate constants of eqs 2 and 3 are 2×10^9 $M^{-1}sec^{-1}$ at pH 7.8 (2), thus the overall reaction (eq 1) proceeds near diffusion-limited rate. Due to this high catalytic activity, SOD dramatically reduces the stady state concentration of superoxide in cells from 10^{-5} M, which is a concentration in the absence of SOD, to 10^{-9} M, and prevent the production of more toxic oxygen speices derived from superoxide.

Three distinct types of superoxide dismutase have been so far isolated from various organisms (5,39). Cu,Zn-superoxide dismutase has been isolated from a wide range of eukaryotes including animals, higher plants and fungi. Mn-SOD has been obtained from mitochondria of eukaryotes and from prokaryotes. Fe-SOD has been purified only from prokaryotes. However, systematic survey for three types of SOD among organisms at different evolutional level has not been conducted yet. A high degree of homology in amino acid sequences of Fe- and Mn-SOD has been reported (40), but Cu,Zn-SOD is sequentially non-homologous to Fe/Mn-SOD (41). Oxygen and superoxide induce the biosynthesis of Cu,Zn-SOD and Mn-SOD, but not Fe-SOD (42-44). Still, little is known in respect of physiological difference among three types of SOD.

This study has been motivated by the simple question why there are

three types of superoxide dismutase which catalyze the same reaction at the same catalytic efficiency. To answer this question, I investigated the distribution among various organisms with a evolutional point of view, the subellular localization and the possible difference in reactivity, of three types of superoxide dismutases. As a result of the study in these directions, the knowledge about the molecular evolution of SOD will not only give a deep insight in one aspect of photosynthesis, but also be a model for the evolution of oxygen metabolizing systems. Furthermore, the elucidation of the structural and catalytic change of three types of SOD during evolution will be a clue to produce more efficient catalyst by molecular engineering.

In this study, the distribution of three types of SOD with a special reference to Cu,Zn-SOD in photosynthetic organisms (Chapter II) and fungi and marine invertebrates (Chapter III) was investigated. Most eukaryotic algae lacked Cu,Zn-SOD, but Spirogyra SOD was characterized to be Cu,Zn-SOD by purification (Chapter IV). On the basis of the distribution studies of SOD in various organisms, each enzyme was characterized after isolation. In Chaper V, spinach contained cytosolic and stromal Cu,Zn-SOD isozymes which were different structurally and immunologically. Two types of Cu,Zn-SOD were also found in rice and were characterized (Chapter VI). To confirm that cells containing chloroplasts have both types of Cu,Zn-SOD, the purification was extended to a fern (Chapter VII). In Chapter VIII, SOD activity was detected in anaerobic bacteria and this was characterized to be Fe-SOD. Presence of only Fe-SOD and absence of Mn-SOD in anaerobes was confirmed in green sulfur bacteria (Chapter IX). Correlation between oxygen concentration in a cell and type of SOD was investigated in aerobic diazotrophs, soybean root-nodule bacteroids and Azotobacter (Chapter X). Purification and characterization of eukaryotic Fe-SOD from Euglena gracilis and its subcellular localization was conducted (Chapter XI). Eukaryotic Fe-SOD was also found in a fern (Chapter XII) and in spinach chloroplasts (Chapter XIII), and the latter was fully characterized. In Chapter XIV, Mn-SOD was isolated from a red alga and its molecular properties were described. Furthermore, gram-scale purification of Fe-SOD was conducted from Azotobacter and its molecular properties were

investigated in details (Chapter XV). Finally, the reactions of Mn- and Fe-SOD with hydrogen peroxide were kinetically investigated (Chapter XVI). The implication from the results of distribution, subcellular localization, molecular and kinetical properties of three types of superoxide dismutase was discussed on a evolutional point of view, and proposed a hypothesis to rationalize the present results (Chapter XVII).

REFERENCES

(1) Ingraham, L.L., and Meyer, D.L. (1985) Biochemistry of Dioxygen, pp. 7-73, Plenum Press, New York.

(2) Gregory, E.M., and Fridovich, I. (1973) J. Bacteriol. 114, 1193-1197.

(3) Dole, M. (1965) J. Gen. Physiol. 49, 5-27.

(4) Berkner, L.V., and Marshall, L.C. (1965) J. Atmos. Sci. 22, 225-261.

(5) Fridovich, I. (1974) Adv. Enzymol. 41, 35-97.

(6) Fridovich, I. (1975) Annu. Rev. Biochem. 44, 147-159.

(7) Fridovich, I. (1978) Science 201, 875-880.

(8) Asada, K. (1976) Seikagaku 48, 226-257.

(9) Draganić, I.G., and Draganić, Z.D. (1971) The Radiation Chemistry of Water, pp. 23-168, Academic Press, New York.

(10) Klug, D., Fridovich, I., and Rabani, J. (1973) J. Amer. Chem. Soc. 95, 2786-2790.

(11) Massey, V., Strickland, S., Mayhew, S.G., Howell, L.G., Engel, P.C., Mattews, R.G., Schuman, M., and Sullivan, P.A. (1969) Biochem. Biophys. Res. Commun. 36, 891-897.

(12) Cohen, H.J., and Fridovich, I. (1971) J. Biol. Chem. 246, 359-366.

(13) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(14) Asada, K., Kiso, K., and Yoshikawa, K. (1974) J. Biol. Chem. 249, 2175-2181.

(15) Haber, F., Weiss, J. (1934) Proc. Roy. Soc. Ser. A 147, 332-351.

(16) Halliwell, B. (1978) FEBS Lett. 96, 238-242.
(17) McCord, J.M., and Day, E.D., Jr. (1978) FEBS Lett. 86, 139-142.
(18) Kasha, M., and Brabham, D.E. (1979) in Singlet Oxygen (Wasserman, H.H., and Murray, R.W., eds.), pp. 1-33, Academic Press, New York.
(19) Takahama, U., and Nishimura, M. (1975) Plant Cell Physiol. 16, 737-748.
(20) Mayeda, E.A., and Bard, A.J. (1973) J. Amer. Chem. Soc. 95, 6223-6226.
(21) Kellog, E.W., and Fridovich, I. (1975) J. Biol. Chem. 250, 8812-8817.
(22) Chance, B., Saronio, C., and Leigh, J.S. (1975) Proc. Natl. Acad. Sci. USA 72, 1635-1640.
(23) Lawrence, G.D., and Sawyer, D.T. (1978) Coordin. Chem. Rev. 27, 173-193.
(24) Asada, K., and Okada, S. (1983) in The Oxygen Evolving System of Photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, N., Renger, G., and Satoh, K., eds.), pp. 245-255, Academic Press, New York.
(25) Misra, H.P. (1974) J. Biol. Chem. 249, 2151-2155.
(26) Orme-Johnson, W.H., and Beinert, H. (1969) Biochem. Biophys. Res. Commun. 36, 905-911.
(27) Misra, H.P., and Fridovich, I. (1971) J. Biol. Chem. 246, 6886-6890.
(28) Nakamura, S., and Kimura, T. (1972) J. Biol. Chem. 247, 6462-6468.
(29) Chance, B., Erecinska, M., and Chance II, E.M. (1971) in Oxidases and Related Redox Systems, Vol. 2 (King, T.E., Mason, H.S., and Morrison, M., eds.), pp. 851-874, University Park Press, Baltimore.
(30) Burns, R.C., and Hardy, R.W.F. (1975) Nitrogen Fixation in Bacteria and Higher Plants, pp. 102-104, Springer-Verlag, Berlin.
(31) Shigeoka, S., Nakano, Y., and Kitaoka, S. (1980) Biochem. J. 186 377-380.
(32) Nakano, Y., and Asada, K. (1981) Plant Cell Physiol. 22, 867-880.
(33) Flohé, L. (1982) in Free Radicals in Biology, Vol. V (Pryor, W.A. ed.), pp. 223-254, Academic Press, New York.
(34) Kearns, D.R. (1971) Chem. Rev. 71, 395-427.

(35) Fragata, M., and Bellemare, F. (1980) Chem. Phys. Lipids 27, 93-99.

(36) Fahrenholtz, S.R., Doleiden, F.H., Trozzolo, A.M., and Lamola, A.A. (1974) Photochem. Photobiol. 20, 505-509.

(37) Anderson, S.M., and Krinsky, N.I. (1973) Photochem. Photobiol. 18, 403-408.

(38) Klug, D., Fridovich, I., and Rabani, J. (1973) J. Amer. Chem. Soc. 95, 2786-2790.

(39) Steinman, H. (1982) in Superoxide Dismutase, Vol. 1, (Oberley, L.W., ed.), pp. 11-68, CRC Press, Florida.

(40) Steinman, H.M., and Hill, R.L. (1973) Proc. Natl. Acad. Sci. USA 70, 3725-3729.

(41) Steinman, H.M., Naik, V.R., Abernethy, J.L., and Hill, R.L. (1974) J. Biol. Chem. 249, 7326-7338.

(42) Hassan, H.M., and Fridovich, I. (1977) J. Bacteriol. 129, 1547-1583.

(43) Hassan, H.M., and Fridovich, I. (1977) J. Biol. Chem. 252, 7667-7672.

(44) Stevens, J.B., and Autor, A.P. (1977) J. Biol. Chem. 252, 3509-3514.

CHAPTER II

SUPEROXIDE DISMUTASES IN PHOTOSYNTHETIC ORGANISMS: ABSENCE OF THE Cu,Zn-ENZYME IN EUKARYOTIC ALGAE

1. INTRODUCTION

It is generally accepted that most molecular oxygen in the biosphere has accumulated due to photosynthetic activity, first by blue-green algae, followed by eukaryotic algae, and then by land plants (1). Though molecular oxygen is an effective electron acceptor for aerobic organisms, oxygen poisons the cells unless they have a protective mechanism. Molecular oxygen itself has rather low toxicity, but its partial reduction products such as O_2^-, H_2O_2, and OH·, as well as O_2 in excited states ($^1O_2$), are highly reactive toward cell components and are responsible for the toxicity of oxygen toward cells.

On illumination, chloroplasts produce these activated oxygen species, O_2^- (2,3), H_2O_2 (4), OH· (5), and $^1O_2$ (6), and, therefore, as a background for the evolution of a system that utilizes light energy in photosynthetic organisms, the cells have acquired a defense mechanism against oxygen toxicity. Superoxide dismutase catalyzes the dismutation of O_2^- to H_2O_2 and O_2 and scavenges O_2^- in the cells. Since O_2^- is a source of OH· by interaction with H_2O_2 (Haber-Weiss reaction) (7), superoxide dismutase, in addition to catalase and peroxidase, has been assumed to play an important role in protection from oxygen toxicity.

Superoxide dismutase (SOD) has been purified from various organisms, and three forms of the enzyme have been found; these differ in the metal in the enzyme (8). Cu,Zn-SOD has been found in eukaryotes including mammalian tissues, higher plants, yeast, and Neurospora. This form of the enzyme has also been found in a prokaryote, Photobacterium leiognathi (9). Mn-SOD occurs in prokaryotes and also in the matrix of mitochondria in yeast and mammalian liver. Fe-SOD has been found only in prokaryotes. A high degree of homology of amino acid sequences of

Mn- and Fe-SOD from mitochondria and bacteria has been reported, which implies that the mitochondrial enzyme arises from prokaryotic cells (10,11). On the contrary, Cu,Zn-SOD is sequentially nonhomologous to the Fe- and Mn-enzymes.

Until recently, Cu,Zn-SOD was detected in all eukaryotes tested. However, this form of the enzyme is absent from Basidiomycete and protozoa (12,13). I also observed that Cu,Zn-SOD is absent from several eukaryotic algae. On the other hand, Fe- and Mn-SOD have been found in photosynthetic bacteria and blue-green algae (14-18). Under these circumstances, I was interested in surveying what form of superoxide dismutase is contained in photosynthetic organisms at different evolutionary or phylogenetic levels. I judged the form of superoxide dismutase using two criteria: (a) Cu,Zn-SOD is sensitive to cyanide, while Fe- and Mn-SOD are insensitive (8), and (b) only Cu,Zn-SOD is sensitive to the antibody against Cu,Zn-SOD from spinach. The results presented in this chapter indicate that superoxide dismutases in photosynthetic bacteria and in both prokaryotic and eukaryotic algae are all Fe- and/or Mn-SOD, and these organisms lack Cu,Zn-SOD. Cu,Zn-SOD appears only in land plants including ferns, mosses, and seed plants. The relation between the oxygen concentration in the atmosphere and the appearance of various forms of superoxide dismutase is discussed.

2. MATERIALS AND METHODS

Photosynthetic bacteria grown under photoautotrophic conditions were supplied by Drs. T. Akazawa, T. Horio, H.G. Truper, and M. Kobayashi. Strains of blue-green algae were provided by Drs. Y. Fujita and T. Katoh, and the cells were cultured as described previously (14). Nostoc verrucosum was the gift of Dr. M. Shin. Chlorella vulgaris, mutant 125, which lacks chlorophyll and was grown in a medium containing glucose (19), was supplied by Dr. S. Ida. Strains of Chlamydomonas reinhardi and Chlorella ellipsoidea were provided by Drs. M. Ishida and S. Hori and the cells were cultured according to Ishida et al. (20), and Tamiya et al. (21), respectively. A strain of Euglena gracilis, strain

Z, was a gift from Dr. K. Hirai, and the cells were grown under photoautotrophic conditions (22). Diatoms were generously supplied by Dr. K. Iwasa. Aerial algae were collected by Mr. K. Madono. Thalli of marine algae were collected in Maizuru Bay and kindly identified by Dr. I. Umezaki. Nitella were supplied by Drs. E. Doi and M. Tazawa. Mosses and ferns were collected in Kyoto and Miyazaki and kindly identified by Mr. Y. Akagi. Chlorophyll-containing tissues, i.e. gametophytes and sporophytes, were employed. Leaf tissues of seed plants were obtained from their seedlings grown in a green house or collected near the campus of Kyoto University.

The materials were stored at -20°C before use. Cells of photosynthetic bacteria and unicellular algae were suspended in 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA, and were disrupted by sonication, usually for a total of 20 min (10 2-min periods at 2-min intervals) in an ice bath to ensure thorough homogenization. Thalli of marine algae, cells of Chlorella ellipsoidea, and land plants were homogenized in a mortar for 30 min with sea sand and 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA. The sonicates or homogenates were centrifuged at 39,000 g for 20 min, and, for concentration, ammonium sulfate was added to the supernatants to give 80% saturation. The sediments obtained by centrifugation were dissolved in and dialyzed against 10 mM phosphate, pH 7.8, containing 0.1 mM EDTA, for 48 h. The cell-free extracts obtained after centrifugation, if necessary, were assayed for superoxide dismutase, peroxidase and catalase.

Superoxide dismutase was assayed by the inhibition of cytochrome c reduction by O_2^- generated with the xanthine-xanthine oxidase system using a modification (23) of the procedure of McCord and Fridovich (24). The cell-free extracts of some organisms reduced or oxidized cytochrome c without the xanthine-xanthine oxidase system. When this interfered with the assay, enzyme activity was determined by the inhibition of the formation of formazane due to the reduction of nitroblue tetrazolium by O_2^- generated with illuminated flavin (25). The reaction mixture contained 25 mM Tricine-KOH buffer, pH 8.5, 25 μM FMN, and 25 μM nitroblue tetrazolium in a total volume of 2 ml. The reaction was carried out using a Shimadzu UV-200 spectrophotometer modified so that

the absorbance change due to formazane could be monitored continuously with an appropriate filter, while the cuvette was being illuminated from the side. The cross-illuminating light provided by a projector was passed through a 480-nm interference filter.

One unit of superoxide dismutase was defined as that amount which caused half-inhibition of cytochrome c reduction under the assay conditions described above. The enzymatic unit is equal to (V/v-1), where V and v are the reduction rates in the absence and presence of the enzyme, respectively (23). Since the reaction volume was one-third, the unit given by this procedure is threefold that of McCord and Fridovich (24). The results in tables I to IV are presented in McCord-Fridovich units. When the method of cytochrome c reduction was interfered with by reducing or oxidizing substances, the enzymatic unit was estimated from the value based on the method of formazane formation by a factor determined with the purified enzyme. Typical results in Fig. 3 indicate that the enzymatic unit estimated from (V/v-1) is proportional to the amount of cell-free extract. The effect of cyanide and antiglobulin on enzyme activity was estimated from (V/v-1) in the presence and absence of the inhibitors by using at least three different amounts of the extract. The effect of the inhibitors, if any, on the O_2^--generating system was corrected for by determination of the O_2^--induced reactions in the absence of the extract. Polyacrylamide disc gel electrophoresis of cell extracts was carried out and location of superoxide dismutase was located on the gels according to the methods of Beauchamp and Fridovich (25).

Peroxidase was assayed using pyrogallol and guaiacol as electron donors, according to Siegel and Siegel (26). Catalase activity was determined by the disappearance of H_2O_2 measured at 240 nm according to Luck (27). All enzymatic assays were carried out at 25°C, and protein in the extracts was determined according to Lowry et al. (28).

Antiserum against Cu,Zn-SOD form spinach (29) was prepared as previously (23). Antiserum against Fe-SOD from the blue-green alga Plectonema boryanum (14) was prepared by immunization of rabbits as follows: The purified enzyme, 0.1 to 0.5 mg in 1 ml of 0.9% NaCl, was emulsified with an equal volume of complete Freund's adjuvant, and for

each rabbit 2 ml of the emulsion were injected one-half into the toe pads and one-half into multiple sites on the back. The injection was repeated four times at weekly intervals. Blood was taken from the ear vein 1 and 2 weeks after the last injection. Control blood was obtained from unimmunized rabbits of the same age. The γ-globulin fraction from immune and control sera was obtained by repeating the precipitation with ammonium sulfate at 33% saturation twice and was dissolved in and dialyzed against 0.15 M NaCl containing 5 mM Tricine-KOH or potassium phosphate, pH 7.8. Further purification of the antibody was performed by passing the γ-globulin fraction through a DEAE-cellulose column equilibrated with 5 mM phosphate, pH 7.8, after dialysis against the same buffer.

The homogeneity of antibodies was confirmed by the following test (the results are shown only for the anti-Cu,Zn-SOD). The double immunodiffusion line between the antibody and the crude spinach extract on the agar plates was fused with that between the antibody and the purified enzyme (Fig. 1). The resutls of quantitative immuoprecipitation are shown in Fig. 2. At the equivalent point, seven molecules of immunoglobulin were found to bind to one molecule of the spinach superoxide dismutase, assuming that the molecular weights of the spinach enzyme and γ-globulin are 32,000 and 140,000, respectively. Anti-Cu,Zn-

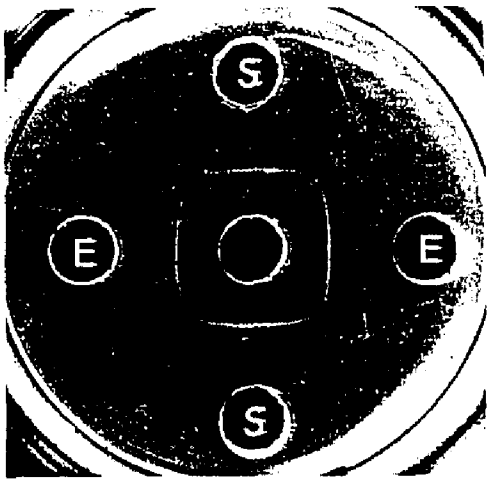

FIG. 1. Ouchterlony double immunodiffusion of spinach superoxide dismutase and its antibody. Center well, immunoglobulin (720 μg of protein); S, crystalline Cu,Zn-superoxide dismutase from spinach; E, crude spinach leaf extract (10 μl).

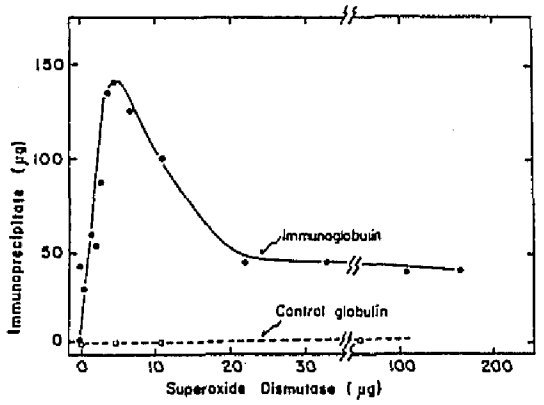

FIG. 2. Precipitation reaction of spinach superoxide dismutase antibody with spinach superoxide dismutase. The immunoglobulin and control globulin (1.41 mg of protein) were incubated with the indicated amounts of spinach superoxide dismutase in 0.15 M NaCl containing 20 mM potassium phosphate, pH 7.8, in a total volume of 0.1 ml, at 5°C for 2 days. The immunoprecipitates formed were collected by centrifugation at 3000*g* for 30 min and washed twice, each time with 0.2 ml of 0.15 M NaCl containing 20 mM potassium phosphate, pH 7.8. The occurrence of unreacted immunoglobulin and antigen in the supernatants was confirmed below and above the equivalent point, respectively, by means of double immunodiffusion on agar plates. The protein of the precipitate was determined according to Lowry *et al.* (28), and a small blank in the absence of the enzyme was subtracted.

SOD completely inhibited the spinach enzyme. Half-inhibition occurred at 40 μg of immunoglobulin for 0.12 μg of purified enzyme. Control globulin neither formed a complex with the enzyme nor inhibited enzymatic activity.

Bovine Cu,Zn-SOD (erythrocuprein) was the generous gift of Dr. F. Hirata. The antibodies against Fe- and Mn-SOD from Mycobacterium were kindly donated by Dr. E. Kusunose. Xanthine oxidase from milk and cytochrome c from horse heart (type III) were obtained from Boehringer and Sigma, respectively.

3. RESULTS

Enzymatic activities of superoxide dismutases in photosynthetic organisms at different evolutionary levels are summarized in Tables I, II, and IV. The effects of cyanide are also included in the tables, and typical effects of both cyanide and the antibody on superoxide dismutase activities of representative photosynthetic organisms are indicated in Figs. 3 and 5.

Seed Plants (Angiospermae and Gymnospermae)

Superoxide dismutases from green peas (Pisum sativum) (30), wheat germ (31), and spinach leaves (29) have been purified and characterized

TABLE I

ACTIVITIES OF SUPEROXIDE DISMUTASES AND THEIR CYANIDE SENSITIVITIES IN EXTRACTS OF SEED PLANTS, MOSSES, AND FERNS

Species	Superoxide dismutase	
	Activity (units/mg of protein)	Cyanide-insensitive activity[a] (%)
Spermatophyta (seed plants)		
Angiospermae		
Monocotyledoneae		
Zea mays	11	24
Triticum aestivum	25	3
Oryza sativa	31	8
Lemna perpusilla	20	18
Halophila ovalis	20	39
Dicotyledoneae		
Cryptotaenia canadensis	4	22
Spinacia oleracea	12	6
Raphanus sativus	11	68
Gymnospermae		
Chamaecyparis obtusa	16	83[b]
Cryptomeria japonica	9	91[b]
Metasequoia glyptostroboides	13	40[b]
Pinus thunbergii	5	55[b]
Ginkgo biloba	14	58[b]
Cycas revoluta	19	67
Bryophyta (mosses)		
Musci		
Polytrichum juniperinum	40	21
Hepaticae		
Marchantia polymorpha	9	33
Conocephalum conicum	41	21
Pteridophyta (ferns)		
Pterophytina		
Azolla imbricata	19	61[b]
Diplazium subsinuatum	4	79[b]
Gleichenia japonica	4	60[b]
Plenasium banksiifolium	7	39
Angiopteris lygodiifolia	14	4
Calamophytina		
Equisetum arvense	10	9
Equisetum ramosissimum	24	12
Lycophytina		
Selaginella nipponica	25	15
Lycopodium cernuum	7	11
Lycopodium serratum	43	20

[a] Cyanide present at 1 mM.

[b] Effect of cyanid was tested using the nitroblue tetrazolium system for the assay of superoxide dismutase.

TABLE II

ACTIVITIES OF SUPEROXIDE DISMUTASES AND THEIR CYANIDE SENSITIVITIES IN EXTRACTS OF EUKARYOTIC ALGAE

Species	Superoxide dismutase	
	Activity (units/mg of protein)	Cyanide-insensitive activity (%)[a]
Charophyta		
Nitella pulchella	27	100
Nitella axilliformis	ND[b]	100
Chlorophyta (green algae)		
Bryopsis plumosa	15	100[c]
Trentepohlia aurea	3	100
Ulva pertusa	3	100
Chlorella ellipsoidea	ND	100
Chlorella vulgaris, mutant 125	ND	100[c]
Chlamydomonas reinhardi	ND	100
Euglenophyta		
Euglena gracilis, strain Z	21	100
Phaeophyta (brown algae)		
Sargassum horneri	28	100[c]
Sargassum thunbergii	6	100[c]
Sargassum tortile	3	100
Colpomenia sinuosa	4	100
Chrysophyta		
Bacillariophyceae (diatoms)		
Navicula pelliculosa	25	100
Phaeodactylum tricornutum	21	100
Rhodophyta (red algae)		
Gratelouoia filicina	11	100[c]
Gymnogongrus flabelliformis	25	100
Gracilaria textorii	29	100
Gelidium amansii	23	100
Porphyra tenera	17	100

[a] Cyanide present at 1 mM.

[b] ND, not determined.

[c] Effect of cyanide was tested using the nitroblue tetrazolium system for the assay of superoxide dismutase.

TABLE III

EFFECT OF GROWTH CONDITIONS ON SUPEROXIDE DISMUTASE CONTENT IN *Euglena gracilis*[a]

Growth conditions	Protein (mg/culture bottle)	Superoxide dismutase (units/mg of protein)
Photoautotrophic[b]	197	10.3
Photoheterotrophic[c]	160	6.0
Heterotrophic[d]	60	3.5

[a] The cells were grown on 400 ml of Cramer and Myers' medium (22) with bubbling 5% CO_2-enriched air at 25°C for 14 days.

[b, c] Cultured under fluorescent lamps (6000 ergs/cm²/sec).

[c, d] To the medium, glucose was added at a concentration of 3.5%.

[d] Cultured in the dark.

as Cu,Zn-SOD. The angiosperm Cu,Zn-SOD closely resemble those from other sources, having only minor differences in amino acid composition. Crystallographic studies on the spinach enzyme have also been carried out (32) for comparison with the mammalian enzyme (33).

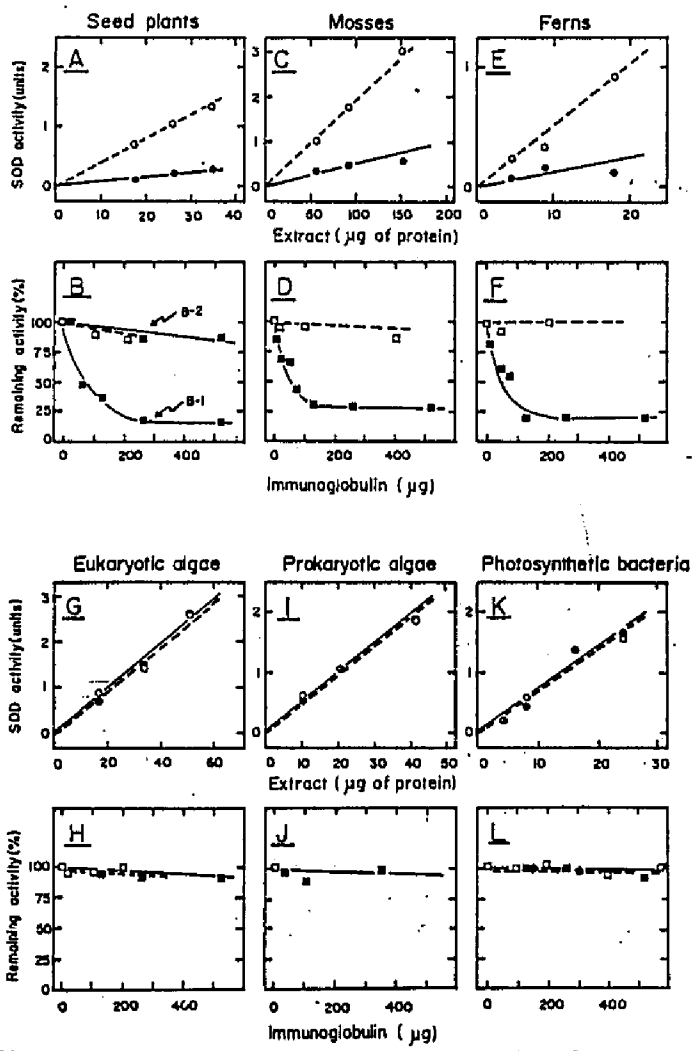

FIG. 3. Effect of cyanide and the antibody against the spinach Cu,Zn-superoxide dismutase on superoxide dismutase activity in cell-free extracts from photosynthetic organisms at different evolutionary levels. Enzymatic activity was calculated from (V/v − 1) using the cytochrome *c* system described in Materials and Methods. The effect of cyanide was tested at different amounts of the extracts in the presence (●——●) and absence (O- - -O) of 1 mM KCN. The effect of the antibody was determined as follows: Extracts containing about 2 units of superoxide dismutase were incubated with various amounts of immunoglobulin or control globulin in 0.15 M NaCl containing 20 mM potassium phosphate, pH 7.8, in a total volume of 0.2 ml, at 5°C. After a 1-h incubation, enzymatic activity was determined under standard conditions. (■——■), Spinach Cu,Zn-superoxide dismutase antibody; (◆——◆), *Plectonema* Fe-superoxide dismutase antibody; (□- - -□), control globulin. A, *Lemna perpusilla*; B-1, *Cryptotaenia canadensis*; B-2, *Cycas revoluta*; C, *Marchantia polymorpha*; D, *Polytrichum juniperinum*; E, *Diplazium subsinuatum*; F, *Lycopodium cernuum*; G, *Nitella pulchella*; H, *Chlamydomonas reinhardi*; I, *Nostoc verrucosum*; J, *Plectonema boryanum*; K, *Chromatium vinosum*; L, *Chlorobium thiosulfatophilum*.

Cu,Zn-SOD is sensitive to cyanide, and this is also the case for the spinach enzyme (23). Table I and Fig. 3A indicate almost complete or partial inhibition of SOD activity by cyanide in extracts of both monocotyledonous and dicotyledonous angiosperms. Disc gel electrophoresis revealed that the major SOD was sensitive to cyanide (Fig. 5). These observations indicate that the major soluble SOD in angiosperms is Cu,Zn-SOD.

Further evidence for the occurrence of the Cu,Zn-enzyme is provided by the inhibition of the enzyme by the antibody against the spinach enzyme (Fig. 3B). In addition to the angiosperms indicated in Table I, SOD activity in extracts from kidney bean (Phaseolus vulgaris) and soybean (Glycine max) was inhibited by the antibody. Whenever the antibody inhibited the enzymatic activity, a precipitin band was

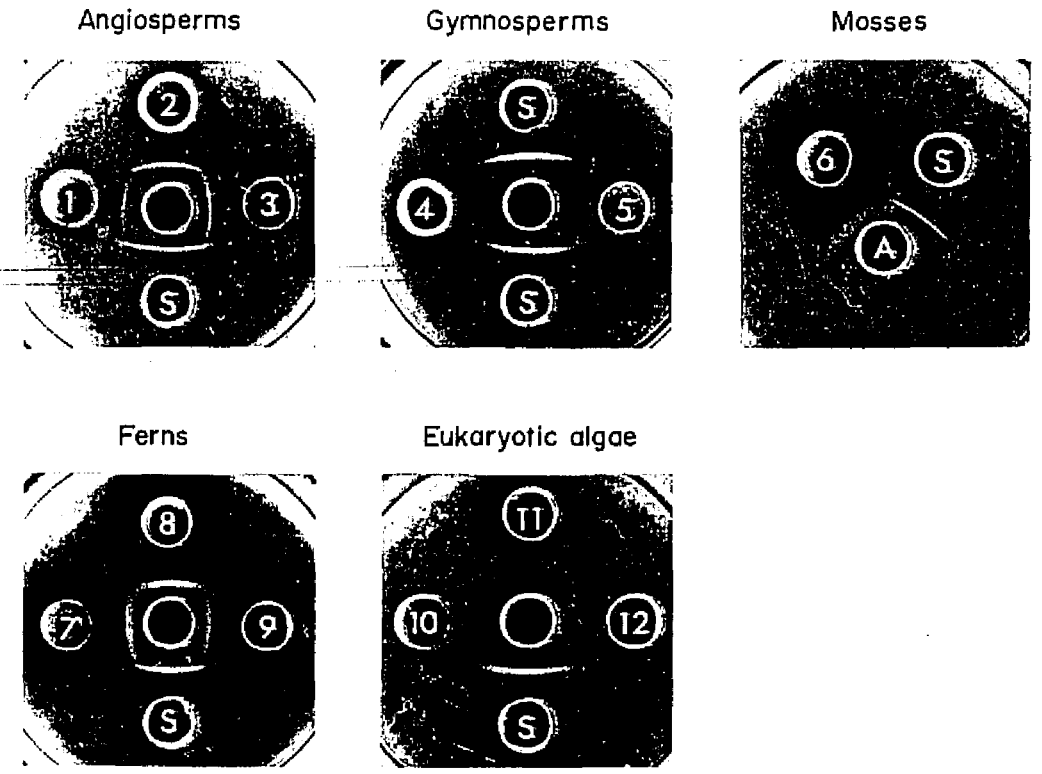

FIG. 4. Ouchterlony double immunodiffusion of the spinach Cu,Zn-superoxide dismutase antibody and superoxide dismutase in cell-free extracts from various photosynthetic organisms. Center well or A, immunoglobulin (260 μg of protein); S, crystalline spinach Cu,Zn-superoxide dismutase (1.1 μg, 10 units); 1-12, cell-free extracts containing 10 to 20 units of superoxide dismutase from: 1, *Lemna perpusilla*; 2, *Cryptotaenia canadensis*; 3, *Raphanus sativus*; 4, *Cycas revoluta*; 5, *Metasequoia glyptostroboides*; 6, *Polytrichum juniperinum*; 7, *Equisetum ramosissimum*; 8, *Equisetum arvense*; 9, *Angiopteris lygodiifolia*; 10, *Ulva pertusa*; 11, *Porphyra tenera*; and 12, *Nitella pulchella*.

observed between the extract and the antibody in the immunoprecipitation test, indicating that the spinach enzyme and the SOD of the other angiosperms have some antigenic determinants in common (Fig. 4). It should be note here that Halophila ovalis and Lemna perpusilla (Figs. 3A, 4, well 1, and 5, strips 2) are aquatic angiosperms.

The localization of Cu,Zn-SOD in chloroplast stroma has been revealed after fractionation of spinach leaf cells by sucrose density centrifugaion (29). I have observed that the chloroplast stroma has a cyanide-insensitive SOD in addition to Cu,Zn-SOD, suggesting the occurrence of Mn- or Fe-SOD (Chapter XIII). However, Lumsden and Hall (34) reported the cyanide-insensitive enzyme in spinach lamellae. In kidney bean leaves, in accordance with spinach, three soluble isozymes of SOD were found. Two of them are sensitive to both cyanide and the antibody, while the other is insensitive to both inhibitors (35). Thus, angiosperms contain both cyanide-sensitive and -insensitive SOD.

Extracts of seven species of gymnosperms were tested for the effect of cyanide on SOD. Since the cytochrome c assay system was disturbed by redox substances in dialyzed extracts of all species except Cycas revoluta, enzymatic activity was measured by the nitroblue tetrazolium system. The results in Table I show partial inhibition by cyanide, suggesting the occurrence of Cu,Zn-SOD in gymnosperms, although in general the percentage of cyanide-insensitive activity was higher than in angiosperms.

The superoxide dismutase activity of gymnosperms was only slightly inhibited by the antibody to spinach enzyme. Figure 3B shows the results of Cycas revoluta using both assay systems. A similar weak inhibition was observed in extracts of the other gymnosprems. Figure 4 shows the absence of or faint immunoprecipitation band between the antibody and an extract of Metasequoia or Cycas. Extracts from the other gymnosperms showed similar results in Ouchterlony tests. This weak interaction with the antibody may be due to a high proportion of cyanide-insensitive SOD in extracts of gymnosperms and may be partly due to the phylogenetic distance between angiosperms and gymnosperms. I observed that Cu,Zn-SOD from bovine erythrocytes (erythrocuprein) did not form a complex and was not inhibited by the spinach Cu,Zn-SOD antibody.

Mosses and Ferns (Bryophyta and Pteridophyta)

Superoxide dismutases in extracts from mosses and ferns were characterized in the same way as those from seed plants. SOD from mosses (three species covering Hepaticae and Musci) were partially inhibited by cyanide and by the antibody (Table I, Fig. 3C and D). Partial inhibition by both inhibitors was also observed in ferns including Lepidophytina, Calmophytina, and Pterophytina (Fig. 3E and F, Table I). Disc electrophoresis indicates the occurrence of cyanide-sensitive and -insensitive isozymes of SOD (Fig. 5). Of the 10 species of fern tested, Azolla imbricata is aquatic. These observations suggest the occurrence of Cu,Zn-SOD in addition to the cyanide-insensitive enzyme in ferns and mosses irrespective of their habitat. Figure 4 shows that extracts of ferns and mosses cross-reacted, with spur formation in the Ouchterlony test.

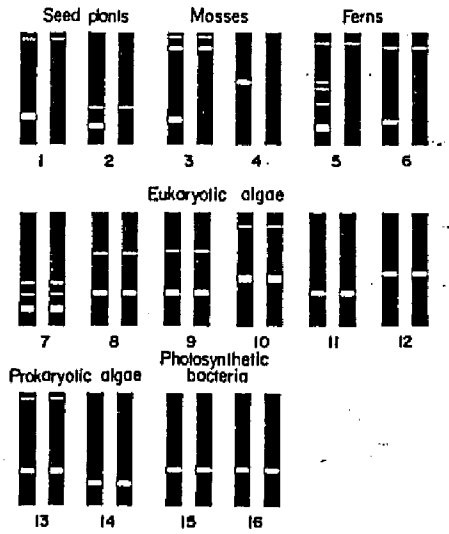

FIG. 5. Polyacrylamide-gel electrophoresis of extracts of photosynthetic organisms and activity patterns on the gels stained for superoxide dismutase in the presence and absence of cyanide. For each pair, the left strips are control and the right were immersed in 5 mM KCN before staining for superoxide dismutase activity. Top: Cathode, bottom: anode. 1, *Spinacia oleracea*; 2, *Lemna perpusilla*; 3, *Polytrichum juniperinum*; 4, *Marchantia polymorpha*; 5, *Angiopteris lygodiifolia*; 6, *Equisetum ramosissimum*; 7, *Nitella pulchella*; 8, *Bryopsis plumosa*; 9, *Chlamydomonas reinhardi*; 10, *Euglena gracilis*; 11, *Colopomenia sinnosa*; 12, *Gymuogongrus flabelliformis*; 13, *Nostoc verrucosum*; 14, *Anabaena variabilis*; 15, *Rhodospirillum rubrum*; and 16, *Rhodopseudomonas capsulata*. The achromatic zones indicate enzymatic activity.

TABLE IV

ACTIVITIES OF SUPEROXIDE DISMUTASES AND THEIR CYANIDE SENSITIVITIES IN EXTRACTS OF BLUE-GREEN ALGAE AND PHOTOSYNTHETIC BACTERIA

Species	Superoxide dismutase	
	Activity (units/mg of protein)	Cyanide-insensitive activity[a] (%)
Cyanophyta (blue-green algae)		
Plectonema boryanum	9	100[b]
Nostoc verrucosum	26	100
Anabaena cylindrica	ND[c]	100
Anabaena variabilis	7	100
Spirulina platensis	61	100
Bacteriophyta		
Rhodospirillaceae (purple nonsulfur bacteria)		
Rhodospirillum rubrum	7	100
Rhodopseudomonas capsulata	10	100
Chlorobiaceae (green sulfur bacteria)		
Chlorobium thiosulfatophilum	13	100
Chromatiaceae (purple sulfur bacteria)		
Chromatium vinosum	7	100

[a] Cyanide present at 1 mM.

[b] Effect of cyanide was tested using the nitroblue tetrazolium system for the assay of superoxide dismutase.

[c] ND, not determined.

Eukaryotic Algae (Charophyta, Euglenophyta, Chlorophyta, Chrysophyta, Phaeophyta and Rhodophyta)

The effects of cyanide and the antibody on SOD in extracts of eukaryotic algae are shown in Table II and Fig. 3G and H. The eukaryotic algae tested covered red algae (five species), Euglena (one species), brown algae (four species), diatoms in Chrysophyta (two species), green algae (five species), and Charophyta (two species). Their habitat is fresh water or marine, except for an aerial green alga, Trentepohlia aurea.

In marked contrast to seed plants, ferns, and mosses, SOD activity in the eukaryotic algae is not affected by cyanide or by the spinach enzyme antibody. In some algae the inhibition of cytochrome c reduction by O_2^- generated by the xanthine-xanthine oxidase system was partially reversed by cyanide, but this was not the case when I assayed for SOD activity using the inhibition of nitroblue terazolium reduction by O_2^- generated by the FMN-light system. Partial reversal of inhibition by cyanide was probably due to the inhibition of cytochrome c oxidase by cyanide. In agreement with the ineffectiveness of the antibody on enzymatic activity, extracts of eukaryotic algae did not form a precipitation band in Ouchterlony tests (Fig. 4).

Immunological studies on ferredoxin (36), ribulose diphosphate carboxylase (37), glycolate oxidase (dehydrogenase) (38), and ferredoxin-NADP reductase (39) have indicated that there are common antigenic determinants among the enzymes of angiosperms, eukaryotic and prokaryotic algae, and photosynthetic bacteria. The sensitivity of the SOD from ferns and mosses and the insensitivity of those from algae to the spinach SOD antibody are probably not due to phylogenetic distance but reflect the presence of different forms of SOD.

The location of SOD activity in polyacrylamide disc gels after electrophoresis provided evidence for the occurrence of several isozymes in extracts of some algae. Some of the results are shown in Fig. 5. In the case of seed plants, mosses, and ferns, at least one achromatic band disappeared on pretreatment of the gel with cyanide, but, in the case of the eukaryotic algae, achromatic bands were not affected by treatment with cyanide. Therefore I may say that eukaryotic algae do not contain

cyanide-sensitive Cu,Zn-SOD, not even as a minor isozyme component.

Adaptive formation of SOD in an environment of high partial pressures of oxygen has been reported in Escherichia coli, Streptococcus feacalis (40), yeast (41), and lungs of rats (42). Since illumination of chloroplasts produces activated oxygen species in photosynthetic organisms (2-6), the level of SOD is expected to be affected by the growth conditions. The results in Table III on the level of SOD in Euglena cultured under different conditions indicate that this is the case. The level of SOD is highest in cells cultured under photoautotrophic conditions. Thus, in addition to oxygen, light also induces adaptive formation of SOD.

Prokaryotic Algae (Cyanophyta)

The absence of Cu,Zn-SOD in prokaryotic algae, as in eukaryotic algae, is evident from the data in Table IV and Figs. 3I and J and 5. The SOD activity in crude extracts of blue-green algae (five species) was not affected by cyanide and the antibody against the spinach Cu,Zn-SOD.

Superoxide dismutases have been purified from Plectonema boryanum (14,18) and Spirulina platensis (17). The major SOD in these blue-green algae is the Fe-containing enzyme having properties similar in several respects to Fe-SOD from Escherichia coli (43) and Pseudomonas ovalis (44). The minor SOD in Plectonema is the Mn-enzyme (35). During purification of the enzyme from Plectonema cells, only the two cyanide-insensitive SOD were detectable (14), supporting the absence of the Cu,Zn-enzyme.

The antibody against Fe-SOD from Plectonema inhibited the Plectonema Fe-enzyme and formed a precipitin line in Ouchterlony tests but neither inhibited nor formed a complex with the SOD from the other algae, not even the enzyme from Nostoc verrucosum. Plectonema Fe-SOD was not inhibited by the antibody against Fe-SOD from Mycobacterium tuberculosis or Mn-SOD from Mycobacterium sp., strain Takeo. It has been shown that antibodies against the Mycobacterium enzyme cross-reacted with several mycobacterial species but did not react with SOD form Escherichia coli, Pseudomonas aeruginosa, Achromobacter xylos-

oxidans and Acinetobacter anitratus (45). In contrast to Cu,Zn-SOD, the antigenic determinants of Fe- or Mn-SOD are apparently highly specific for the enzyme in each organism. Thus, unfortunately, I could not employ the Fe-SOD antibody for the identification of the cyanide-insensitive Fe- or Mn-SOD although a high degree of homology of amino acid sequences has been found between Mn- and Fe-SOD and between the Mn-enzymes from bacteria and mammalian mitochondria (10,11).

Photosynthetic Bacteria (Bacteriophyta)

Superoxide dismutases in extracts from photosynthetic bacteria including purple nonsulfur, purple sulfur, and green sulfur bacteria show the same response to both inhibitors as do those of algae (Table IV, Figs. 3K and L and 5), indicating the occurrence of only cyanide-insensitive SOD and the absence of Cu,Zn-SOD. Antibodies against Plectonema Fe-SOD and Mycobacterium Fe- or Mn-SOD neither inhibited nor formed a complex with the SOD of photosynthetic bacteria (Fig. 3L). The occurrence of cyanide-insensitive SOD in green sulfur and purple nonsulfur bacteria has also been reported by Hewitt and Morris (16) and Lumsden et al.(17).

Sulfur bacteria for the assay of the enzymes were cultured anaerobically under photoautotrophic conditions using sulfide and thiosulfate as electron donors. The contents of SOD in anaerobic photosynthetic bacteria were similar to those in blue-green algae other than Spirulina on the basis of soluble protein. The occurrence of SOD in other anaerobes including sulfate-reducing bacteria, Clostridium (16), and anaerobic protozoa (13) has been also reported.

Purification of SOD form Chromatium by me recently (Chapter VIII) has indicated that Chromatium SOD is the Fe-enzyme. Chromatium cells contain a single superoxide dismutase with properties similar to those of the enzymes from blue-green algae (14,17,18) and Escherichia coi (43): absorption spectra, amino acid compositions, rates of dismutation of O_2^-, and sensitivity to H_2O_2 (Chapter VIII). On the contrary, SOD in a purple nonsulfur bacterium, Rhodopseudomonas spheroides, is the Mn-enzyme (17).

Catalase and Peroxidases

It has been supposed that catalase and peroxidase work as scavengers of hydrogen peroxide formed by the dismutation of O_2^- and by the divalent reduction of molecular oxygen by several oxidases. In addition to SOD, these hydrogen peroxide scavengers in photosynthetic organisms may play a role in defense against light-oxygen toxicity. Levels of catalase and peroxidases showed larger variations between species than those of SOD (data not shown). Catalase was detected in all land plants tested, but some algae (Ulva pertusa, Euglena gracilis, Sargassum tortile and Gratelouoia filicina) were devoid of catalase. Absence of catalase in Euglena was reported previously (46). Those organisms lacking catalase probably scavenged hydrogen peroxide by peroxidases. A brown alga, Sargassum tortile, does not contain catalase or peroxidase, but this does not exclude the possible identification of peroxidases when other electron donors, such as ascorbic acid, glutathione, cytochrome c, or NADPH, are used.

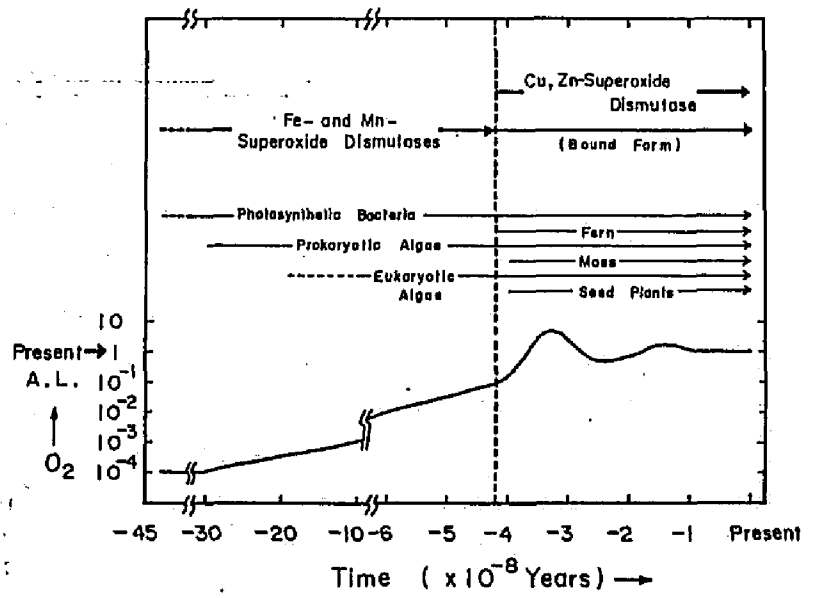

FIG. 6. Evolution of photosynthetic organisms, forms of superoxide dismutase, and oxygen concentration in the earth's atmosphere. Increase in oxygen in the atmosphere is adapted from the results of Berkner and Marshall (1). A. L., atmospheric level.

4. DISCUSSION

This survey of superoxide dismutases in photosynthetic organisms covering photosynthetic bacteria, prokaryotic and eukaryotic algae, ferns, mosses, and seed plants indicates the ubiquitous occurrence of the enzyme. Even when the organism lacks catalase or peroxidase, SOD is contained without exception. Although the level of SOD varied with the species and the growth conditions (Tablel III), the enzyme was found in the range of 5 to 50 units/mg of soluble protein, with a few exceptions (Tables I, II, and IV). Thus SOD accounts for approximately 0.1 to 2% of the soluble protein, depending on the form of the enzyme. Despite various methods of extraction of the enzyme from organisms grown under different conditions, the comparatively narrow variation of the enzyme level over a wide range of organisms suggests an essential and important role of SOD as a defense mechanism against light-oxygen toxicity. The physiological functions of SOD in anaerobic organisms provide interesting problems to be solved.

Among the three forms of SOD differentiable with respect to the metal ion, only Cu,Zn-SOD is sensitive to cyanide. This property is considered to stem from the inner (Cu,Zn-SOD) and outer sphere (Mn- and Fe-SOD) mechanisms of dismutation of O_2^- (47) and allows a distinction to be made between the forms of SOD. In addition to cyanide, the antibody against spinach Cu,Zn-SOD was also employed to characterize SOD. The spinach Cu,Zn-SOD antibody inhibited, more or less, the enzymatic activity in land plant extracts, and a high level of inhibition was observed whenever the inhibition due to cyanide was high. Thus the land plants' Cu,Zn-SOD have some antigen in common, but Cu,Zn-SOD from bovine erythrocytes did not interact with the spinach enzyme antibody. I also prepared antibody against Fe-SOD from Plectonema and tested its reactivity. In contrast to the Cu,Zn-enzyme's antibody, the Plectonema antibody inhibited only the Plectonema enzyme and did not interact with enzymes in other algae and photosynthetic bacteria. A similar narrow reactivity was also observed for antibodies against Mn- and Fe-SOD from Mycobacterium (45), and, in fact, the Mycobacterium antibodies did not inhibit the Plectonema and Chromatium Fe-SOD.

Characterization of SOD according to the above criteria clearly indicates that photosynthetic bacteria, prokaryotic algae, and eukaryotic algae lack Cu,Zn-SOD and contain only the cyanide-insensitive and the spinach Cu,Zn-enzyme antibody-insensitive SOD. In photosynthetic bacteria and blue-green algae cyanide-insensitive SOD has been isolated and characterized as Mn- or Fe-SOD (14,17,18).

The Cu,Zn-SOD appears in the most primitive land plants, ferns and mosses, and extends to higher seed plants. In these plants, in addition to Cu,Zn-SOD, cyanide-insensitive SOD is present in soluble form.

Although an aerial green algae (Trentephohlia aurea) lacks Cu,Zn-SOD, the aquatic fern (Azolla imbricata) and angiosperms (Halophila ovalis and Lemna perpusilla) contain Cu,Zn-SOD in addition of the cyanide-insensitive enzyme. Thus, I may say that the distribution of Cu,Zn-SOD does not reflect the habitat of the orgaisms but rather their phylogenetic position.

In Fig. 6 the forms of SOD, the evolution of photosynthetic organisms, and the accumulation of oxygen in the atmosphere are schematically illustrated. Although I do not yet know when photosynthetic bacteria appeared on the earth, it has been assumed that photosynthetic bacteria appeared before blue-green algae because photosynthetic bacteria are not able to utilize water as an electron donor for photosynthesis. From paleontological studies on microfossils (48), it has been deduced that blue-green algae appeared about 3×10^9 years agao. The geological record indicates the appearance of the first land plants in the late Silurian age, 4.2×10^8 years ago (1). According to Berkner and Marshall (1), during this age the oxygen level in the atmosphere was about 10% of that at present. This level of oxygen formed an ozone layer which caused filtration of lethal ultraviolet light and allowed the organisms to go ashore.

The present results show that those photosynthetic organisms which appeared up to the later Silurian age do not contain Cu,Zn-SOD and have only cyanide-insensitive Fe- and Mn-SOD. The Cu,Zn-SOD is thought to have been acquired first by ferns or mosses, the land plants appearing in the late Silurian age. The plants evolving after that all contain

Cu,Zn-SOD in addition to cyanide-insensitive SOD. From the present results it may be reasonable to assume that SOD of photosynthetic bacteria is an ancestral protein of Fe- and Mn-SOD and that Cu,Zn-SOD of seed plants have evolved from Cu,Zn-SOD of the first land plants, ferns or mosses. Amino acid sequence analyses of SOD in photosynthetic organisms at different evolutionary levels may provide useful information about their phylogenetic positions. Further, sequence studies of chloroplast SOD would give us information on the origin of chloroplasts. A high degree of homology of the amino acid sequences of Fe- and Mn-SOD from bacteria and mitochondria has been shown (10,11).

In addition to eukaryotic algae, several eukaryotes including protozoa (13) and a mushroom, Pleurotus (12), lack Cu,Zn-SOD. I confirmed the absence of Cu,Zn-SOD in a protozoa, Crithidia fasciculata. In contrast, yeast and Neurospora contain Cu,Zn-SOD (8). Therefore, the division Eumycota may be divided into two groups with respect to the form of SOD. It should be mentioned here that Yamanaka and Okunuki have suggested that algae appeared earlier than yeast and Neurospora on the earth; this is based on the reactivity of the respective cytochromes c with cytochrome oxidases (49). Further surveys of the form of SOD in other organisms will test the working hypothesis that the acquisition of Cu,Zn-SOD reflect the time of appearance of the oganisms on the earth.

Osterberg (1) and Egami (51) have suggested that, before oxygen had accumulated in the atmosphere by photosynthetic activity, copper occurred in the form of extremely insoluble Cu(I)-sulfides because of a low environmental redox potential and was not available to organisms, although iron occurred in an available form. For anaerobic photosynthetic organisms the absence of Cu,Zn-SOD is inferred from the unavailability of copper, but this explanation cannot hold for prokaryotic and eukaryotic algae since both contain a copper protein, plastocyanin. At present I can only speculate as to why land plants require a new form of SOD with a different metal and a different amino acid sequence. An increase in the oxygen concentration in the atmosphere is probably one reason, though little difference in the enzymatic dismutation rate of O_2^- has been found between the three forms of SOD except at high pH where Mn- and Fe-SOD have a lower dismutation rate than Cu,Zn-SOD (53). Further

characterization, including sequence analysis, of the three forms of SOD and an understanding of the physiological functions of SOD in anaerobes will provide a clue in solving the above questions.

5. SUMMARY

Superoxide dismutases (SOD) in photosynthetic organisms at different evolutionary levels were characterized using the criterion that Cu,Zn-SOD is sensitive to cyanide while Mn- and Fe-SOD are insensitive. The effect of the antibody against spinach Cu,Zn-SOD was also tested as a means of distinguishing the several forms of the enzyme. SOD activity in extracts from photosynthetic bacteria, prokaryotic algae (blue-green algae), and eukaryotic algae (red, green, and brown algae, diatoms, Euglena, and Charophyta) were insensitive to cyanide and to the antibody, suggesting the presence of Fe- and/or Mn-SOD and the absence of Cu,Zn-SOD. In contrast, ferns, mosses, and seed plants including gymnosperms and angiosperms contained Cu,Zn-SOD in addition to the cyanide-insensitive SOD. Although an aerial green alga lacks Cu,Zn-SOD, aquatic angiosperms and ferns, like other land plants, contain this form of SOD. Thus the distribution of Cu,Zn-SOD does not reflect the habitat but, rather, the phylogeny of the organism. The relation between the oxygen concentration in the atmosphere and the appearance of various forms of SOD during the evolution of photosynthetic organisms is discussed.

6. REFERENCES

(1) Berkner, L.C., and Marshall, L.C. (1965) J. Atmos. Sci. 22, 225-261.

(2) Asada, K., Kiso, K., and Yoshikawa, K. (1974) J. Biol. Chem. 249, 2175-2181.

(3) Elstner, E.F., and Kramer, R. (1973) Biochim. Biophys. Acta 314, 340-353.

(4) Mehler, A.H. (1951) Arch. Biochem. Biophys. 33, 65-77.

(5) Elstner, E.F., and Konze, J.R. (1974) FEBS Lett. 45, 18-21.

(6) Takahama, U., and Nishimura, M. (1975) Plant Cell Physiol. 16, 737-748.

(7) Haber, F., and Weiss, J. (1934) Proc. Roy. Soc. London A 147, 332-351.

(8) Fridovich, I. (1975) Annu. Rev. Biochem. 44, 147-159.

(9) Puget, K., and Michelson, A.M. (1974) Biochem. Biophys. Res. Commun. 58, 830-838.

(10) Steinman, H.M., and Hill, R.L. (1973) Proc. Nat. Acad. Sci. USA 70, 3725-3729.

(11) Bridgen, J., Harris, J.I., and Northrop, F. (1975) FEBS Lett. 49, 392-395.

(12) Lavelle, F., Duroszy, P., and Michelson, A.M. (1974) Biochimie 56, 451-458.

(13) Lindmark, D.G., and Muller, M. (1974) J. Biol. Chem. 249, 4634-4637.

(14) Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y., and Enmanji, K. (1975) J. Biol. Chem. 250, 2801-2807.

(15) Lumsden, J., and Hall, D.O. (1975) Nature (London) 257, 670-672.

(16) Hewitt, J., and Morris, J.G. (1975) FEBS Lett. 50, 315-318.

(17) Lumsden, J., Cammack, R., and Hall, D.O. (1976) Biochim. Biophys. Acta 438, 380-392.

(18) Misra, H.P., and Keele, B.B. (1975) Biochim. Biophys. Acta 379, 418-425.

(19) Schwarz, P., and Frandsen, N.P. (1960) Naturwissenschaften 47, 47.

(20) Ishida, M., Kikuchi, T., Matsubara, T., Ikushima, T., and Mizuma, N.

(1972) Annu. Rep. Res. Reactor Inst. Kyoto Univ. 5, 56-58.

(21) Tamiya, H., Iwamura, T., Shibata, K., Hase, E., and Nihei, T. (1953) Biochim. Biophys. Acta 12, 23-40.

(22) Cramer, M., and Myers, J. (1952) Arch. Mikrobiol. 17, 384-402.

(23) Asada, K., Takahashi, M., and Nagate, M. (1974) Agric. Biol. Chem. 38, 471-473.

(24) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(25) Beauchamp, C.O., and Fridovich, I. (1971) Anal. Biochem. 44, 276-287.

(26) Siegel, B.Z., and Siegel, S.M. (1970) Amer. J. Bot. 57, 285-287.

(27) Luck, H. (1965) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), p. 885, Academic Press, New York.

(28) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

(29) Asada, K., Urano, M., and Takahashi, M. (1973) Eur. J. Biochem. 36, 257-266.

(30) Sawada, Y., Ohyama, T., and Yamazaki, I. (1972) Biochim. Biophys. Acta 268, 305-312.

(31) Beauchamp, C.O., and Fridovich, I. (1973) Biochim. Biophys. Acta 317, 50-64.

(32) Morita, Y., and Asada, K. (1974) J. Mol. Biol. 86, 685-686.

(33) Richardson, J.S., Thomas, K.A., Rubin, B.H., and Richardson, D.C. (1975) Proc. Natl. Acad. Sci. USA 72, 1349-1353.

(34) Lumsden, J., and Hall, D.O. (1975) Biochem. Biophys. Res. Commun. 64, 595-602.

(35) Asada, K., Kanematsu, S., Takahashi, M., and Kono, Y. (1976) in Iron and Copper Proteins (Yasunobu, H.Y., and Hayaishi, O., eds.), pp. 551-564, Plenum Press, New York.

(36) Tel-Or, E., Cammack, R., and Hall, D.O. (1975) FEBS Lett. 53, 135-138.

(37) Akazawa, T., Kondo, H., Shimadzu, T., Nishimura, M., and Sugiyama, T. (1972) Biochemistry 11, 1298-1303.

(38) Codd, G.A., and Stewart, W.D.P. (1974) Plant Sci. Lett. 3, 199-205.

(39) Lazzarini, R.A., and San Pietro, A. (1963) in Studies on Microalgae

and Photosynthetic Bacteria (Japanese Society of Plant Physiologists, eds.), pp. 453-464, University of Tokyo Press, Tokyo.

(40) Gregory, E.M., and Fridovich, I. (1973) J. Bacteriol. 114, 543-548, 1193-1197.

(41) Gregory, E.M., Goscin, S.A., and Fridovich, I. (1974) J. Bacteriol. 117, 456-460.

(42) Crapo, J.D., and Tierney, D.L. (1974) Amer. J. Physiol. 226, 1401-1407.

(43) Yost, F.J., Jr., and Fridovich, I. (1973) J. Biol. Chem. 248, 4905-4908.

(44) Yamakura, F. (1976) Biochim. Biophys. Acta 422, 280-294.

(45) Kusunose, M., Noda, Y., Ichihara, K., and Kusunose, E. (1976) Acrh. Microbiol. 108, 65-73.

(46) Lord, J.M., and Merrett, M.J. (1971) Biochem. J. 124, 275-281.

(47) Villafranca, J.J., Yost, F.J., and Fridovich, I. (1974) J. Biol. Chem. 249, 3532-3536.

(48) Schopf, J.W., and Barghoorn, E.S. (1967) Science 156, 508-512.

(49) Yamanaka, T., and Okunuki, K. (1968) in Structure and Function of Cytochromes (Okunuki, K., Kamen, M.D., and Sekuzu, I., eds.), pp. 390-403, University of Tokyo Press, Tokyo.

(50) Osterberg, R. (1974) Nature (London) 249, 382-383.

(51) Egami, F. (1975) J. Biochem. (Tokyo) 77, 1165-1169.

(52) Forman, H.J., and Fridovich, I. (1973) Arch. Biochem. Biophys. 158, 396-400.

(53) Rigo, A., Tomat, R., and Rotilio, G. (1974) Electroanal. Chem. Interface Electrochem. 57, 291-296.

CHAPTER III

ALGAL Cu,Zn-SUPEROXIDE DISMUTASE: ISOLATION AND CHARACTERIZATION OF THE ENZYME FROM THE GREEN ALGA SPIROGYRA SP.

1. INTRODUCTION

The three types of superoxide dismutase (SOD) show the characteristic distribution among organisms at different stages of evolution (Chapter II). Thus, Mn-SOD occurs in prokaryotes and eukaryotes, while Fe-SOD in prokaryotes, eukarytotic algae, and protozoa. Cu,Zn-SOD has been found in animals, fungi and land plants. Prokaryotes lack Cu,Zn-SOD, but it has been shown that two bacteria, Photobacterium leiognathi(1,2), and Caulobacter crescentus (3) contain Cu,Zn-SOD. Most eukaryotic algae also lack Cu,Zn-SOD (Chapter II), but Henry and Hall (4) reported the occurrence of cyanide-sensitive Cu,Zn-SOD in some green algae, i.e. Charales and Conjugales, whose cell division is a phragmoplast type and contain glycolate oxidase. No cyanide-sensitive SOD is detected in other green algae whose cell division is phycoplast type and contain glycolate dehydrogenase. Amino acid sequence of Photobacterim Cu,Zn-SOD has a homology with those of eukaryotic Cu,Zn-SOD (5-7). However, the characterization of cyanide-sensitive SOD in green algae has not been done.

The algal cyanide-sensitive SOD is supposed to be an ancestor of Cu,Zn-SOD in land plant and I purified it from a Conjugales, Spirogyra sp. (pond scum). In this chapter, the purification and properties of Spirogyra SOD are described.

2. MATERIALS AND METHODS

Cytochrome c (horse heart, type III) and xanthine oxidase (milk) were obtained from Sigma and Boehringer, respectively. Spirogyra sp.

was collected in a pond of the university campus. The cells were thoroughly washed with tap water, then with distilled water, and stored at -20°C until used. Microscopic observation confirmed the samples being free from other algae.

Spinach Cu,Zn-SOD was purified from the leaves as described in Chapter V. Spinach contains two Cu,Zn-SOD isozymes and, the purified enzyme was the stromal one (Chapter V). Cu,Zn-SOD from the fern Equisetum arvense (horse tail) was purified to a homogeneous state (chapter VII). Antibodies against spinach and Equisetum SOD were prepared by immunization of rabbits as described previously (Chapter II).

SOD was assayed by the xanthine-xanthine oxidase-cytochrome c system as described in Chapater II, which is a modification of the method of McCord and Fridovich (8). The assay system gives a threefold activity unit since the reaction volume is reduced to one third of the original method. Enzymatic activity is shown in the McCord and Fridovich unit (8).

Native and SDS-polyacrylamide gel electrophoresis (PAGE), location of SOD activity on the gel, determination of the molecular weight and subunit molecular weight, metal analysis, and Ouchterlony double immunodiffusion were done as in the previous papers (9-12). Protein was determined by the Lowry method using bovine serum albumin as a standard (13). Protein on the gel was stained using a silver staining kit obtained from Daiich Pure Chemicals Co., LTD., Tokyo, Japan.

3. RESULTS

SOD isozymes in Spirogyra extract

Separation of the cell extract of Spirogyra by native PAGE and the location of SOD activity on the gel showed the three major cyanide-sensitive bands and one minor cyanide-insensitive band. Occurrence of cyanide-sensitive SOD in Spirogyra is in agreement with the result of Henry and Hall(4). I conducted purification of the cyanide-sensitive SOD.

Purification of Spirogyra enzyme

Spirogyra (20 g in fresh weight) was homogenized using an electric mortar in 100 ml of 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA with sea sand for 1 hour. After the addition of Triton X-100 to the homogenate to 0.1% (V/V), the homogenate was sonicated for 5 min and was clarified by centrifugation at 39,000 g for 20 min. Ammonium sulfate was added to the supernatant to 30% saturation, and after removal of the precipitate by centrifugation, ammonium sulfate was added to the supernatant to 90% saturation. The precipitate collected by centrifugation at 18,000 g for 20 min was dissolved in and dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA.

The dialyzed enzyme was clarified by centrifugation and applied to a column of DEAE-Sephacel (1.3 x 3.5 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. The column was washed with the same buffer. The adsorbed enzyme was eluted by a linear gradient of KCl (0 to 400 mM, 200 ml) in 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. The active fractions were concentrated through an Amicon PM 10 membrane and charged to a column of Sephadex G-100 (2 x 85 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, containing 0.1 M KCl. SOD was eluted with the same buffer as a single peak. The enzyme was concentrated through the membrane filter and dialyzed against 10 mM potassium phosphate, pH 7.8.

Table I

Summary of the purification of SOD from Spirogyra sp. (20 g)

Purification step	Total protein[a] (mg)	Total activity[b] (units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Extract	75	869	12	100	1
30-90% $(NH_4)_2SO_4$	22	797	36	92	3
DEAE-Sephacel	2.1	692	330	80	28
Sephadex G-100	0.96	445	464	51	39

a) Protein was measured by the method of Lowry et al. (13).

b) McCord and Fridovich unit (8).

Table I summarizes the purification of Spirogyra SOD. The purified SOD showed three activity bands with very close spacing on native-PAGE. Protein bands corresponded to the activity bands (Fig. 1). In contrast to native-PAGE, SDS-PAGE showed a major band with very faint bands (Fig. 2). Thus, the purified SOD was essentially free form other protein. Because of limited amounts of enzyme, further separation of each isozyme was not conducted.

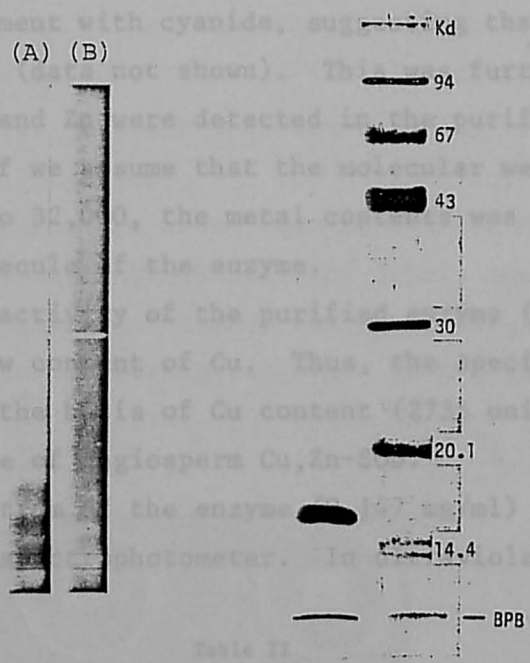

Fig. 1. (left) Native-PAGE of Spirogyra Cu,Zn-SOD. SOD applied was 1 μg for protein staining (A), and 5 units for activity staining (B). Top, cathode; bottom, anode.

Fig. 2. (right) SDS-PAGE of Spirogyra Cu,Zn-SOD. The enzyme (1 μg) was denatured with 4% SDS at 100°C for 3 min. Electrophoresis was carried out using 15% gel slab (1 mm in thick). Protein was localized by the Ag-staining method.

Properties of Spirogyra enzyme

Molecular weight of the enzyme was estimated to be 32,000 by gel filtration using a column of Sephadex G-100 equilibrated with 10 mM potassium phosphate, pH 7.8, and 0.1 M KCl and calibrated with bovine serum albumin, ovalbumin, α-chymotrypsinogen-A, and cytochrome c.

Subunit molecular weight of the enzyme was determined by SDS-PAGE. The enzyme was denatured with 4% SDS in the presence or absence of 4% 2-mercaptoethanol at 100°C for 3 min. The electrophoresis showed a single band corresponding to a molecular weight of 16,500 with faint bands in higher molecular weight region in the absence of mercaptoethanol (Fig. 2). Thus, Spirogyra enzyme is a homodimer having a molecular weight of 32,000, and the subunits are not covalently linked.

Activity staining of native-PAGE showed that all three bands disappeared by treatment with cyanide, suggesting that the purified enzyme was Cu,Zn-SOD (data not shown). This was further supported by metal analysis. Cu and Zn were detected in the purified enzyme, but neither Fe or Mn. If we assume that the molecular weights of the three isozymes are equal to 32,000, the metal contents was 0.17 atom Cu and 0.81 atom Zn per molecule of the enzyme.

A low specific activity of the purified enzyme (Table I) was probably due to a low content of Cu. Thus, the specific activity of the Spirogyra enzyme on the basis of Cu content (2735 units/g atom Cu) was comparable with those of angiosperm Cu,Zn-SOD.

Absorption spectrum of the enzyme (0.147 mg/ml) was recorded using a Shimadzu MPS-2000 spectrophotometer. In ultraviolet region, the

Table II

Effects of inhibitors or treatment on Spirogyra Cu,Zn-SOD

Compound or treatment	Inhibition or inactivation (%)
1 mM Potassium cyanide	97
10 mM Sodium azide	54
1 mM H_2O_2 [a)]	95
Heating (70°C, 30 min) [b)]	55
Heating (80°C, 30 min) [b)]	92

a) The enzyme was incubated in a cuvett with 1 mM H_2O_2 in 200 µl of 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA for 30 min. The assay mixture and 100 units of catalase was added and the remaining activity was determined.

b) The enzyme in a sealed tube was incubated at indicated temperatures.

spectrum showed an absorption peak around 260 nm but no peak around 280 nm, which are characteristic of Tyr and Trp-lacked Cu,Zn-SOD. Although fine structure was not observed, the absorption around 260 nm might be responsible to the Phe residues. Absorption peak around at 680 nm due to Cu-chromophore of Cu,Zn-SOD could not be detected because of its low concentration.

Table II shows the effects of several compounds or treatments on the activity of the purified Spirogyra SOD. The Spirogyra enzyme was inhibited by cyanide and azide, and inactivated by hydrogen peroxide. Heating of the enzyme at 70°C resulted in 45% inactivation, but at 80°C most activity was lost. These properties were similar to Cu,Zn-SOD from other organisms (14).

The Spirogyra enzyme was cross-reacted with both anti-Equisetum and anti-spinach Cu,Zn-SOD sera in Ouchterlony double immunodiffusion (Fig. 3). The results indicate that common antigenic determinant(s) is conserved in the Cu,Zn-SOD form green algae to angiosperms.

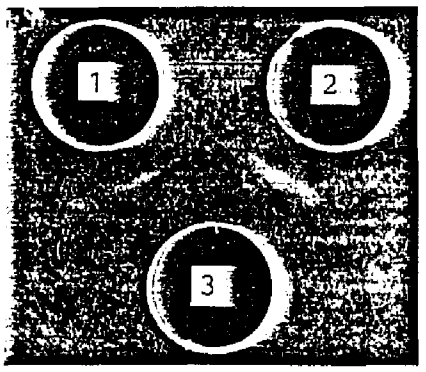

Fig. 3 Ouchterlony double immunodiffusion of Spirogyra Cu,Zn-SOD with anti-Equisetum and anti-spinach Cu,Zn-SOD sera. 1, Anti-spinach SOD serum (390 μg); 2, anti-Equisetum SOD serum (195 μg); 3, Spirogyra Cu,Zn-SOD (2 μg). Each well contained 15 ul of serum or enzyme. Diffusion was conducted at 37°C for 12 hours.

4. DISCUSSION

Cu,Zn-SOD from the eukaryotic green alga Spirogyra resembled those from animals, fungi and plants in respect of molecular properties such as molecular weight, subunit structure, and absorption spectrum. The enzyme was also immunologically similar to Cu,Zn-SOD from fern and angiosperms. Thus, a green algal Cu,Zn-SOD seems to be an ancestor of Cu,Zn-SOD of ferns, mosses and seed plants, since Cu,Zn-SOD distibutes phylogenetically among those organisms at different levels of evolution (Chapter II).

Green algae whose cell division is a phragmoplast type contain the Cu,Zn-SOD, while those whose cell division is a phycoplast type lack Cu,Zn-SOD (4). This distribution of Cu,Zn-SOD overlaps that of glycolate-oxidizing enzymes: the formers contain glycolate oxidase and the laters gycolate dehydrogenase. Glycolate oxidase and the phragmoplast type of cell division are characteristic to land plants. Thus, the present results support the belief that among eukaryotic algae, phragmoplast green algae directly related to land plants in the course of evolution.

The problem remained unclear is that which SOD is closer to ancestral protein of Cu,Zn-SOD, bacterial (1,3) or eukaryotic algal enzyme. If we accept the order of the evolution of organisms, the oldest enzyme would be the bacterial enzymes. However, the limited occurrecne of this enzyme in only two bacteria, i.e. Photobacterium (1) and Caurobacter (3) suggests that this is not the case. Therefore, it may be more probable that phragmoplast green algae acquired the Cu,Zn-SOD during the course of evolution, and then this type of enzyme has been succeded by organisms which have appeared after eukaryotic algae. Comparison of Cu,Zn-SOD sequences of Photobacterium and its host fish has suggested a gene transfer from eukaryote to prokaryote (5-7).

5. SUMMARY

The extract of Spirogyra sp. (pond scum) showed cyanide-sensitive and insensitive superoxide dismutase (SOD) on polyacrylamide gel electrophoresis. Cyanide-sensitive SOD was purified to a mixture of the three isozymes which contained no other protein. It contained Cu and Zn but neither Fe nor Mn, and its molecular weight was determined to be 32,000 by gel filtration. SDS-polyacrylamide gel electrophoresis revealed that it was a homodimer. Absorption spectra showed no peak around 280 nm, indicating the absence of tyrosine and tryptophan residues. Thus, the Spirogyra SOD resembled those of land plant Cu,Zn-SOD. The Spirogyra enzyme cross-reacted with anti-Equisetum and anti-spinach Cu,Zn-SOD sera, indicating the presence of common antigenic determinant between Spirogyra Cu,Zn-SOD, and angiosperm and fern Cu,Zn-SOD.

6. REFERENCES

(1) Puget, K., and Michelson, A.M. (1974) Biochem. Biophys. Res. Commun. 58, 830-838.

(2) Martin, J.P., and Fridovich, I. (1981) J. Biol. Chem. 256, 6080-6089.

(3) Steinman, H.M. (1982) J. Biol. Chem. 257, 10283-10292.

(4) Henry, L.E.A., and Hall, D.O. (1977) in Photosynthetic Organelles (Miyachi, S., Fujita, Y., and Shibata, K., eds.), pp. 377-382, Japanese Soc. Plant Physiol., Kyoto.

(5) Steffens, G.J., Bannister, J.V., Bannister, W.H., Flohe, L., Gunzler, W.A., Kim, S.A., and Otting, F. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364, 675-690.

(6) Rocha, H.A., Bannister, W.H., and Bannister, J.V. (1984) Eur. J. Biochem. 145, 477-484.

(7) Bannister, J.V., and Parker, M.W. (1985) Proc. Natl. Acad. Sci. USA 82, 149-152.

(8) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(9) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(10) Kanematsu, S., and Asada, K. (1978) Arch. Biochem. Biophys. 185, 473-482.

(11) Kanematsu, S., and Asada, K. (1978) FEBS Lett. 91, 94-98.

(12) Kanematsu, S., and Asada, K. (1979) Arch. Biochem. Biophys. 195, 535-545.

(13) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

(14) Steinman, H.M. (1982) in Superoxide dismutase (Oberley, L.W., ed.), pp. 11-68, CRC Press, Florida.

CHAPTER IV

UBIQUITOUS DISTRIBUTION OF Cu,Zn-SUPEROXIDE DISMUTASE IN FUNGI AND MARINE INVERTEBRATES

1. INTRODUCTION

In Chapter II and III, I have systematically surveyed the distribution of the three forms of superoxide dismutase (SOD) among photosynthetic organisms including photosynthetic bacteria, prokaryotic and eukaryotic algae, mosses, ferns and seed plants, and have found that most of eukaryotic algae lack Cu,Zn-SOD. Cu,Zn-SOD is found in only land plants and some phragmoplast algae. Thus, photosynthetic eukaryotes can be divided into two groups by the presence or absence of Cu,Zn-SOD. In this respect, it is interesting to know whether fungi and animals contain Cu,Zn-SOD.

Cu,Zn-SOD in fungi has been isolated from Saccharomyces cerevisiae (1,2), Fusarium oxysporum (3), and Neurospora crassa (4) and Mn-SOD has been purified from a luminescent fungus, Pleutotus olearius (5), and from mitochondria of yeast (6). P. oleraius lacks Cu,Zn-SOD (5).

In animals, although Cu,Zn-SOD from vertebrates have been extensively characterized (7), only a few survey have been done on invertebrates. The enzyme activity has been detected in sipunclulids (8), sea anemone (9) and cuttlefish (10). Cu,Zn-SOD has been isolated from fruit fly (11). I have shown that protozoa is devoid of Cu,Zn-SOD (Chapter II).

In order to clarify the correlation between the type of superoxide dismutase and the phylogeny of organisms, the systematic survey for the enzyme has been extended to fungi and animals. Based on the present results obtained in 57 species of fungi and 24 species of marine invertebrates and other available data, the phylogenetic distribution of the three types of superoxide dismutase is presented.

2. MATERIALS AND METHODS

The strains of slime molds, Dictyostelium discoideum and D. mucoroides were given by Dr. Y. Maeda, Kyoto University. Cells were grown in 0.06% NaCl with Escherichia coli cultured previously in the peptone-sucrose medium by the method of Maeda (12). After 2 days of culture at 21°C slime molds were separated from E. coli by washing with 0.06% NaCl followed by centrifugation. Mastigomycotinous fungi were obtained from Dr. K. Takimoto and cultured in the yeast-starch medium of Emerson (13). The strains of Zygomycotina, Ascomycotina and Deuteromycotina (fungi), and of Ascomycotina and Deuteromycotina (yeasts) were provided by Drs. K. Soda and T. Kido, Kyoto University. They were grown in the 10% malt medium at 30°C with shaking for 1 or 2 days. Several strains were cultured for 5 days. Fruit bodies of Ascomycotina (Aleuria aurantia) and Basidiomycotina were collected from fields in Kyoto and Shiga or obtained from a local market, and kindly identified by Dr. K. Yokoyama, Shiga University. Marine invertebrates including Porifera, Colelenterata, Mollusca, Echinodermata and Arthropoda were obtained from the Seto Marine Biological Laboratory, Kyoto University, or collected near the laboratory and kindly identified by Dr. T. Araga, Kyoto University. The materials were stored at -20°C until use.

About 5-10 g of the materials were homogenized for 10-30 min in a mechanical mortar with 50-100 ml of 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA and sea sand. Yeast cells were homogenized with aluminum oxide for 2 hours. The homogenate was centrifuged at 39,000 g for 30 min and the supernatant was dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA 48 hours. After clarified by centrifugation, the dialyzed solution was used for the assay of SOD and protein. The extracts of fungal fruit bodies were concentrated by adding ammonium sulfate to make 80% saturation and the precipitate dissolved in the buffer was used.

SOD was assayed by the inhibition of cytochrome c reduction by O_2^- generated with the xanthine-xanthine oxidase system as described in Chapter II. Because the extracts from Basidiomycotina interfered with the assay in the cytochrome c-xanthine oxidase system, the enzyme

activity was determined by the inhibition of the formation of formazane due to the reduction of nitroblue tetrazolium by O_2^- generated with the xanthine-xanthine oxidase system. The reaction mixture contained, in a total volume of 1 ml, 50 mM potassium phosphate, pH 7.8, 0.1 mM EDTA, 0.2 mM nitroblue tetrazolium, 0.03% Triton X-100, 0.1 mM xanthine and xanthine oxidase which gave an increase in absorbance at 560 nm at 0.02/min. The addition of Triton X-100 gave a linear absorbance change with time and diminished a lag period due to its stabilization effect on the insoluble formazane formed.

The enzymatic unit of SOD was defined previously (Chapter II) and is presented in the McCord-Fridovich unit. The enzymatic units obtained in the NBT-xanthine oxidase system are also shown in the McCord-Fridovich unit using a factor determined with the purified enzyme. The occurrence of Cu,Zn-SOD was confirmed by its inhibition with 1 mM cyanide. The effect of cyanide and immunoglobulin on the enzyme activity was determined as described in Chapter II. Polyacrylamide gel disc electrophoresis, the location of enzyme active band, and the determination of protein were carried out as before (14-17).

Antiserum against Cu,Zn-SOD (stromal enzyme) from spinach (18) was prepared previously (19). Xanthine oxidase from milk and cytochrome c from horse heart (Type III) were obtained from Boehringer and Sigma, respectively.

3. RESULTS

Myxomycota (slime molds)

The SOD activity in two organisms tested were inhibited by 30-70% in the presence of cyanide, indicating the presence of both cyanide-sensitive Cu,Zn-SOD and cyanide-insensitive Fe- and/or Mn-SOD (Table I-1). The gel disc electrophoresis of the extracts showed several activity bands and one of which was cyanide-sensitive. These observations suggest that slime mold contains Cu,Zn-SOD in addition to the cyanide-insensitive enzyme.

Table I-1

Activities of superoxide dismutases and their cyanide sensitivities in extracts of fungi

Species	Superoxide dismutase	
	Activity (units/mg of protein)	Cyanide-sensitive activity (%)
Myxomycota (Slime molds)		
Acrasiomycetes		
Dictyosteliaceae		
Dictyostelium discoideum	1	43
*Dictyostelium mucoroides**	2	71
Eumycota		
Mastigomycotina		
Oomycetes		
Peronosporales		
Pythiaceae		
*Phytophthora capsici**	11	77
Pythium debaryanum	10	40
Pythium sp. CCR-76	34	78
Zygomycotina		
Zygomycetes		
Mucorales		
Mucoraceae		
Absidia lichteimi	4	34
Absidia orchidis	1	100
Mucor javanicus	2	49
*Mucor racemosus**	5	66
Rhizopus oryzae	1	82

Asterisk indicates the sample used for immunological cross-reaction.

Mastigomycotina (molds)

Mastigomycotina is considered to be the oldest fungi and suggested to have derived from algae in the course of evolutoin. Since most of algae lack Cu,Zn-SOD and contain only the cyanide-insensitive SOD, it was very interesting to know whether Mastigomycotina contain Cu,Zn-SOD. These organisms had both the Cu,Zn-and the cyanide-insensitive enzymes on the basis of the inhibition by cyanide (Table I-1).

Zygomycotina (molds)

No SOD has been isolated from Zygomycotina. Present survey indicates that this group also possesses both Cu,Zn- and the cyanide-insensitive SOD except Absidia orchidis which contained only the cyanide-sensitive Cu,Zn-SOD (Table I-1).

Table I-2 (continued)

Species	Activity	CN-sensitive activity
Ascomycotina		
Hemiascomycetes		
Endomycetales		
Endomycetaceae		
Endomyces decipiens *	27	67
Saccharomycetaceae		
Hansenula mrakii	4	35
Lipomyces starkeyi	17	49
Phichia rhodanensis	6	44
Saccharomyces cerevisiae	2	86
Saccharomyces sake	5	74
Schizosaccharomyces pombe	2	52
Schwanniomyces occidentalis	3	15
Kluyveromyces polysporus	22	79
Pyrenomycetes		
Sphaeriales		
Sordariaceae		
Neurospora crassa *	14	54
Neurospora sitophila	10	45
Hypocreaceae		
Gibberella fujikuroi	23	35
Plectomycetes		
Eurotiales		
Eurotiaceae		
Monascus anka	1	30
Discomycetes		
Pezizales		
Humariaceae		
Aleuria aurantia	1	87

Ascomycotina (molds, yeasts and mushrooms)

Cu,Zn-SOD from Neurospora crassa and from Saccharomyces cerevisiae have been purified and well characterized (1,2,4). Mn-SOD has been isolated from the mitochondria of yeast (6). The SOD activity and its cyanide-sensitivity in Ascomycotina are shown in Table I-2. The enzyme activity fell in a range of 1-27 units/mg protein and was inhibited by 15-87% varying from species to species. Thus, Ascomycotina contains both the Cu,Zn- and cyanide-insensitive SOD.

Table I-3 (continued)

Species	Activity[a]	CN-sensitive activity
Basidiomycotina		
Hymenomycetes		
Agaricales		
Tricholomataceae		
Flammulina velutipes	5	32
Laccaria laccata	5	78
Laccaria laccata var. *proxima**	6	86
Lyophyllum decastes	4	61
*Tricholoma matsutake**	9	46
Hygrophoraceae		
Camarophyllus pratensis	1	50
Cortinariaceae		
Cortinarius purpurascens	4	63
Rozites caperata	11	82
Coprinaceae		
Psathyrella velutina	2	32
Rhodophyllaceae		
Rhodophyllus sinuatus	8	55
Russulaceae		
Lactarius chrysorrheus	4	26
Russula emetica	2	51
Russula sanguinea	9	40
Aphyllophorales		
Polyporaceae		
*Coriolus versicolor**	3	60
Ganoderma lucidum	1	51
Gasteromycetes		
Lycoperdales		
Calostomataceae		
Calostoma japonicum	18	53

a) Assayed in NBT-xanthine oxidase system.

Basidiomycotina (mushrooms)

Mn-SOD, which was a tetramer and contained 1 g-atom of Mn/mol of enzyme, has been isolated from a luminescent mushroom, Pleurotus olearius by Lavelle et al. (5). They detected only the Mn-enzyme in this organism. Table I-3, however, shows the ubiquitous distribution of Cu,Zn-SOD in addition to the cyanide-insensitive enzyme among Basidiomycotina.

Table I-4 (continued)

Species	Activity	CN-sensitive activity
Deuteromycotina		
Blastomycetes		
Cryptococcales		
Cryptococcaceae		
Cryptococcus neoformans	3	53
Kloeckera magna	4	82
Torulopsis candida	5	27
Torulopsis colliculosa	1	55
Trichosporon cutaneum *	3	74
Rhodotorula flava	18	38
Rhodotorula rubra	14	27
Sporobolomycetales		
Sporobolomycetaceae		
Sporobolomyces coprophilus	9	28
Hyphomycetes		
Hyphomycetales		
Aspergillus oryzae *	5	53
Gliocladium deliquescens	1	46
Penicillium chrysogenum	3	50
Penicillium rubrum	2	30
Trichophyton mentagrophytes	3	41
Trichophyton tonsurans var. *sulfureum*	2	79
Verticillium albo-atrum	2	29
Stilbellales		
Stilbellaceae		
Isaria kogane	3	55
Tuberculariales		
Tuberculariaceae		
Fusarium lini	2	35

Deuteromycotina (molds and yeasts)

The classification of Deuteromycotina is artificial rather than phylogenetic, and these organisms essentially belong to Ascomycotina. As expected from the results of Ascomycotina and Basidiomycotina, Deuteromycotina contained both Cu,Zn-SOD and the cyanide-insensitive enzyme (Table I-4).

Table II

Activities of superoxide dismutases and their cyanide sensitivites in extracts of marine invertebrates

Species	Superoxide dismutase	
	Activity (units/mg of protein)	Cyanide-sensitive activity (%)
Porifera		
Halichondria okadai	9	68
Spirastrella insignis	28	100
Colelenterata		
Alcyonium gracillimum	2	56
Anthopleura japonica	5	87
Anthoplexaura dimorpha	2	71
Cladiella digitulata	4	52
Dendronephthya gigantea	3	48
Dendrophyllia arbuscula	87	83
Epizoanthus ramosus	12	79
Halocordyla disticha	21	45
Stereonephthya japonica	4	15
Stereonephthya rubriflora	3	33
Mollusca		
Aplysia sp.	6	86
Prepostera hyotis imbricata	4	61
Spondylus barbatus	10	62
Echinodermata		
Anthocidaris crassispina	16	86
Astropecten scoparium	4	81
Ehinometra mathaei	6	74
Holothuria leucospilota	3	88
Holothuria parvicax	2	85
Tripneustes gratilla	7	60
Arthropoda		
Pollicipes mitella	10	40
Tetraclita squamosa japonica	4	64
Pagurus sp.	1	30

Porifera, Colelenterata, Mollusca, Echinodermata and Arthropoda (marine invartebrates)

No SOD has been isolated from invertebrates except the Cu,Zn-SOD from a fruit fly, Drosophila melanogaster (11), although Cu,Zn- and Mn-SOD have been purified from many vertebrates (7). Table II indicates that all marine invertebrates at different level of evolution including Porifera, Colelenterata, Mollusca, Echinodermata ad Arthropoda contain both the Cu,Zn- and cyanide-insensitive SOD. Although the ratio of both the enzymes varied from species to species, the Cu,Zn-enzyme was a major form in marine invertebrates. Among invertebrates tested the cyanide-insensitive SOD was not detected in a Porifera, Spirastrella insignis, as in a mold, Absidia orchidia

Effects of anti-plant Cu,Zn-SOD serum

Antiserum against spinach stromal Cu,Zn-SOD cross-reacts with the stromal Cu,Zn-enzymes from land plants including ferns, mosses, and seed plants (Chapter II). Antiserum against the Cu,Zn-enzyme did not neither inhibit the enzymatic activity nor form precipitin in Ouchterlony double immunodiffusion test with the extracts from fungi and invertebrates tested (Table I and II). These resutls indicate that Cu,Zn-SOD of fungi and invertebrates are immunologically distinguished from the plant Cu,Zn-SOD. It has been also shown that bovine Cu,Zn-SOD is immunologically different from plant Cu,Zn-enzymes (Chapter II).

4. DISCUSSION

SOD was found in all fungi and invertebrates tested. This adds further evidence for its ubiquitous distribution in organisms to those obtained previously in photosynthetic organisms covering photosynthetic bacteria, blue-green algae, eukaryotic algae and land plants (Chapter II). The enzyme contents in fungi and invertebrates centered within 1-30 units/mg of protein, which comprize 0.1-0.5% of soluble protein assuming a specific activity of Cu,Zn-SOD of 7,000-8,000 units/mg of protein. A narrow variation of the enzynme level was also observed in photosynthetic orgnisms (Chapter II). Exceptionally, among invertebrates tested, Dendrophyllia arbuscula had the prominent activity (Table II). The ubiquitous occurrence and small variation of the SOD contents in fungi and invertebrates support a proposed role of the enzyme as a defense against oxygen toxicity (20).

All fungi and invertebrates contained Cu,Zn-SOD. The cyanide-insensitive SOD was also found in most fungi and invertebrates, but not detected in fungus, Absidia orchidis and a Porifera, Spirastrella insignis (Table I-1 and II). Although SOD activity in the extracts from Absidia orchidis and Spirastrella insignis were completely inhibited by cyanide, it might be safe to say that these organisms contain a small amount of the cyanide-insensitive enzyme, because the subcellular distribution study have revealed that the cyanide-insensitive Mn-SOD is ubiquitously localized in mitochondria matrix of eukaryotes (21). The failure of the detection of the cyanide-insensitive SOD is probably due to the incomplete extraction or unstability of the enzyme. It is well known that Mn-SOD less stable than the Cu,Zn-enzyme.

In the present experiments, the cyanide-insensitive SOD has not been characterized. Fe-SOD has not been isolated from fungi and animals whereas Mn-SOD was purified from several fungi and animals (7). Under these circumstances, it is reasonable to postulate that the cyanide-insensitive SOD in fungi and invertebrates is the Mn-enzyme.

Lavelle et al. isolated Mn-SOD from a mushroom, Pleutotus olearius, but did not detect any Cu,Zn-SOD in this organism (5). I tested the cyanide-sensitivity of SOD in extracts from five organisms which belong

to the same family, Tricholomataceae. The enzymatic activity was inhibited 32-86% by cyanide, indicating that these organisms contain both the Cu,Zn-enzyme and the cyanide-insensitive enzyme (Table I-3). It should be noted that the amount and type of Cu,Zn-SOD isozymes in higher plants differs depending on their age and tissues (Chapter V and VI).

In conclusion, ubiquitous distribution of Cu,Zn-SOD among invertebrates and fungi (present results), and land plants (Chapter II) indicates that the acquisition of Cu,Zn-SOD by ancestral organism might have occurred prior to the divergence of animals, fungi and plants in the course of evolution. Thus, evolutional distances of fruit fly (23), yeast (24,25), and spinach stromal (Chapter V) Cu,Zn-SOD based on the amino acid sequences are similar degree. Therefore, phragmoplast algal Cu,Zn-SOD (Chapter III) might be their ancestral protein.

5. SUMMARY

Cu,Zn-SOD and cyanide-insensitive Fe/Mn-SOD were systematically surveyed in 57 species of fungi including slime molds, molds, yeasts and mushrooms, and 24 species of marine invertebrates covering Porifera, Colelenterata, Mollusca, Echinodermata, and Arthropoda at the different levels of evolution. Cyanide-sensitive Cu,Zn-SOD activity was detected in all fungi and invertebrates tested. Ubiquitous distribution of the Cu,Zn-SOD among invertebrates, fungi and land plants suggest that the acquisition of this enzyme by ancestral organism might have occurred prior to the divergence of animals, fungi and plants in the course of evolution.

6. REFERENCES

(1) Weser, U., Fretzdorff, A., and Prinz, R. (1972) FEBS Lett. 27, 267-269.

(2) Goscin, S.A., and Fridovich, I. (1972) Biochim. Biophys. Acta 289, 276-283.

(3) Rapp, U., Adams, W.C., and Miller, R.W. (1973) Can. J. Biochem. 51, 158-171.

(4) Misra, H.P., and Fridovich, I. (1972) J. Biol. Chem. 247, 3410-3414.

(5) Puget, K., Lavelle, F., and Michelson, A.M. (1977) in Superoxide and Superoxide Dismutases (Michelson, A.M., McCord, J.M., and Fridovich, I., eds.), pp. 139-150, Academic Press, New York.

(6) Ravindranath, S.D., and Fridovich, I. (1975) J. Biol. Chem. 250, 6107-6112.

(7) Steinman, H.M. (1982) in Superoxide Dismutase (Oberley, L.W., ed.), pp. 11-68, CRC Press, Florida.

(8) Manwell, C. (1977) Comp. Biochem. Physiol. 58B, 331-338.

(9) Lin, A.L. (1979) Comp. Biochem. Physiol. 62B, 425-431.

(10) Wolf, G.H., Heyneman, R., and Decleir, W. (1981) Comp. Biochem. Physiol. 69B, 865-867.

(11) Lee, Y.M., Ayala, F.J., and Misra, H.P. (1981) J. Biol. Chem. 256, 8506-8509.

(12) Maeda, Y., and Takeuchi, I. (1969) Development 11, 232.

(13) Emerson, R. (1958) Mycologia 50, 589-621.

(14) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(15) Kanematsu, S., and Asada, K. (1978) Arch. Biochem. Biophys. 185, 473-482.

(16) Kanematsu, S., and Asada, K. (1978) FEBS Lett. 91, 94-98.

(17) Kanematsu, S., and Asada, K. (1979) Arch. Biochem. Biophys. 195, 535-545.

(18) Asada, K., Urano, M., and Takahashi, M. (1973) Eur. J. Biochem. 36, 257-266.

(19) Asada, K., Takahashi, M., and Nagate, M. (1974) Agric. Biol. Chem.

38, 471-473.
(20) Fridovich, I. (1975) Annu. Rev. Biochem. 44, 147-159.
(21) Weisiger, R.A., and Fridovich, I. (1973) J. Biol. Chem. 248, 3582-3592.
(22) Weisiger, R.A., and Fridovich, I. (1973) J. Biol. Chem. 248, 4793-4796.
(23) Lee, Y.M., Friedman, D.J., and Ayala, F.J. (1985) Proc. Natl. Acad. USA 82, 824-828.
(24) Johansen, J.T., Overballe-Petersen, C., Martin, B., Hasemann, V. and Svendsen, I. (1979) Carlsberg Res. Commun. 44, 201-217.
(25) Steinman, H.M. (1980) J. Biol. Chem. 255, 6758-6765.

CHAPTER V

NH_2-TERMINAL SEQUENCE OF CYTOSOLIC AND STROMAL Cu,Zn-SUPEROXIDE DISMUTASE ISOZYMES FROM SPINACH

1. INTRODUCTION

Superoxide dismutase (SOD) which catalyzes the disproportionation of the superoxide anion radicals to molecular oxygen and hydrogen peroxide constitutes a defense system against deleterious action of the superoxide with catalase and peroxidase (1). In plant, the superoxide radical is produced mainly in chloroplasts under illumination (2,3). Since the production of superoxide in chloroplast is inevitable, chloroplast possesses elaborate defense system against photooxidative damage consisted of superoxide dismutase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase (4). Chloroplasts contain Cu,Zn-SOD in stroma (5,6), and mitochondria also this type of SOD (7). However, it is not clear whether chloroplast Cu,Zn-SOD is identical to mitochondrial Cu,Zn-SOD.

Isozymes of Cu,Zn-SOD have been found in many higher plants, and purified and characterized from wheat (8) and maize (9,10). Beauchamp and Fridovich indicated that two Cu,Zn-SOD isozymes from wheat are different in respect of molecular weight, stability against heat and sensitivity to hydrogen peroxide (8). Their amino acid compositions showed that they were neither charge variant nor the product due to point mutation. Furthermore, Baum et al. showed that antiserum against one of the isozymes in maize did not cross-react with another (10), although the antiserum against Cu,Zn-SOD from spinach leaves cross-reacted with SOD in the extracts from plants covering angiosperms, gymnosperms, ferns, and mosses (11). These observations suggested that at least two Cu,Zn-SOD isozymes occur in plants, and prompted me to isolate Cu,Zn-SOD isozymes and characterize further from spinach in which the Cu,Zn-SOD from leaf tissues have been isolated (5). I found

three Cu,Zn-SOD isozymes in spinach seeeds, and two of them were purified to a homogeneous state. One of them was the stromal Cu,Zn-SOD similar to the enzyme isolated from the leaves (5), but the other one was distinguished immunologically and physicochemically from the stromal Cu,Zn-SOD. The NH_2-terminal sequence of two Cu,Zn-SOD are also different. Here, I describe the physicochemical and immunological properties of Cu,Zn-SOD isozymes with NH_2-terminal sequence.

2. MATERIALS AND METHODS

Materials

Cytochrome c (horse heart, type III) and DFP-treated carboxypeptidase-A were obtained from Sigma, xanthine oxidase (bovine milk) from Boehringer, and Percoll and standard proteins for isoelectric point and molecular weight were form Pharmacia. Ampholine was obtained from LKB, EDAE Affi-Gel Blue and Chelex 100 from Bio-Rad, polyamide sheets from Schleicher and Schuell, silica gel plates (G-60, without fluorescent indicator, 0.25 mm) from Merck, N-ethyl morpholine from Pierce, 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate (DABITC) from Dojin and phenylisothiocyanate (PITC) form Wako. Organic solvents for sequencing were a sequencial grade or the highest grade available.

Plant

Spinach seeds (Spinacia oleracea L. cv. King of Denmark) were obtained from Takii Seeds Co., LTD, Kyoto, Japan. Spinach was water-cultured using the medium of Walker (12) in a glass house, or obtained from a local market. Etiolated seedlings were obtained by a culture on a moist varmuculite for a week under darkness.

Isolation of intact chloroplasts

Intact chloroplasts were isolated from three-week old spinach leaves at 4°C. The leaves (30 g) were homogenized using a Polytron blender for 2 sec at the setting of maximum power in 120 ml of the medium containing 0.33 M D-sorbitol, 1 mM $MgCl_2$, 1 mM EDTA, 10 mM NaCl,

0.2% BSA and 50 mM HEPES-NaOH, pH 7.6. The homogenate was filtered through four layers of gauze and centrifuged at 2,500 g for 70 sec. The pellets were suspended in the isolation medium and centrifuged at 500 g for 1 min to remove cell debris. The supernatant was further centrifuged at 1,700 g for 2 min, and the chloroplasts were resuspended in the isolation medium. The chloroplasts (2 ml) were further purified in Percoll by discontinuous gradient centrifugation at 7,700 g for 15 min using Sorval SS-34 rotor (9 ml of 85%, 9 ml of 65%, 12 ml of 45%, and 9 ml of 20% Percoll, each containing 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM EDTA, 1% BSA and 50 mM HEPES-NaOH, pH 7.6). After the centrifugation, the intact chloroplast fraction at the interface between 65% and 85% Percoll was collected, diluted 4-fold with the isolation medium omitting BSA (washing medium), and sedimented at 1,700 g for 2 min. The pellet was then resuspended in the washing medium and washed once by centrifugation. The intact chloroplasts thus obtained were free from mitochondria as judged from negligible cytochrome c oxidase activity. The chloroplasts were ruptured osmotically by resuspending in 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA for 1 hour. Stroma fraction was obtained by centrifugation at 27,000 g for 30 min, dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA, concentrated by ultrafiltration using an Amicon YM 5 membrane.

Purification of "stromal" Cu,Zn-SOD from leaves

Cu,Zn-SOD was purified from spinach leaves using acetone and ammonium sulfate fractionations, DEAE-Sephadex chromatography and Sephadex G-100 gel filtration, and crystallized by ammonium sulfate (5). I found that by omitting acetone fractionation, the enzyme having higher specific activity than the previous preparation (3100 units/mg protein) was obtained. The outline of purification for Cu,Zn-SOD from spinach leaves is as follows: Spinach leaves (10 kg) were homogenized with 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA using an electric chopper. The leaf extract was obtained by centrifugation after squeezing the homogenate through 2 layers of gauze. The extract was fractionated by ammonium sulfate between 45-80% saturation. The precipitate formed at 45% saturation was removed by filtration through a filter

paper with an aid of Hyflo Super-Cell and Whatman fibrous CF11 cellulose powder, and the precipitate at 80% saturation was collected by centrifugation after removal of the supernatant by decantation.

The enzyme was further purified by the elution with 100 mM potassium phosphate, pH 7.8, on DEAE-Sephadex, and a linear gradient elution of potassium phosphate (10 to 100 mM) on DEAE-Sephacel. After gel filtration through Sephadex G-100, the enzyme was purified to homogeneity by passing through Bio-Gel HTP as described for SOD-I purification. The purified enzyme had a specific activity of about 8,000 units/mg protein.

Preparation of monospecific antibodies

Monospecific antibodies to purified SOD-I and -II were raised in white rabbits. Purified SOD-I and -II (1 mg each) in 1 ml of 10 mM potassium phosphate, pH 7.8, were emulsified with 1 ml of complete Freund's adjuvant and the emulsion was injected into multiple sites of the back of rabbit. The injection (0.5-1 mg enzymes) was repeated three times more at an interval of week. The rabbits were bled a week after the last injection, and monthly thereafter. Booster injection was given to rabbits which decreased in their specific antibody titer. IgG fractions were obtained by passing through a column of DEAE Affi-Gel Blue according to the manufacture's manual (Catalog No. 153-7307) after ammonium sulfate precipitation at 50% saturation. The IgG fractions were dialyzed against 10 mM potassium phosphate, pH 7.8, and 0.15 M NaCl, and stored at -80°C. Ouchterloy double immunodiffusion was conducted in a 1.5% agar plate containing 0.15 M NaCl, 20 mM potassium phosphate, pH 7.8, and 0.02% NaN_3 at 37°C for 9-12 hours. Crossed-immunoelectrophoresis using a polyacrylamide gel slab in the first direction and an agrose gel containing antibody in the second direction was performed essentially by the method of Ludahl and Liljas (13).

SOD assay and protein measurement

SOD was assayed by the procedure of McCord and Fridovich (14) with a slight modification (15). One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction of cytochrome c by

50%. Since my reaction volume was 1 ml compared with 3 ml of the McCord and Fridovich method, the activity is divided by three and presented by the McCord-Fridovich unit. Protein was determined according to Lowry et al. (16) using bovine serum albumin as a standard, but the purified enzyme was by $A^{1\%}_{1\ cm}$ at 258 nm.

Electrophoresis and isoelectric focusing

Native polyacrylamide gel electrophoresis was performed by the method of Davis (17) using a 1 mm-thick gel slab (9 cm x 13.5 cm) at 4°C with a constant current of 25 mA per gel slab. The gel (7.5%) was polymerized by ammonium persulfate one day before use. Proteins were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol and 7% acetic acid for 1 hour.

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (18) using a 15% gel slab (1 mm-thick). The enzyme was sealed in a small glass tube with 5% SDS in the presence or absence of 5% 2-mercaptoethanol, and denatured at 100°C for 3 min. Electrophoresis was carried out at room temperature with a constant voltage of 100 V per gel slab. The following molecular weight standards were used: phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,4000).

Isoelectric focusing was carried out with 2% Ampholine (pH 3.5-10) on 5% polyacrylamide gel using a vertical mini-slab gel apparatus (5 x 9 cm) for electrophoresis. The septums of sample wells which directly connected with Ampholine-containing polyacrylamide gel were made by 15% polyacrylamide gel containing 0.02 M phosphoric acid to avoid a curvature of protein band near the cathode. Focusing was conducted at 4°C at a constant voltage of 200 V for 4.5 hours with phosphoric acid and NaOH as the cathodic and anodic electrolytes. The pI standards were amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin-acidic band (6.85), horse myoglobin-basic band (7.35), lentil lectin-acidic band (8.15), lentil lectin-middle band (8.45), lentil lectin-basic band (8.65) and trypsinogen (9.30). Protein was stained with 0.1% Coomassie brilliant blue G-250 in

25% methanol and 5% acetic acid after fixing with 10% trichloroacetic acid and 5% sulfosalicylic acid for 1 hour.

SOD activity on the gel for native electrophoresis and isoelectric focusing was located by the photochemical method of Beauchamp and Fridovich (19) with a slight modification. After electrophoresis, the gel plate was washed in 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA with several chenges of buffer for 5 min. The gel was soaked in 2.5 mM NBT for 7 min, and then immersed in 100 μM riboflavin containing 30 mM TEMED for 5 min. The NBT and riboflavin solutions contained 50 mM potassium phosphate, pH 7.8, and 0.5 mM EDTA. For characterization of the types of SOD, the gel was treated by cyanide or hydrogen peroxide. Cu,Zn-SOD is sensitive to cyanide, but Mn-SOD and Fe-SOD are not (1). Hydrogen peroxide inactivates Cu,Zn-SOD and Fe-SOD, but not Mn-SOD (20). For cyanide treatment, 2 mM potassium cyanide was added in the riboflavin solution. For H_2O_2 treatment, the gel was soaked in 3 mM H_2O_2 containing 50 mM potassium phosphate, pH 7.8, and 0.5 mM EDTA for 30 min after washing with the buffer.

Molecular weight determination

Molecular weight of the enzymes were determined by gel filtration using a Sephadex G-100 column (2.5 x 85 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, and 0.1 M KCl. The column was calibrated with BSA (67,000), ovalbumin (43,000), bovine Cu,Zn-SOD (31,000), α-chymotrypsinogen A (25,000), myoglobin (17,800), and cytochrome c (12,400).

The molecular weight of the enzymes were also determined by the sedimentation equilibrium according to Yphantis (21) using a Hitachi UCA-1A analytical ultracentrifuge equipped with an interference optics. Purified enzyme at 0.2, 0.5, 0.8 mg/ml in 5 mM potassium phosphate, pH 7.8, containing 0.1 M KCl were equilibrated using a three-column double sectered cell at 28,400 rpm, 20°C. Partial specific volume of the enzymes were calculated from the amino acid composition (Table II) by the method of Cohn and Edsall (22).

Spectroscopy

Optical spectra were taken using a Shimadzu MPS-2000 spectrophotometer, and circular dichroism (CD) spectra were recorded using a JASCO J-500C spectropolarimeter equipped with a data processor Model DP-501. In ultraviolet region, the ellipticity was expressed in terms of mean residue ellipticity, $[\theta]_R$, which was based on the mean amino acid residue weights of 101 (SOD-I) and 103 (SOD-II), and in visible region, molar ellipticity, $[\theta]_M$, with molecular weights of 30,800 (SOD-I) and 31,800 (SOD-II). The contents of α-helix were estimated using the method of Chen and Yang (23).

Metal analysis

The metal contents of the enzymes were determined using a Hitachi Perkin-Elmer 303 atomic absorption spectrophotometer with a flame-less graphite atomizer. The enzymes were dialyzed against 5 mM Tris-HCl, pH 7.8, which was passed through a Chelex 100 column to remove metals. Aliquot (10 µl) of the samples was directly atomized in a graphite atomizer.

Amino acid analysis

The enzyme in duplicate was hydrolyzed in 6 M HCl in a evacuated and sealed tube for 24, 48, 72 hours at 110°C after dialysis against distilled water and then subjected to a Hitachi 835 amino acid analyzer. Half-cystine and methionine were determind as cysteic acid and methionine sulfone after performic acid oxidation followed by hydrolysis in 6 M HCl for 18 hours according to Moore (24). Tryptophan was estimated by the method of Edelhoch (25). The loss or increase in amino acid during hydrolysis were corrected by extrapolation to zero time or taking the values at 72 hours.

Sequencing

Apoenzyme was prepared by the procedure of McCord and Fridovich (14,26) with a slight modification. SOD-I was dialyzed against 50 mM sodium acetate, pH 3.8, 10 mM EDTA at 4°C for one day, and then against distilled water, and freeze-dried. For carboxymethylation the apoenzyme

(2.3 mg) was reduced for 4 hours at 25°C, with 45 μmol of 2-mercapto-ethanol in 1 ml of 6 M guanidine-HCl and 0.2 M Tris-HCl buffer, pH 8.5. Iodoacetic acid (42 μmol in 50 μl of 1 M NaOH) was then added, and after 10 min, during which pH changed from 8.0 to 8.5, the reaction was quenched by the addition of 45 μmol of 2-mercaptoethanol. The alkylated SOD-I was dialyzed against distilled water, and freeze-dried.

The NH_2-terminal sequence was determined manuallly by the DABITC/PITC double coupling method of Chang (27). Ten nmol of carboxy-methylated SOD-I was dissoloved in 80 μl of 50% pyridine. The enzyme was reacted with 400 nmol of DABITC in 40 μl of pyridine for 50 min and then with 10 μl of PITC for 15 min at 52°C. DABTH-amino acid derivatives were identified by TLC of polyamide sheets (2.5 x 2.5 cm) after exposure to HCl vapor (28). Leucine and isoleucine were identified by TLC of silica gel (29).

The COOH-terminal sequence was determined by the time-dependent liberation of amino acid with carboxypeptidase A. The carboxymethylated SOD-I (800 μg) was incubated with 35 μg of carboxypeptidase A in 0.2 M N-ethylmorpholine-acetate buffer, pH 8.5, at 37°C. Aliquot (corresponding to 10 nmol of the enzyme) was withdrawn at intervals, and the reaction was quenched by the addition of 10 μl of 6 M HCl. The supernatant obtained by centrifugation was freeze-dried, and amino acids were analyzed by a amino acid analyzer.

3. RESULTS

Isozymes

It has been shown that in spinach leaves Cu,Zn-SOD is localized in chloroplast stroma (5). I found electrophoretically distinct Cu,Zn-SOD isozymes in spinach leaves and seeds (Fig. 1). The seeds contained one major Cu,Zn-SOD (fastest moving band) and minor two Cu,Zn-SOD (middle two bands) as well as Mn-SOD (slowest band), while the leaf buffer extract showed two Cu,Zn-SOD isozymes which have the same mobility as those of seed Cu,Zn-SOD isozymes. I refer the bands of Cu,Zn-SOD isozymes as Cu,Zn-SOD-I, -II, and -III from anodic side. Cu,Zn-SOD-III and Mn-SOD

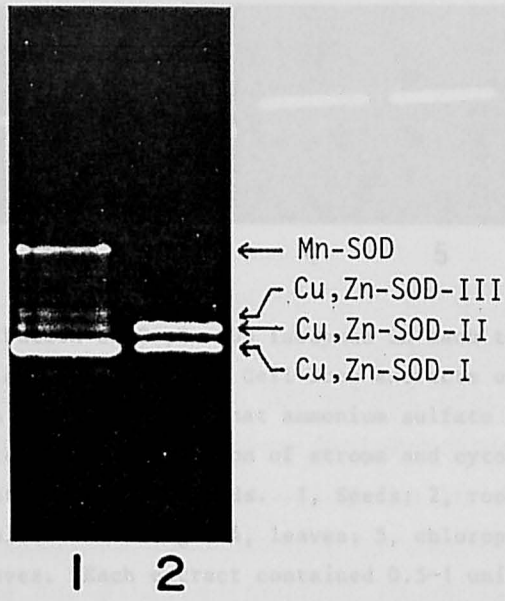

Fig. 1. SOD isozyme patterns of spinach seeds and leaves revealed by native-polyacrylamide gel electrophoresis. Cell-free extracts of spinach seeds and leaves were prepared by homogenizing with 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA. The homogenate clarified by centrifugation was fractionated by ammonium sulfate (30-80%). The precipitate obtained by centrifugation was dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. 1, Seeds (1.9 units); 2, leaves (1.1 units). Electrophoresis and activity staining were described in Materials and Methods.

activity bands were also found in the leaves, when a large amount of the extract was subjected to the electrophoresis. The "stromal" Cu,Zn-SOD purified previously from the leaves (5) coincided with the band of Cu,Zn-SOD-II (Fig. 1). Thus, SOD-I is a major isozyme in the seeds and SOD-II is a major one in the leaves.

The change of isozyme pattern of Cu,Zn-SOD during germinaiton and growth under the dark and light condition was followed electrophoretically. In the 6-day old etiolated seedlings, isozyme pattern was almost identical to that of seed; SOD-I was major, and SOD-II was minor. Non-green tissues such as roots, etiolated hypocotyls also showed the similar isozyme pattern, indicating that SOD-I is characteristic to non-green tissues. (Fig. 2) Under the light SOD-II band increased progressively for 3-4 days after the germination with the concomitant increase

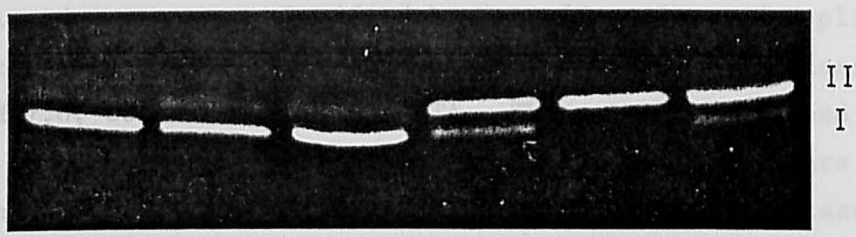

Fig. 2. Distribution of Cu,Zn-SOD isozymes in each tissue of spinach and their subcellular distribution. Cell-free extracts of each tissue were prepared as in Fig. 1, except that ammonium sulfate fractionation (0-80%) was conducted. Fractionation of stroma and cytosol from leaves was described in Materials and Methods. 1, Seeds; 2, roots; 3, hypocotyls from 6-day old etiolated seedlings; 4, leaves; 5, chloroplast stroma; 6, cytosol from leaves. Each extract contained 0.5-1 unit of SOD. I, Cu,Zn-SOD-I; II, Cu,Zn-SOD-II.

of chlorophyll, while SOD-I was gradually decreased (data not shown). These results indicate that biosynthesis of SOD-II is correltate with the biogenesis of chloroplasts.

The stroma prepared from the Parcoll-purified intact chloroplasts contained SOD-II but lacked SOD-I. SOD-I was detected in cytosol fraction. (Fig. 2). Thus, SOD-II is a stromal enzyme and SOD-I may be a cytosolic enzyme.

Purification of Cu,Zn-SOD isozymes from spinach seeds

Spinach seeds (10 kg) were homogenized in 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA using an electric mortar for 1.5 hours with sea sands (one batch, 300 g seeds in 1.5 liters buffer). The homogenate was successively squeezed through one layer, and then four layers of gauze.

Ammonium sulfate was added to the extract to 30% saturation. The pH was maintained at 7.8 by adding ammonia. After removal of insoluble materials by centrifugation at 11,000 g for 30 min, ammonium sulfate was added to the supernatant to 80% saturation. The precipitate collected by centrifugation at 11,000 g for 30 min, was dissolved in and dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA.

The dialyzed enzyme was clarified by centrifugation and applied to a DEAE-Sephacel column (10 x 22 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. The column was washed with 15 liters of 10 mM potassium phosphate, and eluted wiht 10 liters of 50 mM, and 200 mM KCl and then 5 liters of 500 mM KCl in 50 mM potassium phosphate, pH 7.8. All buffers contained 0.1 mM EDTA. Washing, 50 mM, 200 mM, and 500 mM KCl fractions contained 5.8, 83.6, 10.0 and 0.7% of SOD activity, respectively. The SOD in washing and 50 mM KCl fractions were cyanide-sensitive Cu,Zn-SOD. The 200 mM KCl fraction contained one third of Cu,Zn-SOD and two thirds of cyanide-insensitive SOD. Further purification of Cu,Zn-SOD was conducted using the 50 mM KCl fraction.

The 50 mM KCl fraction was concentrated by ultrafiltration through an Amicon PM 10 membrane and dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. The concentrated enzyme was applied to a DEAE-Sephacel column (2.5 x 23 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. After washing the column with 3 liters of the equilibrating buffer, the enzyme was eluted by 3 liters of a linear gradient of KCl (0 to 80 mM) in 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. Cu,Zn-SOD-II and -I were eluted at 25 mM and 40 mM KCl (Fig. 3). Each fraction was pooled and concentrated by ultrafiltration.

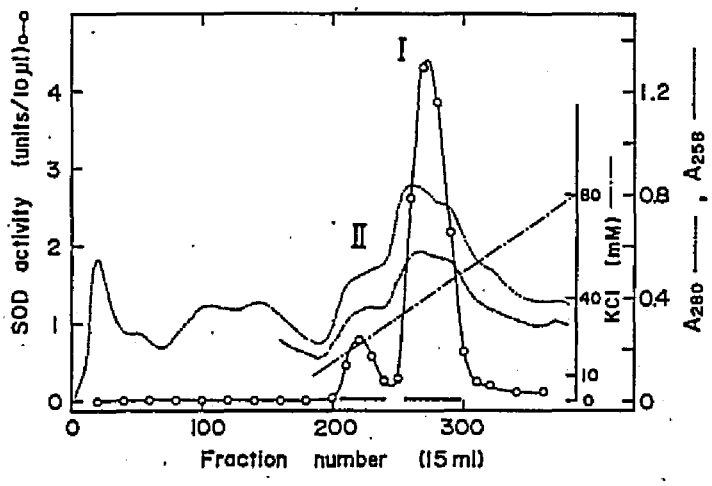

Fig. 3. Linear gradient chromatography of SOD isozymes from spinach seeds on DEAE-Sephacel. I, Cu,Zn-SOD-I; II, Cu,Zn-SOD-II. Details are given in the text.

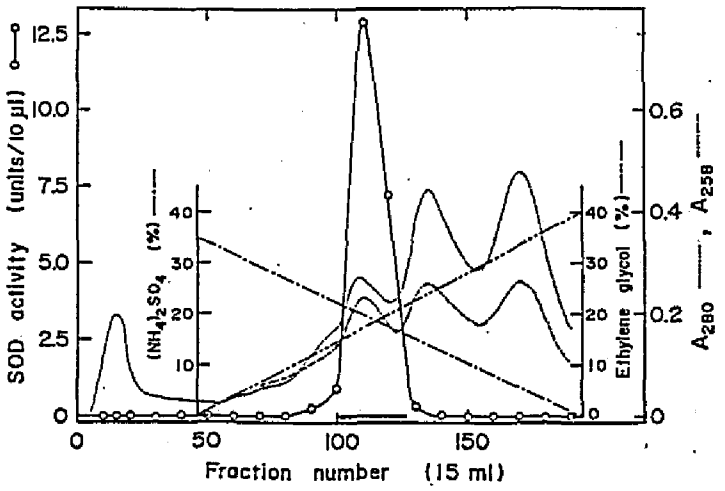

Fig. 4. Phenyl-Sepharoase hydrophobic chromatography of Cu,Zn-SOD-I. Details are given in the text.

Ammonium sulfate was added to 35% saturation to Cu,Zn-SOD-I and -II fractions and the enzymes were subjected to Phenyl-Sepharose hydrophobic chromatography. Cu,Zn-SOD-I was applied to a Phenyl-Sepharose column (4 x 7 cm) equilibrated with 35% ammonium sulfate in 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. After wahing with the starting buffer, the enzyme was eluted by a simultaneous cross linear gradient of ammonium sulfate (35 to 0%) and ethylene glycol (0 to 40%) in 1 liter each of 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. SOD-I was eluted at 20 % ammonium sulfate and 18 % ethylene glycol (Fig. 4). Cu,Zn-SOD-II was also purified by the similar procedure as above on Phenyl-Sepharose column (2.5 x 10 cm) using a linear gradient of ammonium sulfate (35 to 0%) and ethylene glycol (0 to 40%) in 750 ml each of 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. SOD-II appeared at 22% ammonium sulfate and 13% ethylene glycol. Each fraction was pooled and concentrated by ultrafiltration.

The concentrated enzymes (SOD-I, 20 ml; SOD-II, 10ml) were separately gel-filtered through Sephadex G-100 column (4 x 86 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, containing 0.1 M KCl using the equilibrating buffer as an elution buffer. At this step, the absorbance at 258 nm of Cu,Zn-SOD-II was higher than that at 280 nm. The active fractions were pooled and concentrated by ultrafiltration

during which the buffer was changed to 5 mM potassium phosphate, pH 7.8.

Cu,Zn-SOD-II was finally purified by passing through a column of Bio-Gel HTP (1.5 x 1.5 cm) equilibrated with 5 mM potassium phosphate, pH 7.8. SOD-II was not adsorbed, and obtained from washings with a high yield. SOD-I was also passed through a Bio-Gel HTP column (1.5 x 7 cm) equilibrated with the same buffer to remove a pink pigment exhibiting at 550 nm.

The concentrated Cu,Zn-SOD-I was adsorbed onto DEAE-Sephacel column (4 x 8 cm) equilibrated with 10 mM potassium phosphate, pH 7.8. The column was washed with 500 ml of the equilibrating buffer containing 10 mM KCl, and then eluted with a linear gradient of KCl (10 to 80 mM, 2 liters) in 10 mM potassium phosphate, pH 7.8. The active fractions having a constant absorbance ratio at 258 and 280 nm were pooled an concentrated. The purification of Cu,Zn-SOD-I and -II is summarized in Table I. Both isozymes had a specific activity of 8,000 units/mg protein.

Table I

Summary of the Purification of Cu,Zn-SOD Isozymes from Spinach Seeds

Purification step	Total protein (mg)	Total activity[a] (10^{-3} units)	Specific activity (units/mg protein)	Yield (%)	Purification (n-fold)
Homogenate	105,000	613	6	100	1
30 to 80% $(NH_4)_2SO_4$	38,700	570	15	93	2.5
1st DEAE-Sephacel	3,400	457	134	75	22
2nd DEAE-Sephacel					
SOD-I[c]	506	414	818	68	136
SOD-II	279	40	143	7	24
Phenyl-Sepharose					
SOD-I	105.0	391	3,724	64	620
SOD-II	10.1	37	3,663	6	611
Sephadex G-100					
SOD-I	71.6	331	4,623	54	771
SOD-II	7.0	37	5,286	6	881
Bio-Gel HTP					
SOD-I	60.0[b]	325	5,417	53	903
SOD-II	4.4[b]	36.4	8,273	6	1379
3rd DEAE-Sephacel					
SOD-I	32.1[b]	270.5	8,427	44	1405

a) McCord and Fridovich unit (14).

b) Protein was measured by the method of Lowry et al. (16) and in the final step the enzymes were determined using an $A_{1\ cm}^{1\%}$ at 258 nm of 4.20 and 4.08 for SOD-I and -II, respectively.

c) SOD-I, Cu,Zn-SOD-I; SOD-II, Cu,Zn-SOD-II.

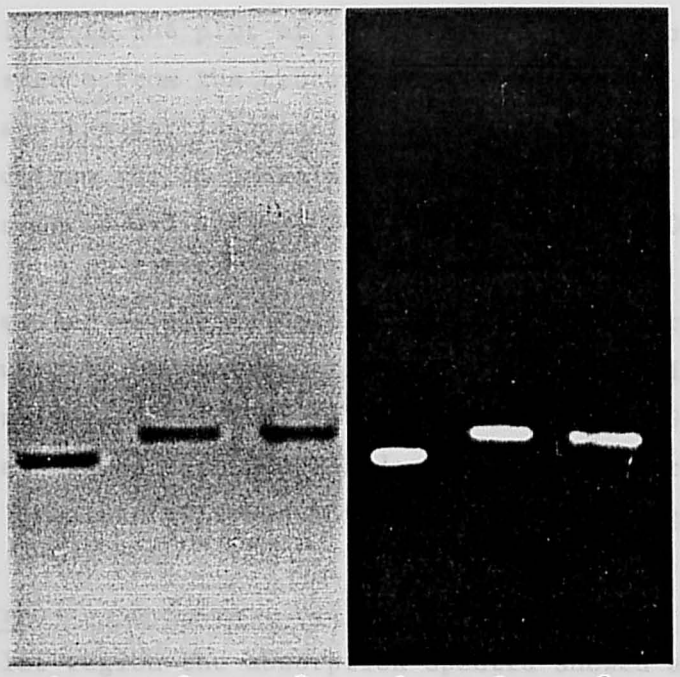

Fig. 5. Polyacrylamide gel electrophoresis of the purified spinach Cu,Zn-SOD isozymes. Left panel, protein staining (each 3 μg); right panel, activity staining (each 6 units). 1, Cu,Zn-SOD-I; 2, Cu,Zn-SOD-II; 3, leaf "stromal" Cu,Zn-SOD.

Purity of the enzyme

Both Cu,Zn-SOD-I and -II gave a single protein band on native polyacrylamide gel (Fig. 5), SDS-polyacrylamide gel, and isoelectric focusing gel. Protein band of SOD-I and -II on native polyacrylamide gel coincided with SOD activity band of purified enzyems and of crude extract, indicating no modification of the enzymes during the purification (Fig. 5)

The protein band of seed Cu,Zn-SOD-II was coincided with that of leaf "stromal" Cu,Zn-SOD (Fig. 5). Since the present preparation of Cu,Zn-SOD-II had a higher specific activity than that of previous preparation (5), Cu,Zn-SOD-II was characterized in parallel with Cu,Zn-SOD-I.

Molecular weight and subunit structure

Analytical ultracentrifugation for molecular weight determination

gave a straight line in the plot of log(fringe displacement) against square of the distance from the center of rotation. From the slopes of the above plots and the partial specific volumes (SOD-I, 0.721; SOD-II, 0.727) calculated from the amino acid compositions (Table II), the molecular weights for SOD-I and -II were 30,800 and 31,800, respectively.

Subunit molecular weight as determined by SDS-polyacrylamide gel electrophoresis were 17,000 for SOD-I and 20,000 for SOD-II in the presence of 2-mercaptoethanol, and 17,000 for SOD-I and 19,000 for SOD-II in its absence. Thus, both SOD-I and -II are homodimers and the subunits are not covalently linked through disulfide bridge.

Absorption spectra

In ultraviolet region the absorption spectra showed a difference between Cu,Zn-SOD-I and -II (Fig. 6). SOD-I exhibits absorption shoulder around 275 nm indicating the presence of tyrosine in addition to the peaks (252, 258, and 264 nm) characteristic to phenylalanine. SOD-II lacked absorption at 275 nm but showed the peaks due to phenylalanine. The molar absorbance coefficient and $A^{1\%}_{1\,cm}$ at 258 nm were 12,900 $M^{-1}cm^{-1}$ and 4.20 for SOD-I, and 12,900 $M^{-1}cm^{-1}$ and 4.08 for SOD-II. The spectra in visible region showed absorption peak at 670 nm for SOD-I, and 680 nm for SOD-II, with a molar absorbance coefficient of 422 $M^{-1}cm^{-1}$ and 476 $M^{-1}cm^{-1}$.

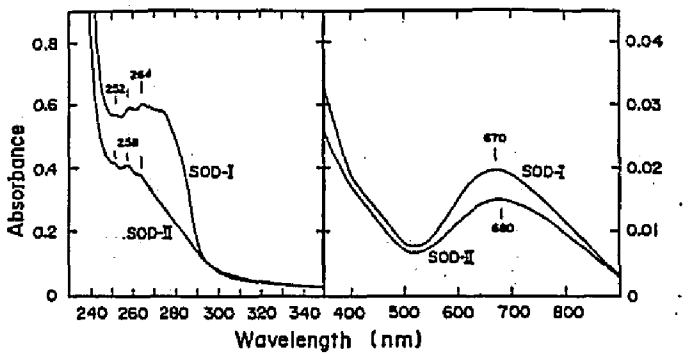

Fig. 6. Absorption spectra of spinach Cu,Zn-SOD isozymes. Spectra were recorded at a concentraiton of 1.39 mg/ml (SOD-I), and 1.00 mg/ml (SOD-II) in 10 mM potassium phosphate, pH 7.8, at 25°C. The light path was 10 mm. SOD-I, Cu,Zn-SOD-I; SOD-II, Cu,Zn-SOD-II.

Metal analysis

SOD-I contained 2.13 g-atoms of Cu and 1.98 g-atoms of Zn, and SOD-II 2.08 g-atoms of Cu and 1.94 g-atoms of Zn per mol of enzyme. Iron and manganese were not detected or lower than the limit of determination.

Amino acid compositions

Table II shows the amino acid compositions of spinach seed Cu,Zn-SOD-I and -II as well as the leaf "stromal" Cu,Zn-SOD (5). The amino acid compositions of seed Cu,Zn-SOD-II and leaf "stromal" Cu,Zn-SOD were identical within experimental error. Cu,Zn-SOD-I and -II were composed

Table II

Amino Acid Compositions of Spinach Superoxide Dismutase Isozymes

The number of residues of Cu,Zn-SOD-I was calculated for 30,800 g of the enzyme. The correction was made for metals (2 Cu and 2 Zn) and two water at terminals. The number of residues of Cu,Zn-SOD-II was calculated for a molecular weight of 31,381 which was obtained from the sequence data for spinach "stromal" Cu,Zn-SOD.

Amino acid	Cu,Zn-SOD-I		Cu,Zn-SOD-II		"Stromal" Cu,Zn-SOD[e]
	Number of residues	Nearest integer	Number of residues	Nearest integer	Sequence
Asp	34.76	35	37.88	38	38[a]
Thr[c]	25.86	26	31.18	31	32
Ser[c]	20.33	20	13.58	14	10
Glu	19.00	19	22.93	23	22[b]
Pro	16.55	17	17.14	17	18
Gly	60.41	60	46.35	46	46
Ala	20.40	20	24.39	24	24
1/2Cys	3.77	4	4.14	4	4
Val[d]	30.12	30	33.25	33	36
Met	1.58	2	1.66	2	2
Ile[d]	13.08	13	8.72	9	8
Leu	18.74	19	24.58	25	24
Tyr	2.28	2	-	0	0
Phe	6.41	6	6.13	6	6
Lys	8.58	9	14.18	14	14
His	15.87	16	14.41	14	16
Arg	8.10	8	8.19	8	8
Trp	-	0	-	0	0
Total		306		308	308

a) Aspartic acid + asparagine. b) Glutamic acid + Glutamine.

c) 0-time. d) 72 hours hydrolysis.

e) Kitagawa et al. (personal communication).

of almost the same numbers of amino acid residues. SOD-I contained two residues of tyrosine as deduced from its UV absorption spectrum (Fig. 6), but SOD-II lacked tyrosine. Both enzymes did not contain tryptophan. Significant difference in the contents of serine, glycine and leucine was found between SOD-I and -II, indicating that they are products of different genes.

The difference in the contents of serine, glycine and leucine has been also observed in Cu,Zn-SOD isozymes of maize (10) and wheat (7). Higher contents of serine and glycine and lower content of leucine of cytosolic spinach SOD-I are found in maize SOD-2 and -4, and wheat isozyme I. Lower contents of serine and glycine and higher content of leucine of stromal Cu,Zn-SOD-II are found in maize SOD-1 and wheat isozyme II. The difference in specific amino acid residues among Cu,Zn-SOD isozymes from angiosperms suggests an early divergence of cytosolic and stromal Cu,Zn-SOD from an ancestral protein.

Isoelectric points

Isoelectric points of Cu,Zn-SOD-I and -II were 4.7 and 5.2, respectively.

NH_2- and COOH-terminal sequences of SOD-I

Amino acid sequences of Cu,Zn-SOD have been determined for the enzymes from bovine (30), human (31,32,33), horse (34), yeast (35,36), swordfish (37), fruit fly (38), and bacteria (39). Kitagawa et al. have determined the sequence of spinach leaf "stromal" Cu,Zn-SOD (i.e. Cu,Zn-SOD-II) (personal communication). I determined the NH_2-terminal sequence of spinach Cu,Zn-SOD-I using the micro-sequencing method. The NH_2-terminal sequence of SOD-I upto 21 residues and those of spinach SOD-II, and bovine, human, horse, and yeast Cu,Zn-SOD are shown in Fig. 7. The position number is based on spinach SOD-II. It is evident that spinach SOD-I is evolutionally related to other Cu,Zn-SOD, since positions 5, 6, 8, 9, 15, and 17 were common among these Cu,Zn-SOD. In addition, positions 4, 19, and 23 were shared by both spinach Cu,Zn-SOD isozymes. From X-ray diffraction data (40,41), the residues from position 1 to 11, and from 15 to 23 form first and second β-strands

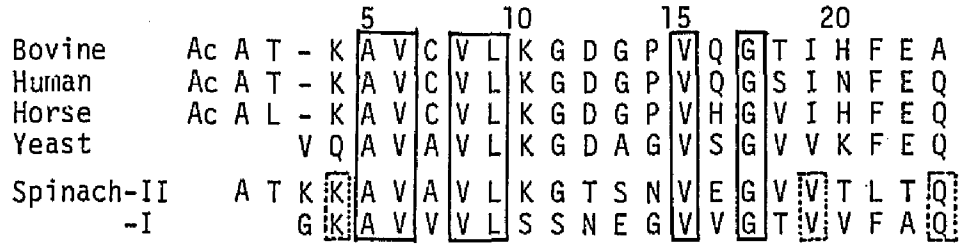

Fig. 7. NH_2-terminal sequence of spinach Cu,Zn-SOD-I. The sequence (21 residues) of spinach SOD-I was aligned with those of bovine (30), human (31,32), horse (34), yeast (35,36) Cu,Zn-SOD, and spinach Cu,Zn-SOD-II (Kitagawa et al. personal communication). The residue mumber was based on that of spinach SOD-II.

consisting eight-stranded β-barrel structure. The residues position 12-14 corresponds β-turn. High homology between the sequences of spinach Cu,Zn-SOD isozymes was found in the first and second β-strands, however, the region of β-turn was not conserved. Secondary strucutre of spinach SOD-I predicted by the method of Chau and Fasman (42) revealed the β-strand → β-turn → β-strand structure similar to that of spinach SOD-II.

The NH_2-terminal amino acid of mammalian Cu,Zn-SOD are N-acetylated, while spinach Cu,Zn-SOD isozymes were not as well as those of fish, fruit fly, yeast, and bacteria. It is evident that SOD-I lacks the first two residues when aligned with SOD-II, and in this respect, spinach SOD-I resembles yeast Cu,Zn-SOD. The percentage of homology of NH_2-terminal sequence (residue 1-23) between SOD-I and -II was 39%, which is the same degree between angiosperm and mammalian, and between angiosperm and yeast (40-50%), indicating an early divergence of SOD-I and -II.

The COOH-terminal sequence of spinach Cu,Zn-SOD-I was analyzed using carboxypeptidase-A. The terminal sequence was -Leu-Ser-Gly-Gly-COOH. The counterpart of SOD-II has been reported as -Leu(151)-Thr-Pro-Val(154)-COOH. The numbers of total residue were 306 for SOD-I, which was calculated by amino acid composition (Table II), and 308 for SOD-II, which was determined by the sequence. Alignment of the COOH-terminal region of SOD-I and -II indicated homology at position 151 (SOD-II) as

Leu, it is resonalbe to assume that the delation of only two residues at the NH_2-terminal region would contribute the difference in total residue amounts.

Circular dichroism spectra

The circular dichroism (CD) spectra of spinach Cu,Zn-SOD isozymes were similar in the 190-240 nm region and exhibited single negative band centered at 208 nm indicating the few α-helical structure in the both enzymes (Fig. 8). The mean residue ellipticities $[\theta]_R$ at 208 nm of SOD-I and -II were -6,300 and -10,600 deg·cm^2·dmol^{-1}, respectively. The $[\theta]_R$

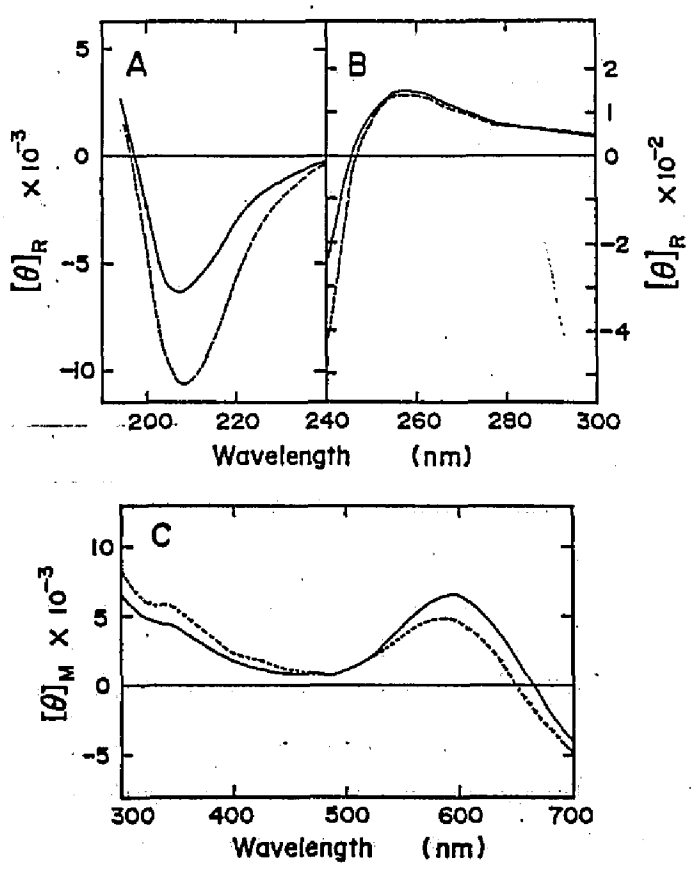

Fig. 8. Circular dichroism spectra of spinach Cu,Zn-SOD isozymes. The concentrations of SOD-I and SOD-II in 10 mM potassium phosphate, pH 7.8, and light path were (A), 0.136 mg/ml, 0.198 mg/ml, 1 mm; (B), 1.36 mg/ml, 1.98 mg/ml, 1 mm, (C) 1.36 mg/ml, 1.98 mg/ml, 10 mm, respectively. $[\theta]_R$, Mean residue ellipticity (A and B); $[\theta]_M$, molar ellipticity (C). ———, Cu,Zn-SOD-I; – – – –, Cu,Zn-SOD-II. The spectra were recorded at 25°C.

of SOD-I and -II at 222 nm indicates at most 10% α-helical contents of the both enzymes, which is almost same as that of bovine Cu,Zn-SOD (40). Thus, the chain foldings of SOD-I and -II might be similar or identical. Spinach "stromal" Cu,Zn-SOD (i.e. SOD-II) has eight-stranded β-barrel structure similar to that of bovine Cu,Zn-SOD (Kitagawa et al., personal communicaiton).

The spectra in the 240-300 nm region showed a single positive band at 260 nm ($[\theta]_R$ = 149 for SOD-I, $[\theta]_R$ = 140 deg·cm^2·dmol^{-1} for SOD-II) with a broad shoulder reaching visible region. No prominent difference between both enzymes was observed.

In visible region (300-700 nm), both enyzmes showed positive bands at 340, 450, and 600 nm, and a negative band at more than 700 nm. A little difference was observed in the positions and the intensities of the maximum bands: SOD-I, molar ellipticity $[\theta]_M$ at 592 nm = 6,570; SOD-II, $[\theta]_M$ at 582 nm = 4,820 deg·cm^{-1}·dmol^{-1}, in accordance with the small difference in optical spectra of Cu chromophore (Fig. 6). This might reflect the difference in configuration of ligands for Cu site of both enzymes.

Immunological properties

It has been reported that anti-maize cytosolic Cu,Zn-SOD isozyme did not react with the maize stromal Cu,Zn-SOD isozyme (10). I prepared both antibodies against spinach Cu,Zn-SOD-I (cytosolic) and SOD-II (stromal), and tested their antigenic specificities in Ouchterlony double immunodiffusion. Although anti-SOD-I and anti-SOD-II formed a single precipitin line with the purified SOD-I and SOD-II, respectively, anti-SOD-I did not cross-react with SOD-II, and anti-SOD-II not with SOD-I (Fig 9). Thus, both SOD-I and -II are immunologically distinct enzymes, in accordance with a large difference in the NH_2-terminal sequences. Cross-reaction of SOD-III from spinach seed extract with anti-SOD-I but no reaction with anti-SOD-II in crossed-immunoelectrophoresis indicates that SOD-III is a cytosolic enzyme (data not shown).

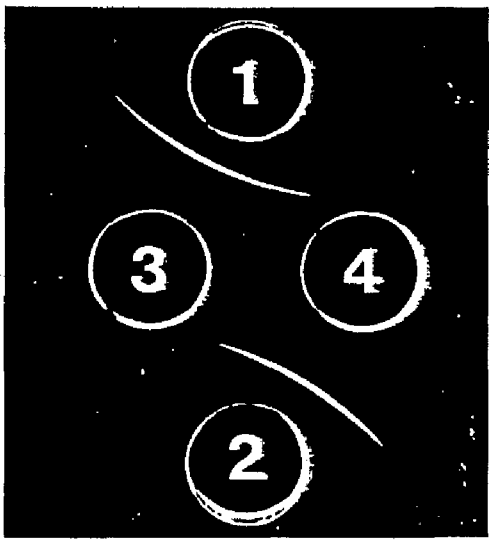

Fig. 9. Ouchterlony double immunodiffusion of anti-spinach SOD-I and anti-spinach SOD-II with spianch Cu,Zn-SOD isozymes. Diffusion was carried out at 37°C for 9-12 hours. 1, Anti-spinach Cu,Zn-SOD-I (56 μg/15 μl); 2, anti-spinach Cu,Zn-SOD-II (73 μg/15 μl); 3, spianch Cu,Zn-SOD-I; 4, spianch Cu,Zn-SOD-II. One μg/15 μl of the purified SOD isozymes were used.

Inactivation by hydrogen peroxide

Maize and wheat Cu,Zn-SOD isozymes differ in sensitivity against hydrogen peroxide: Stromal Cu,Zn-SOD isozyme is more resistant against H_2O_2 than cytosolic one (10). Spinach Cu,Zn-SOD-I and -II were inactivated by H_2O_2 in 10 mM potassium phosphate, pH 7.8, at 25°C. The second order rate constants for inactivation were determined to be 0.64 $M^{-1}sec^{-1}$ for SOD-I and 0.28 $M^{-1}sec^{-1}$ for SOD-II, indicating that spinach stromal Cu,Zn-SOD is also more resistant to H_2O_2 than cytosolic Cu,Zn-SOD.

4. DISCUSSION

It has been previously shown that Cu,Zn-SOD is localized in chloroplast stroma of spinach levaves (5). Here, I found three Cu,Zn-SOD isozymes in spinach seeds and leaves, and purified two isozymes from the seeds. One of the purified isozyme, Cu,Zn-SOD-II, was structurally identical with the "stromal" Cu,Zn-SOD previously purified from the leaves (5), as indicated by the mobility on native (Fig. 1 and 5) and SDS-polyacrylamide gel electrophoresis and on isoelectric focusing, and amino acid compositions (Table II).

Subcellular distribution of Cu,Zn-SOD isozymes in spinach leaves indicated that SOD-II is a stromal enzyme (Fig. 2). Although in leaves SOD-II is localized in stroma, the occurrence of a small amount of SOD-II in non-green tissues does not necessarily indicate that SOD-II occurs in cytosol of of such tissues. Rather, SOD-II might be localized in proplastid in non-green cells, since stromal SOD-II should be synthesized as a precursor in cytoplasm and imported into the organelles, based on the import mechanism of organelle proteins (43). So far no data indicate that SOD is encoded in chloroplast genome.

Cu,Zn-SOD-I was absent in stromal fraction but present in cytosolic fraction of the leaves, and occurs dominantly in non-green tissues (Fig. 2). Thus, SOD-I seems to be a cytosolic enzyme. However, I cannot still exclude the possibilities of the localization of either type of Cu,Zn-SOD isozymes or of the occurrence of distinct novel Cu,Zn-SOD in mitochondria, since it has been reported that yeast mitochondria contain Cu,Zn-SOD as well as Mn-SOD (7). Absence of Cu,Zn-SOD in mitochondria of higher plant is also possible. Characterization of SOD in spinach mitochondria is now underway.

The purified Cu,Zn-SOD-I and -II from spinach seeds had gross properties characteristic to Cu,Zn-SOD so far reported (44) in respect of molecular weight, subuint structure, metal content, and CD spectra. In spite of these similar properties, animo acid compositions and NH_2-terminal sequences of Cu,Zn-SOD-I and -II indicated that both enzymes are products of different genes. Comparison of amino acid compositions between both spinach SOD-I and SOD-II, and Cu,Zn-SOD isozymes from wheat

and maize suggest the occurrence of two types of Cu,Zn-SOD in terms of the contents of glycine, serine and leucine. Thus, wheat isozyme-I for which subcellular localization has not been reported could be assinged to be a cytosolic enzyme, and wheat isozyme-II to be a stromal enzyme as judged from the amino acid composition.

Alignment of NH_2-terminal sequence of Cu,Zn-SOD-I and -II revealed that first and second β-strands were conserved but β-turn was not (Fig. 7). This is not surprising because the turn region of proteins having a β-barrel structure would be more changeable than β-strand which might be received restriction by a pairing strand. It should be noted that the regions of β-turn are the most probable candidates for antigenic determinants, since these regions are located at marginal positions of the protein. Non-conservation of residues in the turn region between spinach Cu,Zn-SOD isozymes predicts the absence of common antigenic determinants. This was the case (Fig. 9).

Antigenic determinants characteristic to each type of spinach Cu,Zn-SOD isozyme are conserved by cytosolic and stromal Cu,Zn-SOD isozyme among angiosperms. Anti-spinach SOD-I (cytosolic enzyme) cross-reacted with cytosolic Cu,Zn-SOD isozymes from rice and maize, but not with stromal isozymes. To the contrary, anti-spinach Cu,Zn-SOD-II (stromal enzyme) recognized stromal Cu,Zn-SOD isozymes from rice and maize, but not cytosolic isozymes (data not shown). Furthermore, immunological crosss-reactivity suggested that the green alga Spirogyra sp. which is the first photosynthetic organism acquired Cu,Zn-SOD (Chapter III) contained two types of Cu,Zn-SOD isozyme (data not shown). Thus, the present results suggest that photosynthetic organisms having Cu,Zn-SOD contain two types of structurally and immunologically different Cu,Zn-SOD, i.e. cytosolic and stromal Cu,Zn-SOD.

A small differnce of active site configuration between spinach Cu,Zn-SOD-I and -II was indicated by the differences in position and intensity of visible absorption peak and CD band: SOD-I (cytosolic enzyme) exhibited absorption maximum at 670 nm in visible absorption spectrum and positive band at 590 nm in visible CD spectrum, while SOD-II (stromal enzyme) at 680 nm and 580 nm, respectively (Fig. 6 and 8). Beauchamp and Fridovich have reported that wheat Cu,Zn-SOD isozymes

could be distinguished by the difference of superhyper fine details in EPR spectra (8). Inspection of their absorption spectra revealed maximum peak at 680 nm of isozyme-II (stromal enzyme as judged from amino acid composition), although maximum peaks at 670 nm for both enzymes have been described (8). The fern Equisetum arvense stromal Cu,Zn-SOD also exhibited the maximum peak of Cu-chromophore at 680 nm (Chapter VII). Thus, it seems that the active site configuration of cytosolic and stromal Cu,Zn-SOD differs to some extent. It should be noted that spinach SOD-II was more resistant than SOD-I against hydrogen peroxide by which one of the histidine residue ligated to Cu is destroyed. Difference in sensitivity against hydrogen peroxide has been also reported between Cu,Zn-SOD isozymes of wheat and maize (8,10). In all cases stromal Cu,Zn-SOD were more resistant to hydrogen peroxide than cytosolic Cu,Zn-SOD. Thus, the configuration of active site of stromal Cu,Zn-SOD resulting in red shift of Cu-chromophore (about 10 nm) as compared with that of cytosolic Cu,Zn-SOD in optical spectra may correlate to the resistance against hydrogen peroxide without affecting its catalytic efficiency.

It is a interesting problem to estimate the time when stromal and cytosolic Cu,Zn-SOD isozymes separated form their ancestral protein during evolutional process. Since the green alga Spirogyra sp. contained two types of immunologically distinguishable Cu,Zn-SOD isozymes (data not shown), divergence of ancestral gene for Cu,Zn-SOD to stromal and cytosolic Cu,Zn-SOD occurred before or at the time when eukaryotic algae acquired Cu,Zn-SOD.

Another problem to be solved is the regulation of protein synthesis of Cu,Zn-SOD isozymes and their organelle localization. There is no data which indicate that Cu,Zn-SOD biosynthesis is controlled by non-nuclear gene such as chloroplast or mitochondrial gene. To the contrary, Mn-SOD in mitochondria of yeast was shown to be encoded on genomic gene (7). Therefore, it is reasonable to postulate that spinach SOD-II localizing in chloroplast stroma is encoded on genomic gene, and synthesized in cytoplasm like spinach SOD-I. If so, organelle specificity of Cu,Zn-SOD isozymes may correlate to their structure. In this respect, alignment of complete sequences of amino acid and DNA for both

Cu,Zn-SOD isozymes are needed.

I could obtain specific antibodies against cytosolic and stromal Cu,Zn-SOD. These will be useful tools for analysis of Cu,Zn-SOD biosynthesis under specific conditions such as light induction, methyl viologen induction, and developmental gene expression.

5. SUMMARY

Spinach contained three Cu,Zn-superoxide dismutase (SOD) isozymes as well as a small amount of Mn-SOD. Cu,Zn-SOD-I was present dominantly in non-green tissues such as seeds, roots, and hypocotyls of etiolated seedlings, and localized in cytosolic fraction of leaf, but not in chloroplast stromal fraction. On the other hand, Cu,Zn-SOD-II was localized in chloroplast stroma.

Two Cu,Zn-SOD isozymes were purified from spinach seeds. The purified SOD-I and -II were almost identical in respects of molecular weight, subunit structure, metal contents and circular dichroism spectra. Isoelectric points were 4.7 for SOD-I and 5.2 for SOD-II. Absorption spectrum in UV region of SOD-I showed the presence of tyrosine. Amino acid compositions of both isozymes were distinguishable, indicating that SOD-I and -II were products of different genes. Alignment of NH_2-terminal sequence of SOD-I (1-21 residues) with those of SOD-II (Kitagawa et al., personal communication), and yeast and animal Cu,Zn-SOD revealed that the degree of homology (40%) between SOD-I and -II was similar to those between plant and yeast, and between plant and animal, indicating early divergence of SOD-I and -II from an ancestral protein.

6. REFERENCES

(1) Fridovich, I. (1975) Annu. Rev. Biochem. 44, 147-159.

(2) Asada, K., and Kiso, K., and Yoshikawa, K. (1974) J. Biol. Chem. 249, 2175-2181.

(3) Elstner, E.F., and Kramer, R. (1973) Biochim. Biophys. Acta 314, 340-353.

(4) Asada, K., Kanematsu, S., Nakano, Y., Hossain, M.A., and Hayakawa, T. (1986) in Molecular Basis of Biological Evolution, pp. 55-57, Report of Special Research Project Sponsored by MESC.

(5) Asada, K., Urano, M., and Takahashi, M. (1973) Eur. J. Biochem. 36, 257-266.

(6) Jackson, C., Dench, J., Moore, A.L., Halliwell, B., Foyer, C.H., and Hall, D.O. (1978) Eur. J. Biochem. 91, 339-344.

(7) Weisiger, R.A., and Fridovich, I. (1973) J. Biol. Chem. 248, 4793-4796.

(8) Beauchamp, C.O., and Fridovich, I. (1973) Biochim. Biophys. Acta 317, 50-64.

(9) Baum, J.A., and Scandalios, J.G. (1981) Arch. Biochem. Biophys. 206, 249-264.

(10) Baum, J.A., Chandlee, J.M., and Scandalios, J.G. (1983) Plant Physiol. 73, 31-35.

(11) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(12) Walker, D.A. (1980) Methods Enzymol. 69, 94-104.

(13) Lundahl, P., and Liljas, L. (1975) Anal. Biochem. 65, 50-59.

(14) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(15) Asada, K., Takahashi, M., and Nagate, M. (1974) Agric. Biol. Chem. 38, 471-473.

(16) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

(17) Davis, B.J. (1964) Ann. New York Acad. Sci. 121, 404-427.

(18) Laemmli, U.K. (1970) Nature (London) 277, 680-685.

(19) Beauchamp, C.O., and Fridovich, I. (1971) Anal. Biochem. 44,

276-287.

(20) Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y., and Enmanji, K. (1975) J. Biol. Chem. 250, 2801-2807.

(21) Yphantis, D.A. (1964) Biochemistry 3, 297-317.

(22) Cohn, E.J., and Edsall, J.T. (1941) Proteins, Amino Acids and Peptides, p. 375, Reinhold, New York.

(23) Chen, Y.H., and Yang, J.T. (1971) Biochem. Biophys. Res. Commun. 44, 1285-1291.

(24) Moore, S. (1963) J. Biol. Chem. 238, 235-237.

(25) Edelhoch, H. (1967) Biochemistry 6, 1948-1954.

(26) Abernethy, J.L., Steinman, H.M., and Hill, R.L. (1974) J. Biol. Chem. 249, 7339-7347.

(27) Chang, J.Y., Brauer, D., and Wittmann-Liebold, B. (1978) FEBS Lett. 93, 205-214.

(28) Chang, J.Y. (1983) Methods Enzymol. 91, 455-466.

(29) Chang, J.Y., Creaser, E.H., and Hughes, G.J. (1977) J. Chromatogr. 140, 125-128.

(30) Steinman, H.M., Naik, V.R., Abernethy, J.L., and Hill, R.L. (1974) J. Biol. Chem. 249, 7326-7338.

(31) Jabusch, J.R., Farb, D.L., Kerschensteiner, D.A., and Deutsch, H.F. (1980) Biochemistry 19, 2310-2316.

(32) Barra, D., Martini, F., Bannister, J.V., Schinina, M.E., Rotilio, G., Bannister, W.H., and Bossa, F. (1980) FEBS Lett. 120, 53-56.

(33) Sherman, L., Dafni, J., Lieman-Hurwitz, J., and Groner, Y. (1983) Proc. Natl. Acad. Sci. USA 80, 5465-5469.

(34) Lerch, K., and Ammer, D. (1981) J. Biol. Chem. 256, 11545-11551.

(35) Johansen, J.T., Overballe-Peterson, C., Martin, B., Hasemann, V., and Svendsen, I. (1979) Carlsberg Res. Commun. 44, 201-217.

(36) Stein an, H.M. (1980) J. Biol. Chem. 255, 6758-6762.

(37) Rocha, H.A., Bannister, W.H., and Bannister, V.B. (1984) 145, 477-484.

(38) Lee, Y.M., Friedman, D.J., and Ayala, F.J. (1985) Proc. Natl. Acad. Sci. USA (1985) 82, 824-828.

(39) Steffens, G.J., Bannister, V.J., Bannister, W.H., Flohe, L., Gunzler, W.A., Kim, S.-M.A., and Otting, F. (1983) Hoppe-Seyler's

Z. Physiol. Chem. 364, 675-690.

(40) Richardson, J.S., Thomas, K.A., Rubin, B.H., and Richardson, D.C. (1975) Proc. Natl. Acad. Sci. USA 72, 1349-1353.

(41) Tainer, J.A., Getzoff, E.D., Beem, K.M., Richardson, J.S., and Richardson, D.C. (1982) J. Mol. Biol. 160, 181-217.

(42) Chou, P.Y., and Fasman, G.D. (1974) Biochemistry 13, 211-222; 222-245.

(43) Kreil, G. (1981) Ann. Rev. Biochem. 50, 317-348.

(44) Steinman, H.M. (1982) in Superoxide dismutase (Oberley, L.W., ed.), pp. 11-68, CRC Press, Florida.

CHAPTER VI

Cu,Zn-SUPEROXIDE DISMUTASE ISOZYMES IN RICE: OCCURRENCE OF CYTOSOLIC AND STROMAL ENZYMES, AND MONOMERIC ENZYME

1. INTRODUCTION

In Chapter V, I isolated the cytosolic Cu,Zn-superoxide dismutase (SOD) from spinach seeds in addition to stromal Cu,Zn-SOD, and compared their physicochemical and immunological properties. Cytosolic and stromal Cu,Zn-SOD isozymes were significantly differ in their NH_2-terminal amino acid sequences and distinguished immunologically, but their higher structures deduced from circular dichroism spectra were similar.

Occurrence of Cu,Zn-SOD isozymes has been found in many plants, and the isozymes were isolated and characterized from wheat (1), maize (2,3) and spinach (Chapter V). However, a relation of each isozyme among these plants is not known. I describe here the purification and characterization of rice Cu,Zn-SOD isozymes, and their immunological properties using anti-spinach cytosolic and stromal Cu,Zn-SOD. I found first that one isozyme of rice Cu,Zn-SOD is a monomer, and that rice also contain cytosolic and stromal Cu,Zn-SOD.

2. MATERIALS AND METHODS

Cytochrome c (horse heart, type III) and xanthine oxidase (bovine milk) were purchased from Sigma and Boehringer, respectively, marker proteins for isoelectric point and molecular weight from Pharmacia, and Ampholine (pH 3.5-10) and DEAE Affi-Gel Blue from LKB and Bio-Rad, respectively.

Rice (Oryza sativa L. cv. Sasanishiki) was water-cultured (4) in a glass house. The leaves were harvested two months after sowing and

stored at -20°C.

Antibodies against rice Cu,Zn-SOD-I, spinach stromal and cytosolic Cu,Zn-SOD were raised in rabbits, and the immunoglobulins were obtained by passing the sera through a column of DEAE Affi-Gel Blue as described previously (Chapter V). Ouchterlony double immunodiffusion, and crossed-immunoelectrophoresis using a polyacrylamide slab gel in the first direction and an agarose gel containing antibody in the second direction were performed as before (Chapter V).

SOD was assayed by the xanthine-xanthine oxidase-cytochrome c system of McCord and Fridovich (5) using a 1 ml reaction volume. The enzymatic activity is shown with the McCord and Fridovich unit by dividing the observed unit by three (Chapter II). Protein was determined by the method of Lowry et al. (6) using bovine serum albumin as a standard.

Native and SDS-polyacrylamide gel electrophoresis, activity staining for SOD on gel, determination of the molecular weight and subunit molecular weight, metal analysis, amino acid analysis, isoelectric focusing on gel were performed as described in Chapter V.

Discrimination of three types of SOD in a gel by cyanide and hydrogen peroxide was carried out as before (Chapter V).

3. RESULTS

Isozymes

SOD isozymes of rice leaves and seed embryos were surveyed by activity staining after polyacrylamide gel electrophoresis at pH 9.5 (Fig. 1). Rice leaves and embryos contained four cyanide-sensitive Cu,Zn-SOD, and one Mn-SOD as judged from the insensitivity to both cyanide and H_2O_2. Cyanide-insensitive band was not affected by the treatment with H_2O_2, indicating the absence of Fe-SOD in the buffer extract (data not shown). I refer the activity bands from the anode to cathode as Cu,Zn-SOD-I, Mn-SOD-I, Cu,Zn-SOD-II, Cu,Zn-SOD-III, and Cu,Zn-SOD-IV (Fig. 1). Isoelectric focusing (pH 3.5-10) on polyacrylamide gel showed another Mn-SOD-II having a pI of about 9 in addition to

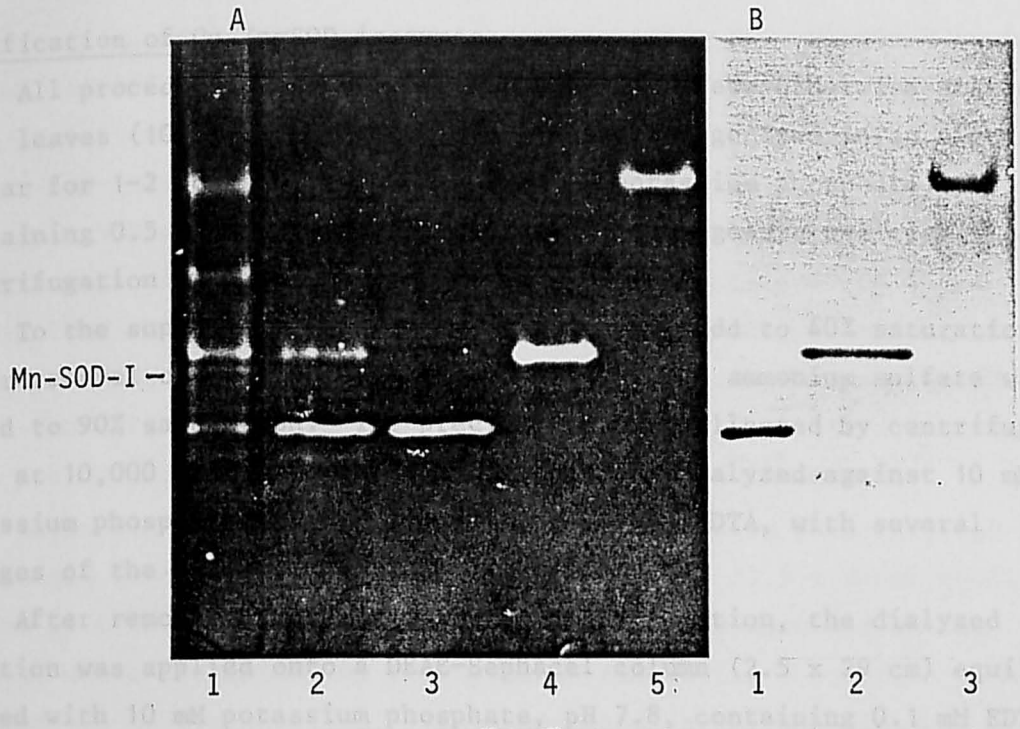

Fig. 1. Native-polyacrylamide gel electrophoresis of rice SOD isozymes. (A) Activity staining: 1, germ extract (3 units); 2, leaf extract (3 units); 3, purified Cu,Zn-SOD-I (1 unit); 4, purified Cu,Zn-SOD-II (1 unit); 5, purified Cu,Zn-SOD-IV (1 unit). (B) Protein staining: 1, Cu,Zn-SOD-I; 2, Cu,Zn-SOD-II; 3, Cu,Zn-SOD-IV, each 3 μg. Electrophoresis was performed at 4°C. Top, cathode; bottom, anode. Protein was stained by Coomassie Brilliant Blue R-250.

the five SOD (data not shown).

Cu,Zn-SOD-I was a major enzyme in the leaves, while Cu,Zn-SOD-II, -III, and -IV as well as Mn-SOD-I were dominant in the seed embryos (Fig. 1). Low contents of Cu,Zn-SOD-I was also observed in whole seeds. Cu,Zn-SOD-I increased during growth after germination with greening of seedlings, indicating that the SOD-I is a stromal enzyme like spinach Cu,Zn-SOD-II (Chapter V). The increase of Cu,Zn-SOD-I was not observed in etiolated seedlings. To the contrary, Cu,Zn-SOD-II, -III, and -IV showed different changes: the SOD-II was almost constant, and the SOD-III and -IV was decreased after germination (data not shown). Since these SOD isozymes did not relate with greening process, it is reasonable to assume that Cu,Zn-SOD-II, -III, and -IV are cytosolic enzyme (data not shown).

Purification of Cu,Zn-SOD isozymes

All procedures were carried out at 4°C, unless otherwise stated. Rice leaves (100 g batch, total 500 g) were homogenized in an electrical mortar for 1-2 hours with 800 ml of 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA with sea sands. The homogenate was clarified by centrifugation at 10,000 g for 20 min.

To the supernatant, ammonium sulfate was addd to 40% saturation. After removal of precipitates by centrifugation, ammonium sulfate was added to 90% saturation. The precipitate was collected by centrifugation at 10,000 g for 20 min, dissolved in and dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA, with several changes of the buffer.

After removal of precipitates by centrifugation, the dialyzed solution was applied onto a DEAE-Sephacel column (2.5 x 29 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA (Fig. 2). About 15% of SOD activity was not adsorbed. Unadsorbed fraction contained Cu,Zn-SOD-III, -IV, and Mn-SOD-II. The column was washed by 1.9 liters of equilibrating buffer until Cu,Zn-SOD-II was eluted. After the second SOD was eluted, the column was eluted by 2 liters of a linear gradient of potassium phosphate, pH 7.8, (10 to 60 mM) containing 0.1 mM EDTA. The third SOD (Cu,Zn-SOD-I) was eluted at a

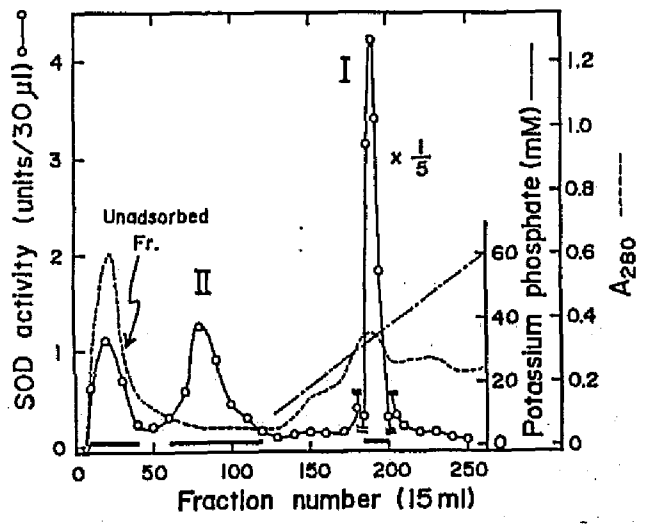

Fig. 2. Linear gradient chromatography of rice SOD isozymes on DEAE-Sephacel. I, Cu,Zn-SOD-I; II, Cu,Zn-SOD-II. Details are given in the text.

concentration of 34 mM potassium phosphate (Fig. 2). Mn-SOD-I could be eluted by 0.16 M KCl in 10 mM potassium phosphate, pH 7.8. High ionic strength was also required to elute spinach Fe-SOD (Chapter XIII) and pea Mn-SOD (7).

Each SOD was pooled and concentrated to about 10 ml by ultrafiltration through an Amicon PM 10 membrane and then subjected to a gel filtration. The concentrated enzyme was applied onto a Sephadex G-100 column (4 x 86 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, and 0.1 M KCl, and eluted with the same buffer. The active fractions were pooled and concentrated through an Amicon PM 10 membrane.

Cu,Zn-SOD-I was dialyzed against 5 mM potassium phosphate, pH 6.8, and passed through a small column of Bio-Gel HTP (1.5 x 2 cm) equilibrated with the same buffer. Cu,Zn-SOD-I was not adsorbed on the column, while contaminating flavoprotein exhibiting absorption peaks at 386 and 458 nm was adsorbed. The unadsorbed enzyme was dialyzed against 10 mM potassium phosphate, pH 7.8.

Cu,Zn-SOD-II was further purified by a linear gradient elution on DEAE-Sephadex column. The concentrated enzyme was dialyzed against 10 mM Tris-HCl, pH 7.8, and applied onto a column of DEAE-Sephadex (1.5 x 6 cm) equilibrated with the same buffer. After washing the column with 25 ml of equilibrating buffer, the enzyme was eluted by 200 ml of a linear gradient of Tris-HCl, pH 7.8 (10 to 100 mM). Cu,Zn-SOD-II was eluted at 75 mM of the buffer. The active fractions were pooled, concentrated using an Amicon PM 10 and dialyzed against 10 mM potassium phosphate, pH 7.8.

The Cu,Zn-SOD-IV fraction was dialyzed against 2.5 mM Tris-HCl, pH 7.8, and adsorbed onto a column of DEAE-Sephadex (1.5 x 9 cm) equilibrated with the same buffer, and eluted by 200 ml of a linear gradient of Tris-HCl, pH 7.8 (2.5 to 100 mM). Cu,Zn-SOD-IV appeared at 38 mM of the buffer. The active fractions were pooled and concentrated using an Amicon YM 5 membrane instead of PM 10 to about 1 ml. Cu,Zn-SOD-IV was finally purified by gel filtration operated at a flow rate of 0.5 ml/min using a TSK G3000SW HPLC column (7.5 mm x 60 cm) equilibrated with 50 mM sodium phosphate, pH 7.2, and 0.1 M Na_2SO_4. Each 100 µl of the enzyme was injected. Cu,Zn-SOD-IV was baseline-separated from contaminating

Table I

Summary of the Purification of Cu,Zn-SOD Isozymes from Rice Leaves

Purification step	Total[a] protein (mg)	Total[b] activity (10^{-3} units)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
Homogenate	5,938	141	24	100	1
40 to 90% $(NH_4)_2SO_4$	1,329	135	102	96	4
DEAE-Sephacel					
Cu,Zn-SOD-I	63	53	841	38	35
Cu,Zn-SOD-II	13	15	1153	11	48
Cu,Zn-SOD-IV[c]	77	13	169	9	7
Sephadex G-100					
Cu,Zn-SOD-I	12.83	52.5	4091	37	170
Cu,Zn-SOD-II	2.96	11.1	3750	8	156
Cu,Zn-SOD-IV[c]	29.29	12.4	425	9	18
Cu,Zn-SOD-I					
Bio-Gel HTP	7.36	48.7	6617	35	276
Cu,Zn-SOD-II					
DEAE-Sephadex	1.31	9.2	7022	7	293
Cu,Zn-SOD-IV					
DEAE-Sephadex	5.32	8.7	1635	6	68
TSK Gel G3000SW	0.79	5.3	6709	4	280

a) Protein was measured by the method of Lowry et al. (6).

b) McCord and Fridovich unit (5).

c) This fraction contained Cu,Zn-SOD-III, and Mn-SOD-II as well as Cu,Zn-SOD-IV.

lower molecular weight protein. Active fractions were pooled, concentrated through a YM 5 membrane, and dialyzed against 10 mM potassium phosphate, pH 7.8.

Table I summarizes the purification of Cu,Zn-SOD-I, -II, and -IV from 500 g of rice leaves. Specific activities of rice Cu,Zn-SOD isozymes were same and approximately 7,000 units/mg protein. Cu,Zn-SOD-III, and Mn-SOD-I and -II were not purified due to their small amount in rice leaves.

Purity

The purified Cu,Zn-SOD-I, -II and -IV were homogeneous as judged

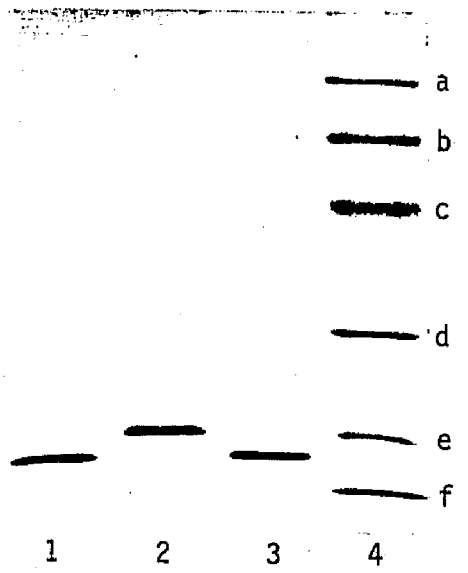

Fig. 3. SDS-polyacrylamide gel electrophoresis of rice Cu,Zn-SOD isozymes. 1, Cu,Zn-SOD-I; 2, Cu,Zn-SOD-II; 3, Cu,Zn-SOD-VI, each 3 μg; 4, marker proteins. The following marker proteins were used: a, phosphorylase b (94,000); b, bovine serum albumin (67,000); c, ovalbumin (43,000); d, carbonic anhydrase (30,000); e, soybean trypsin inhibitor (20,000); f, α-lactalbumin (14,400). The enzyme was sealed in a small glass tube, and denaturated in 5% SDS in the presence of 5% 2-mercaptoethanol at 100°C for min. Electrophoresis was carried out at room temperature. Top, cathode; bottom, anode. Protein was stained by Commassie Brilliant Blue R-250.

from native polyacrylamide gel electrophoresis (Fig. 1). No contamination was observed when 20 μg of each enzyme was separated by polyacrylamide disc gel electrophoresis and stained by Coomassie Brilliant Blue (data not shown). The SOD activity bands of the crude extract of rice leaves after electrophoresis corresponded to the purified Cu,Zn-SOD-I, -II, and -IV, indicating no modification of the enzymes during purification (Fig. 1). The purity of Cu,Zn-SOD-I, -II, and -IV was further confirmed by a single band in SDS-polyacrylamide gel electrophoresis both in the presence and absence of 2-mercaptoethanol (Fig. 3), and isoelectric focusing on polyacrylamide gel (data not shown). Linearity of the slope obtained from a plot of log(fringe displacement) vs. square of the distance from the center of rotation in ultracentrifugation analysis also indicates the homogeneity of Cu,Zn-SOD-I and -II (Fig. 5).

Molecular weights

The molecular weights of the purified enzymes were determined by gel filtration and ultracentrifugation methods. A gel filtration column of Sephadex G-100 (2 x 85 cm) was equilibrated and eluted with 10 mM potassium phosphate, pH 7.8, and 0.1 M KCl, and calibrated with bovine serum albumin, ovalbumin, α-chymotrypsinogen-A, myoglobin and cytochrome c. The molecular weights of Cu,Zn-SOD-I, -II, and -IV were 31,600, 30,900, and 17,400, respectively (Fig. 4). It should be noted that the molecular weight of Cu,Zn-SOD-IV was half as those of Cu,Zn-SOD-I and -II, indicating that the SOD-IV is a monomer.

The sedimentation equilibrium centrifugation of the enzymes in 5 mM potassium phosphate, pH 7.8, and 0.1 M KCl was performed by the meniscus depletion method of Yaphantis (8) at 20°C. The partial specific volume of the enzyme was estimated from its amino acid composition (Table II) by the method of Cohn and Edsall (9): 0.726 for Cu,Zn-SOD-I and 0.711 for SOD-II. From the slopes (Fig. 5) and the partial specific volumes of the enzymes, the molecular weights of Cu,Zn-SOD-I and -II were calculated to be 31,900, and 30,900, respectively, which are in fair agreement with those obtained by the gel filtration method.

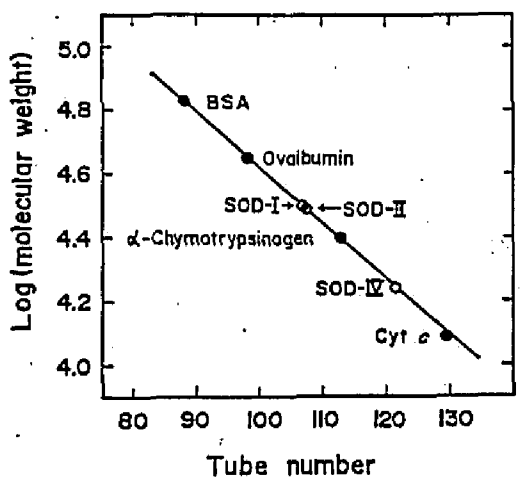

Fig. 4. Molecular weight determination of rice Cu,Zn-SOD isozymes by Sephadex G-100. One ml of each sample (1-2 mg) was applied onto a Sephadex G-100 column (2 x 85 cm) which was equilibrated with 10 mM potassium phosphate, pH 7.8, and 0.1 M KCl, and eluted with the same buffer. The following marker proteins were used: bovine serum albumin (67,000), ovalbumin (43,000), α-chymotrypsinogen A (25,000), cytochrome c (12,400).

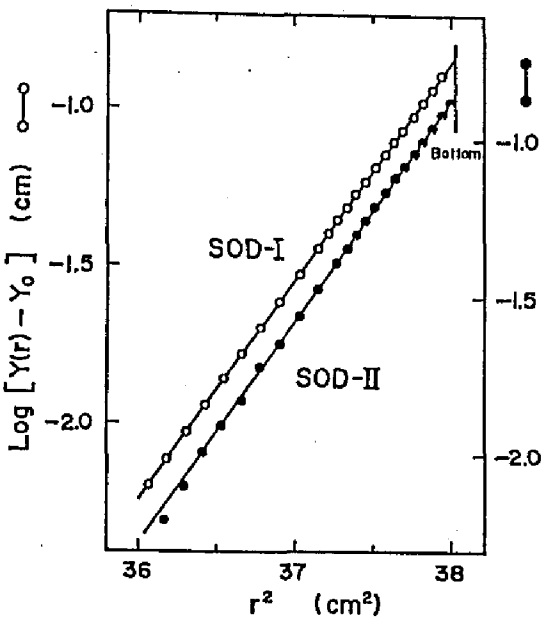

Fig. 5. Sedimentation equilibrium of rice Cu,Zn-SOD-I and SOD-II. Purified enzymes at concentrations of 0.64 (Cu,Zn-SOD-I) and 0.63 mg/ml (Cu,Zn-SOD-II) in 5 mM potassium phosphate, pH 7.8, and 0.1 M potassium chloride were equilibrated at 28,441 and 28,455 rpm, respectively, at 20°C. The ultracentrifuge was equipped with interference optics, and the logarithm of fringe displacement, $Y(r) - Y_o$, was plotted as a function of the square of the distance from the center of rotation (r^2).

I determined the molecular weight of Mn-SOD-I, which was separated on DEAE-Sephacel column and freed from Cu,Zn-SOD isozymes (see above), by gel filtration following its activity. The results indicated that rice Mn-SOD-I has a molecular weight of 100,000 like tetrameric pea Mn-SOD (7). The molecular weight of spinach (10) and two red algae (see Chapter XIV) Mn-SOD are 52,000 and 40,000-45,000, respectively.

Subunit structure

The subunit molecular weights of the purified enzymes were estimated by SDS-polyacrylamide gel electrophoresis according to Laemmli (11) using 15% gel. The enzyme was denaturated by 5% SDS in the presence or absence of 5% 2-mercaptoethanol at 100°C for 3 min as before (Chapter V). The enzyme gave a single band in the presence and absence of 2-mercaptoethanol (Fig. 3). The subunit molecular weights of

Cu,Zn-SOD-I, -II, and -IV were 18,100, 20,100, 18,300 in the presence of the reductant, and 17,200, 18,500, and 17,300 in the absence of the reductant, respectively. Thus, Cu,Zn-SOD-IV is a monomer, while Cu,Zn-SOD-I and -II are homodimers without a disulfide linkage. To my knowledge, monomeric Cu,Zn-SOD has not been isolated. Therefore, this is the first isolation of monomeric Cu,Zn-SOD.

Absorption spectra

Fig. 6 shows the absorption spectra of Cu,Zn-SOD-I and -II. In ultraviolet region, both enzyme showed the similar spectra having absorption peaks at 252, 258, 262 nm. In addition to these peaks, Cu,Zn-SOD-II exhibited small shoulders around at 280 and 290 nm, resembling that of one of maize Cu,Zn-SOD isozymes (2), while Cu,Zn-SOD-I had no shoulder in these wavelength regions like spinach stromal Cu,Zn-SOD (12). Since both SOD lacked tryosine and tryptophan (Table II), the origin of the shoulders at 280 and 290 nm of Cu,Zn-SOD-II is not known. The molar absorbance coefficients and $A^{1\%}_{1\ cm}$ at 258 nm were 12,600 $M^{-1}cm^{-1}$ and 3.93 for Cu,Zn-SOD-I, and 12,400 $M^{-1}cm^{-1}$ and 4.02 for Cu,Zn-SOD-II.

In visible spectra, both enzymes showed absorption peak around 670-680 nm, suggesting the presence of copper. The absorption maxima were not identical: Cu,Zn-SOD-I at 680 nm and Cu,Zn-SOD-II at 670 nm.

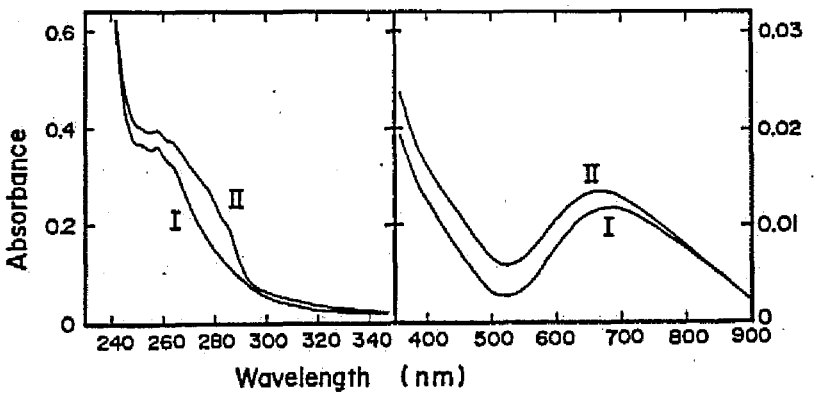

Fig. 6. Absorption spectra of rice Cu,Zn-SOD-I and -II. Spectra were recorded at 0.93 (Cu,Zn-SOD-I), and 0.99 mg/ml (Cu,Zn-SOD-II), respectively, in 10 mM potassium phosphate, pH 7.8, at 25°C. The light path was 10 mm. I, Cu,Zn-SOD-I; II, Cu,Zn-SOD-II.

Such difference in the absorption maxima was also observed in wheat (1), and spinach (Chapter V) Cu,Zn-SOD isozymes, and seems to be characteristic of cytosolic and stromal Cu,Zn-SOD. The molar absorbance coefficients of Cu,Zn-SOD-I and -II at respective visible peaks were 379 and 406 $M^{-1}cm^{-1}$, respectively.

Absorption spectrum of Cu,Zn-SOD-IV also resembled that of Cu,Zn-SOD-II, having an absorption maximum around 260 nm, and indicating the absence of tyrosine and tryptophan.

Metal analysis

Metal contents of Cu,Zn-SOD-I, -II, and -IV were determined using an atomic absorption spectrophotometer equipped with a graphite atomizer. After dialysis against 5 mM Tris-HCl, pH 7.8, the enzymes were diluted with the same buffer, and the aliquots (10 μl) were atomized in a graphite atomizer. Cu,Zn-SOD-I, -II, and -IV contained 2.20, 2.19, and 0.98 g-atoms of Cu, and 1.96, 1.78, and 0.98 g-atoms of Zn per mol of enzyme, respectively. The contents of Fe and Mn were the levels of background.

Amino acid compositions

The enzymes were hydrolyzed in duplicate in 6 M HCl in an evacuated and sealed tube for 24, 48, and 72 hours at 110°C after dialysis against distilled water. Half-cystine and methionine were determined as cysteic acid and methionine sulfone after performic acid oxidation followed by hydrolysis in 6 M HCl for 18 hours (13). Tryptophan was estimated by the method of Edelhoch (14). The loss or increase in amino acids during hydrolysis was corrected by extrapolation to zero time or taking the values at 72 hours. Amino acid compositions of rice Cu,Zn-SOD-I and -II were significantly differ in aspartic acid, serine, glutamic acid, valine, and leucine contents (Table II). Cu,Zn-SOD-II has lower content of aspartic acid than that of the SOD-I, but this was almost compensated by higher content of glutamic acid. No prominant difference in basic amino acids between the two isozymes was observed. These results clearly indicate that both enzymes are products of different genes. The difference in serine and leucine contents of rice Cu,Zn-SOD-I and -II

Table II

Amino Acid Compositions of Rice Cu,Zn-SOD-I and -II[a)]

Amino acid	Cu,Zn-SOD-I		Cu,Zn-SOD-II	
	Number of residues	Nearest integer	Number of residues	Nearest integer
Asp	30.09	30	11.88	12
Thr[b)]	26.74	27	22.28	22
Ser[b)]	23.83	24	55.69	56
Glu	31.89	32	42.38	42
Pro	18.23	18	17.74	18
Gly	46.00	46	50.00	50
Ala	24.04	24	23.07	23
1/2 Cys[d)]	4.31	4	3.55	4
Val[c)]	31 24	31	17.19	17
Met[d)]	2.26	2	3.79	4
Ile[c)]	8.08	8	10.26	10
Leu	26.33	26	15.42	15
Tyr	0	0	0	0
Phe	6.08	6	5.52	6
Lys	11.90	12	11.58	12
His	12.76	13	11.65	12
Arg	7.17	7	6.13	6
Trp	0	0	0	0
Total		310		309

a) The number of residues was calculated for 31,900 g of Cu,Zn-SOD-I and 30,900 g of Cu,Zn-SOD-II, respectively. The correction was made for 2 atoms each of Cu and Zn and two molecules of water at the terminals.

b) 0-time.

c) 72 hours-hydrolysis.

d) Determined as cysteic acid and methionine sulfone after performic acid oxidation.

resembled that of spinach stromal and cytosolic Cu,Zn-SOD (Chapter V). In this respect, rice Cu,Zn-SOD-I more resembled spinach stromal Cu,Zn-SOD than rice Cu,Zn-SOD-II, and rice Cu,Zn-SOD-II is similar to spinach cytosolic Cu,Zn-SOD.

Isoelectric points

Isoelectric points of Cu,Zn-SOD isozymes were determined by

isoelectric focusing on a vertical polyacrylamide slab gel containing Ampholine (pH 3.5-10). The pI of Cu,Zn-SOD-I, -II, and -IV were 5.0, 5.3, and 5.7, respectively, based on protein staining. The bands of purified enzymes were corresponded with those of the buffer extracts of rice leaves and seed embryos after activity staining, indicating no modification during purification. The order of isozymes on the gel were the same as in native polyacrylamide gel electrophoresis. The pI of Mn-SOD-I and Cu,Zn-SOD-III were estimated to be 5.2, and 5.5, respectively, by activity staining. Furthermore, isoelectric focusing revealed Mn-SOD-II having a pI of approximatry 9.

Effect of temperature on the activity

The molecular weight determination indicated that Cu,Zn-SOD-IV is a monomer, while Cu,Zn-SOD-I and -II are dimers. It has been reported that subunit-subunit interaction through catalytic cycle dose not take place and each active site copper catalyzes independently the disproportionation of the superoxide (15,16). The same specific activity of rice monomeric and dimeric Cu,Zn-SOD also confirmed the independent catalysis of each active site of dimeric Cu,Zn-SOD. In order to get insight into the difference between monomeric and dimeric SOD in terms of catalytic activity, I investigated the response of the both monomeric and dimeric

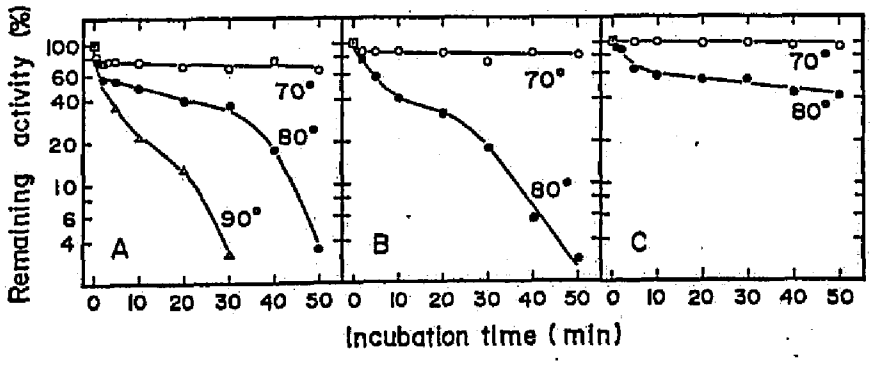

Fig. 7. Heat stability of rice Cu,Zn-SOD isozymes. Each enzyme (2 units/20 µl) in 10 mM potassium phosphate, pH 7.8, was sealed in glass tubes and incubated at the indicated temperatures. Aliquots (10 µl) were withdrawn at the indicated time and then assayed for the remaining enzyme activity under standard assay conditions at 25°C. A, Cu,Zn-SOD-I; B; Cu,Zn-SOD-II; C, Cu,Zn-SOD-IV.

SOD activity upon denaturating stress. Cu,Zn-SOD-I and -II started to inactivate at 70°C, and about 20-30% of the initial activity was lost within 5 min, but further inactivation was not observed upon longer incubation at 70°C (Fig. 7A and B). To the contrary, Cu,Zn-SOD-IV was quite stable at 70°C, at least, up to 50 min (Fig. 7C). At 80°C, the activity of Cu,Zn-SOD-I and -II was lost with three phases and most activity was lost within an hour. Cu,Zn-SOD-IV was more stable against heat than Cu,Zn-SOD-I and -II at 80°C, and about 40% of the initial activity was rapidly lost first 10 min. Thereafter, the remaining activity was decreased very slowly with time. Thus, monomeric Cu,Zn-SOD-IV is more stable against heat than dimeric Cu,Zn-SOD-I, and -II, and the difference of their susceptibility to heat may be attributed to the difference in their subunit structure.

Immunological properties

Antibody against the purified Cu,Zn-SOD-I distinguished rice Cu,Zn-SOD isozymes: anti-rice Cu,Zn-SOD-I reacted with Cu,Zn-SOD-I, but did not form any precipitin line with Cu,Zn-SOD-II and -IV in Ouchterlony double immunodiffusion (Fig. 8A). No reactivity of Cu,Zn-SOD-III with anti-Cu,Zn-SOD-I was confirmed by crossed-immunoelectrophoresis of

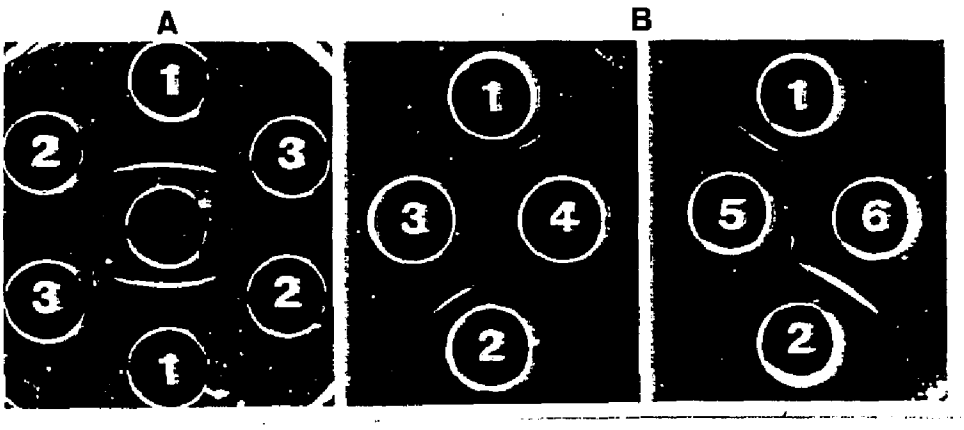

Fig. 8. Ouchterlony double immunodiffusion of anti-rice Cu,Zn-SOD-I, anti-spinach stromal Cu,Zn-SOD, and anti-spinach cytosolic Cu,Zn-SOD with rice Cu,Zn-SOD isozymes. (A) Center well, anti-rice Cu,Zn-SOD-I (258 μg); 1, Cu,Zn-SOD-I; 2, Cu,Zn-SOD-II; 3, Cu,Zn-SOD-IV (each 2 μg). (B) 1, Anti-spinach cytosolic Cu,Zn-SOD (56 ug); 2, anti-spinach stromal Cu,Zn-SOD (73 μg); 3, Cu,Zn-SOD-I; 4, Cu,Zn-SOD-II; 5, Cu,Zn-SOD-IV; 6, Equisetum Cu,Zn-SOD (each 2 μg).

the extract from rice seed embryos (data not shown).

Wide-range of cross-reactivity of anti-spinach stromal Cu,Zn-SOD with SOD in extracts from angiosperms, gymnosperms, ferns and mosses has been shown (17). I tested the immunological cross-reactivity using anti-rice Cu,Zn-SOD-I and anti-spinach stromal and cytosolic Cu,Zn-SOD sera with purified spinach and rice Cu,Zn-SOD isozymes in Ouchterlony double immunodiffusion. Anti-rice Cu,Zn-SOD-I cross-reacted with spinach stromal Cu,Zn-SOD, but did not recognize the spinach cytosolic Cu,Zn-SOD (data not shown). On the other hand, anti-spinach stromal Cu,Zn-SOD cross-reacted with rice Cu,Zn-SOD-I, but not with rice Cu,Zn-SOD-II and -IV, while anti-spinach cytosolic Cu,Zn-SOD cross-reacted with rice Cu,Zn-II, and -IV, but not with rice Cu,Zn-SOD-I (Fig. 8B). Cross-reaction of Cu,Zn-SOD-III from rice seed embryos extract with anti-spinach cytosolic Cu,Zn-SOD but no reaction with anti-spinach stromal Cu,Zn-SOD was confirmed by crossed-immunoelectrophoresis (data not shown). Thus, it is evident that rice Cu,Zn-SOD-I is a stromal enzyme, while rice Cu,Zn-SOD-II, -III, and -IV are cytosolic enzymes. The classification for the type of Cu,Zn-SOD using these antibodies was also applicable to the purified Equisetum Cu,Zn-SOD: it was characterized to be a stromal enzyme (Fig. 8B, Chapter VII).

4. DISCUSSION

The buffer extract of rice leaves and seed embryos showed four Cu,Zn-SOD and one Mn-SOD on polyacrylamide gel after electrophoresis (Fig. 1). Moreover, isoelectric focusing on polyacrylamide gel revealed the occurrence of one more Mn-SOD having a pI of 9. Cu,Zn-SOD-I was a major isozyme in the leaves, while Cu,Zn-SOD-II, -III, and -IV were dominant in the embryos and seeds. Content of Cu,Zn-SOD-I was very low in the dry seeds and increased with the greening process after germination. The content of Cu,Zn-SOD-II, -III, and -IV were constant or rather decreased after germination. These results suggest that Cu,Zn-SOD-I is a chloroplast enzyme while other isozymes are not.

The purified Cu,Zn-SOD-I and -II were very similar in respects of molecular weight (Fig. 4, 5), subunit structure (Fig. 3), absorption spectrum (Fig. 6) and metal content. Amino acid composition (Tabe II) cleary indicated that Cu,Zn-SOD-I and -II were products of different genes.

All Cu,Zn-SOD so far isolated are homodimers having a molecular weight of 30,000-33,000. Only one cyanide-sensitive mammalian SOD has a high moleucular weight (18), but its relation in amino acid sequence of typical Cu,Zn-SOD is not known. Cu,Zn-SOD is unique in structure: β-barrel structure composed with eight anti-pararel β-sheets (19,20) which results in extreme stability against denaturation stresses such as heat and detergents. Subunit association is also stable against denaturating agents and it is impossible to prepare the monomer without loss of activity. Very low activity has been shown to associate with monomer of wheat Cu,Zn-SOD on SDS-polyacrylamide gel electrophoresis (21,22). Subunit exchange of maize Cu,Zn-SOD isozymes has been demonstrated (23). However, no monomeric SOD bas been isolated which has activity. Rice Cu,Zn-SOD-IV purified here was a monomer; the SOD-IV was eluted at the position of half molecular weight of rice Cu,Zn-SOD-I and -II on Sephadex G-100 column (Fig. 4), but it migrated to the same position as that of the SOD-I on SDS-polyacrylamide gel electrophoresis (Fig. 3). The specific activity of Cu,Zn-SOD-IV was almost the same as those of Cu,Zn-SOD-I and -II (7,000 units/mg protein).

Monomer-dimer equilibrium of Cu,Zn-SOD-IV was unlikely, because no dimerization was observed in Sephadex G-100 gel filtration. Furthermore, the following observations indicate that Cu,Zn-SOD-IV was not a subunit of other Cu,Zn-SOD isozymes: 1) Anti-rice Cu,Zn-SOD-I did not react with the SOD-IV. 2) The subunit molecular weight of SOD-II was different with the molecular weight of SOD-IV (Fig. 3). And 3) the relative activity ratio of Cu,Zn-SOD-IV to -III on polyacrylamide gel differed in both seed embryo and leaf extracts, suggesting that Cu,Zn-SOD-IV was not in equilibrium with the SOD-III. These results suggest that the SOD-IV is a monomeric Cu,Zn-SOD distincted from Cu,Zn-SOD-I, -II, and -III.

It has been shown that spinach stromal and cytosolic Cu,Zn-SOD isozymes are immunologically distinguishable (Chapter V). In the present work, anti-spinach stromal and cytosolic Cu,Zn-SOD also distinguished rice Cu,Zn-SOD isozymes and classified them into stromal and cytosolic enzymes: Cu,Zn-SOD-I is a stromal enzyme and the others are cytosolic enzymes (Fig. 8). I tested the cross-reactivity of anti-spinach Cu,Zn-SOD isozymes with the extracts of the gymnosperm Cycas revoluta and the eukaryotic green alga Spirogyra sp., and found that these gymnosperm and green alga also cotained two types of immunologically distinguishable Cu,Zn-SOD like angiosperms (data not shown). These specific antisera allowed me to determine the type of Cu,Zn-SOD in photosynthetic plants.

The occurrence of two types of immunologically distinguishable Cu,Zn-SOD isozymes in angiosperms, gymnosperms, and green alga, and large difference in amino acid sequences of spinach Cu,Zn-SOD isozymes (Chapter V) indicates that both groups of Cu,Zn-SOD isozymes had been separated at an early phase of the evolution, i.e. before the emergence of invertebrates, fungi and land plants from their ancestral organisms (Chapter III). It would be resonable to assum that the divergence of both types of enzyme from ancestral protein might take place when phragmoplast green algae acquired Cu,Zn-SOD.

5. SUMMARY

Rice leaves and seed embryos contained four Cu,Zn-superoxide dismutase (SOD) and two Mn-SOD as judged from the activity staining on a polyacrylamide gel after electrophoresis and isoelectric focusing. Three Cu,Zn-SOD (Cu,Zn-SOD-I, -II, and -IV) were purified from the leaves to a homogeneous state. The purified Cu,Zn-SOD-I and -II had similar properties in respect of molecular weight, dimeric structure, absorption spectrum, and metal content, but the amino acid composition was different. Cu,Zn-SOD-IV was a monomer based on the molecular weight as determined by gel filtration and SDS-polyacrylamide gel electrophoresis. Antibody against Cu,Zn-SOD-I did not react with Cu,Zn-SOD-II and -IV. Anti-spinach stromal Cu,Zn-SOD cross-reacted with rice Cu,Zn-SOD-I, but not with Cu,Zn-SOD-II and -IV. On the contrary, anti-spinach cytosolic Cu,Zn-SOD (Chapter V) cross-reacted with rice Cu,Zn-SOD-II and -IV, but not with Cu,Zn-SOD-I. Thus, rice Cu,Zn-SOD-I is a stromal enzyme and rice Cu,Zn-SOD-II and -IV are cytosolic enzymes.

6. REFERENCES

(1) Beauchamp, C.O., and Fridovich, I. (1973) Biochim. Biophys. Acta 317, 50-64.

(2) Baum, J.A., and Scandalios, J.G. (1981) Arch. Biochem. Biophys. 206, 249-264.

(3) Baum, J.A., Chandlee, J.M., and Scandalios, J.G. (1983) Plant Physiol. 73, 31-35.

(4) Yoshida, S., Forno, D.A., Cock, J.H., and Gomez, K.A. (1976) Laboratory Mannual for Physiological Studies of Rice, 3rd ed, pp. 61-66, The International Rice Research Institute, Philippins

(5) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(6) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

(7) Sevilla, F., Lopez-Gorge, J., Gomez, M., and del Rio, L.A. (1980) Planta 150, 153-157.

(8) Yphantis, D.A. (1964) Biochemistry 3, 297-317.

(9) Cohn, E.J., and Edsall, J.T. (1941) Proteins, Amino Acids and Peptides, p. 375, Reinhold, New York.

(10) Hayakawa, T., Kanematsu, S., and Asada, K. (1985) Planta 166, 111-116.

(11) Laemmli, U.K. (1970) Nature (London) 277, 680-685.

(12) Asada, K., Urano, M., and Takahashi, M. (1973) Eur. J. Biochem. 36, 257-266.

(13) Moore, S. (1963) J. Biol. Chem. 238, 235-237.

(14) Edelhoch, H. (1967) Biochemistry 6, 1948-1954.

(15) Cockle, S.A., and Bray, R.C. (1977) in Superoxide and Superoxide Dismutases (Michelson, A.M., McCord, J.M., and Fridovich, I., eds.), pp. 215-216, Academic Press, London.

(16) Malinowski, D.P., and Fridovich, I. (1979) Biochemistry 18, 237-244.

(17) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(18) Marklund, S.L. (1982) Proc. Natl. Acad. Sci. USA 79, 7634-7638.

(19) Richardson, J.S., Thomas, K.A., Rubin, J.H., and Richardson, D.C. (1975) Proc. Natl. Acad. Sci. USA 72, 1349-1353.

(20) Tainer, J.A., Getzoff, E.D., Beem, K.M., Richardson, J.S., and Richardson, D.C. (1982) J. Mol. Biol. 160, 181-217.

(21) Marmocchi, F., Venardi, G., Caulini, G., and Rotilio, G. (1974) FEBS Lett. 44, 337-339.

(22) Rigo, A.R., Marmocchi, F., Cocco, D., Viglino, P., and Rotilio, G. (1978) Biochemistry 17, 532-537.

(23) Baum, J.A., and Scandalios, J.G. (1982) J. Hered. 73, 95-100.

CHAPTRE VII

Cu,Zn-SUPEROXIDE DISMUTASE FROM THE FERN EQUISETUM ARVENSE: ITS PURIFICATION, AND PHYSICOCHEMICAL AND IMMUNOLOGICAL PROPERTIES

1. INTRODUCTION

As described previous chapters, Cu,Zn-superoxide dismutase (SOD) occurs in animals, fungi and land plants. This type of SOD had been considered to occur only in eukaryotes, but recently isolated from two bacteria, Photobacterium leiognathi (1,2) and Caulobacter crescentus (3). The Photobacterium Cu,Zn-enzyme has been shown to relate to the eukaryotic Cu,Zn-SOD on the basis of amino acid sequence alignment, and this is interpreted by a gene transfer from eukaryotes to prokaryotes (4-6). On the other hand, the Caulobacter Cu,Zn-enzyme has been considered to be the case of divergence and to be rather the most primitive Cu,Zn-SOD (3). Among eukaryotes, most eukaryotic algae lack Cu,Zn-SOD except phragmoplast green algae (7,8). I have shown that Spirogyra contains Cu,Zn-SOD by its purification (chapter III).

Under these circumstances, it is desirable to compare bacterial Cu,Zn-SOD with the Cu,Zn-SOD from primitive eukaryotes. Since prokaryotes except above two bacteria, and most of eukaryotic algae lack the Cu,Zn-SOD but contain Fe- and/or Mn-SOD, the most primitive eukaryotes possesing the Cu,Zn-SOD seems to be green algae (Chapter III). Although Cu,Zn-SOD has been isolated from vertebrates, invertebrates, fungi, angiosperms, and green alga, the enzyme has not been isolated yet from lower plants, i.e. fern or moss. I purified the Cu,Zn-SOD from the fern Equisetum arvense and report its properties.

2. MATERIALS AND METHODS

Materials

Cytochrome c (horse heart, type III) and xanthine oxidase (bovine milk) were obtained from Sigma Chemical Co., St. Louis, MO, USA, and Boehringer Mannheim, Germany, respectively. The standard proteins for isoelectric points and molecular weights, DEAE-Sephadex, DEAE-Sephacel, and Sephadex G-100 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Ampholine was obtained from LKB-Produkter, Bromma, Sweden. DEAE Affi-Gel Blue and Bio-Gel HTP were products of Bio-Rad Laboratories, Richmond, CA, USA. Bovine Cu,Zn-SOD was obtained from Diagnostic Data, USA.

Equisetum arvense was collected in the campus in May and August, and stored at -20° C. Spinach Cu,Zn-SOD was purified from the leaves as described Chapter V. Spinach contains two isozymes of Cu,Zn-SOD, and the purified enzyme was a one in the leaves localized in chloroplast stroma (Chapter V). Porphyra yezoensis Mn-SOD and Azotobacter vinelandii Fe-SOD were purified to a homogeneous state (Chapter XIV and XV).

Antibodies

Anti-Equisetum Cu,Zn-SOD anti-spinach stromal and cytosolic Cu,Zn-SOD sera were prepared as described in Chapter II and V. Ouchterlony double immunodiffusion was conducted in a 1.5 % agar plate containing 0.15 M NaCl and 20 mM potassium phosphate, pH 7.8, at 37°C for 9-12 hours. Crossed-immunoelectrophoresis was carried out as described in Chapter V.

SOD assay

SOD was assayed by the inhibition of the reduction of cytochrome c with the O_2^- generated by the xanthine-xanthine oxidase system (Chapter II). This procedure is a modification of that of McCord and Fridovich (9). The modified procedure gives a threefold yield, in terms of units, as the reaction volume is reduced to one third of the original method. The enzyme activity is presented by the McCord and Fridovich unit (9).

Other methods

Polyacrylamide gel electrophoresis (PAGE), location of SOD activity on polyacrylamide gel, and the determination of the molecular weight by

gel filtration and ultracentrifugation were done as described previously (Chapter V). SDS-PAGE was conducted by the method of Laemmli (10). Isoelectric focusing was carried out with Ampholine (pH 3.5-10) in a 5% polyacrylamide slab gel. Metal contents were determined by a Hitachi-Perkin Elmer 303 spectrometer with a graphite atomizer. Amino acid composition was determined using a Hitachi 835 amino acid analyzer. Optical and Circular dichroism spectra were recorded using a Shimadzu MPS-2000 spectrophotometer and a JASCO J-500C spectropolarimeter equipped with a data processor Model DP-501.

3. RESULTS

Purification of Equisetum SOD

Horse tail (300 g batch, total 9.8 kg) was thoroughly ground with one liter of 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA, 300 g of wet Polyclar AT (polyvinylpyrrolidone) and sea sand for 1-2 hours using an electric mortar. Polyclar AT had been washed with 1 M HCl and 1 M KOH, then with distilled water (11), and equilibrated with the buffer before use. Addition of Polyclar AT caused twofold increase of activity. 2-Mercaptoethanol was not included in the homogenizing medium because of the possible reduction of disulfide bond of the enzyme.

The homogenate was clarified by centrifugation at 11,000 g for 30 min, and ammonium sulfate was added to make 45% saturation to the supernatant. After overnight standing, the sediment was removed by centrifugation, and ammonium sulfate was added to the supernatant to 90% saturation. The precipitate collected by centrifugation at 11,000 g for 30 min was dissolved in and dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. The dialyzed enzyme was applied to a DEAE-Sephadex column (10 x 28 cm) equilibrated with the same buffer. After washing the column with 5 liters of the equilibrating buffer, the enzyme was eluted successively with 5 liters each of 100 mM, 200 mM and 500 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. SOD was found in the 100 mM and 200 mM fractions.

The enzyme was concentrated through an Amicon PM 10 membrane, and

then dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. The concentrated enzyme was adsorbed to a DEAE-Sephadex column (2.5 x 42 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA and eluted by a linear gradient of potassium phosphate, pH 7.8 (10 to 150 mM, 3 liters), containing 0.1 mM EDTA. SOD was eluted at 46 mM potassium phosphate. Faint blue enzyme was concentrated through the membrane filter as above to 10 ml and applied to a Sephadex G-100 column (4 x 87 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, and 0.1 M KCl. The enzyme was eluted with the same buffer as a symmetrical peak.

The enzyme was concentrated through an Amicon YM 5 membrane instead of PM 10, because a portion of SOD was leaked in the filtrate through a PM 10 membrane. During the concentration, the buffer was changed to 5 mM potassium phosphate, pH 7.8. The concentrated enzyme was adsorbed onto a column of DEAE-Sephacel (1.5 x 10 cm) equilibrated with 5 mM potassium phosphate, pH 7.8, and then eluted with a linear gradient of potassium phosphate, pH 7.8 (5 to 60 mM, 500 ml). The enzyme was eluted at 33 mM potassium phosphate and most of brown components remained on the top of the gel.

Table I

Summary of the Purification of Cu,Zn-SOD form Equisetum arvense

Purification step	Total protein (mg)	Total[1] activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Crude extract	240,000	89 x 10^4	3.7	100	1
45 - 90% $(NH_4)_2SO_4$	14,840	62	42	70	11
Stepwise DEAE-Sephadex	489	39	792	44	214
Linear gradient DEAE-Sephadex	98	27	2755	30	828
Sephadex G-100	38	17	4474	19	1209
DEAE-Sephacel[2]	27	15	5591	17	1511
Hydroxylapatite[2]	21	15	7076	17	1904

1) McCord & Fridovich unit

2) $A^{1\%}_{1\ cm}$ at 258 nm = 3.80

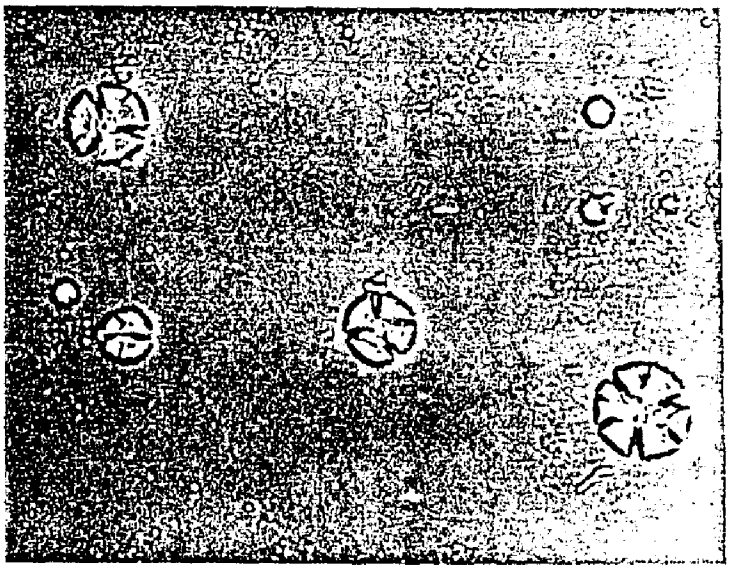

Fig. 1. Crystals of Equisetum Cu,Zn-SOD (x 400).

The active fractions were concentrated using an Amicon YM 5 membrane and the buffer was changed to 2 mM potassium phosphate, pH 6.8. The enzyme was applied to a column of hydroxylapatite (Bio-Gel HTP) (1.5 x 2 cm) equilibrated with 2 mM potassium phosphate, pH 6.8. Blue-green SOD was not adsorbed to the column but brown components were retained on the gel. The enzyme was eluted with 2 mM potassium phosphate, pH 6.8. The purified SOD was dialyzed against 10 mM potassium phosphate, pH 7.8, and stored at -20°C, or at 4°C after addition of ammonium sulfate to 80% saturation. Table I summarizes the purification of enzyme from 9.8 kg of horse tail.

Powdered ammonium sulfate was gradually added until turbidity appeared, and kept at 4°C. After several days, fine crystals in irregular shape were obtained (Fig. 1).

Purity

The purified enzyme showed a single protein band which corresponded to the activity band of SOD on native PAGE (Fig. 2, left panel). The location of the purified enzyme in the gel was identical with the activity band of cell extract, indicating no modification of the enzyme during purification (Fig. 2, right panel). SDS-PAGE in the presence or absence of 2-mercaptoethanol, and isoelectric focusing in Ampholine-

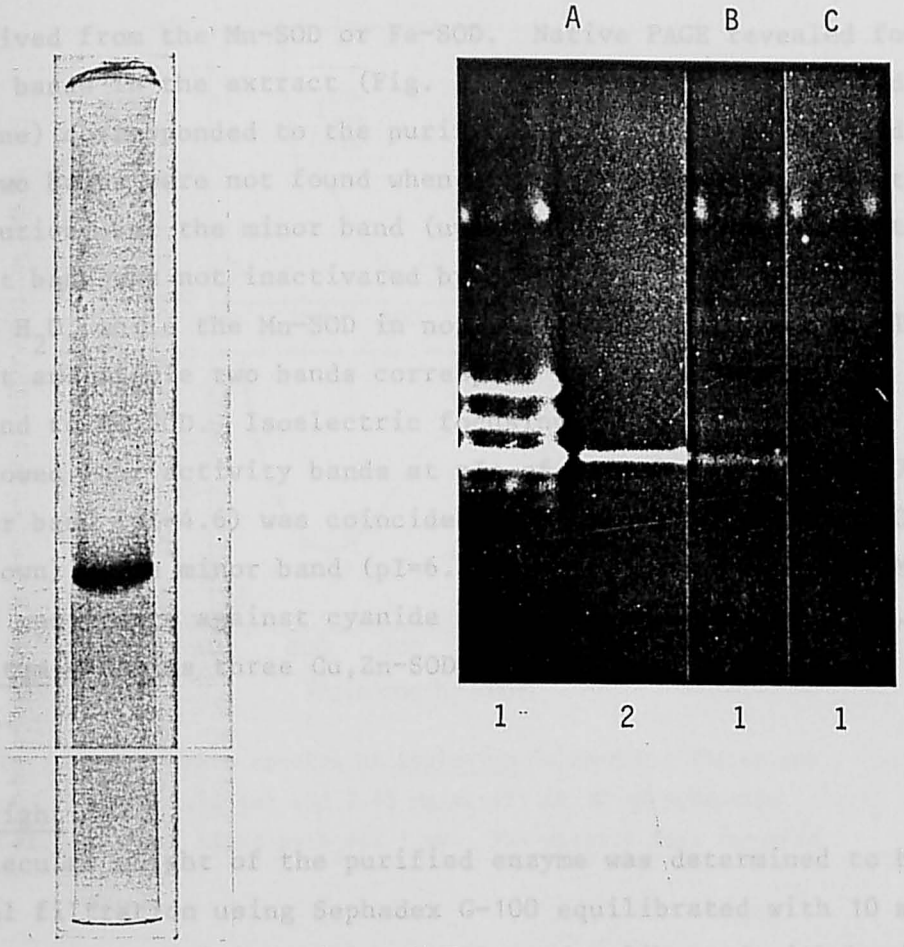

Fig. 2. Native polyacrylamide gel electrophoresis of Equisetum Cu,Zn-SOD. (Left panel): Purified Equisetum Cu,Zn-SOD (40 μg). Protein was stained with Coomassie Brilliant Blue R-250. (Right panel): 1, cell extract (3 units); 2, purified Equisetum Cu,Zn-SOD (1 unit) (A); stained in the presence of 2 mM KCN (B); stained after the treatment with 3 mM H_2O_2. Achromatic band shows the SOD activity. Top, anode; bottom, cathode.

polyacrylamide gel also gave a single band (data not shown). The purity of the enzyme was also confirmed by sedimentation pattern in ultracentrifugation (Fig. 4).

Isozymes

The SOD activity in the buffer extract of Equisetum was inhibited by 91 % with 1 mM cyanide, indicating that most SOD activity was responsible to the Cu,Zn-SOD, and minor cyanide-insensitive activity

might be derived from the Mn-SOD or Fe-SOD. Native PAGE revealed four SOD activity bands in the extract (Fig. 2, right panel). Major band (lowermost one) corresponded to the purified enzyme. The major band and the middle two bands were not found when 1 mM cyanide was added in the staining solution, but the minor band (uppermost one) was not affected. The uppermost band was not inactivated by 3 mM H_2O_2. The Fe-SOD is sensitive to H_2O_2 while the Mn-SOD in not (12, see Chapter XIII). Thus, the lowermost and middle two bands correspond to the Cu,Zn-SOD, and the uppermost band to Mn-SOD. Isoelectric focusing of the extract of Equisetum showed four activity bands at pIs of 4.6, 5.1, 5.4 and 6.7, and the major band (pI=4.6) was coincided with the purified Cu,Zn-SOD (data not shown). The minor band (pI=6.7) was characterized to be Mn-SOD from its resistance against cyanide and the treatment with H_2O_2. Thus, Equisetum contains three Cu,Zn-SOD isozymes and Mn-SOD in a soluble form.

Molecular weight

The molecular weight of the purified enzyme was determined to be 32,400 by gel filtration using Sephadex G-100 equilibrated with 10 mM potassium phosphate, pH 7.8, and 0.1 M KCl, and calibrated with bovine serum albumin, ovalbumin, α-chymotrypsinogen, and cytochrome c. The molcular weight of the enzyme was also determined by the sedimentation equilibrium in 5 mM potassium phosphate, pH 7.8, and 0.1 M KCl according to Yphantis (13). Analytical ultracentrifugation operated at 28,453 rpm gave a linear line in the plot of log(fringe displacement) vs. square of the distance from the center of rotation (Fig. 4). The slope (0.662) and a partial specific volume (0.730) of the enzyme calculated from the amino acid composition (Table II) by the method of Cohn and Edsall (14) gave a molecular weight of 31,100.

Subunit structure

The subunit molecular weight of the enzyme was estimated by SDS-PAGE using 15% gel. When the enzyme was incubated in the presence or absence of 1% 2-mercaptoethanol with 1% SDS for 1 hour at 60°C, the electrophoresis showed two bands corresponding to molecular weights of

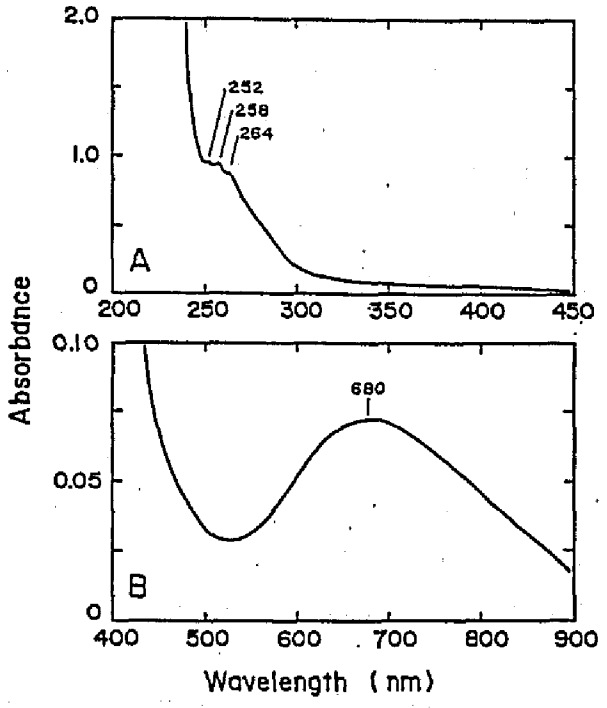

Fig. 3. Absorption spectra of Equisetum Cu,Zn-SOD. The enzyme concentration was 2.48 (A) and 7.45 mg/ml (B) in 10 mM potassium phosphate, pH 7.8. Light path was 1 cm. The spectra were recorded at 25°C.

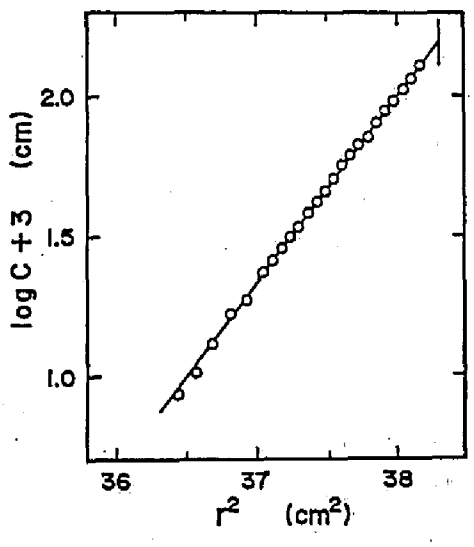

Fig. 4. Sedimentation equilibrium of Equisetum Cu,Zn-SOD. The purified enzyme (0.83 mg/ml) in 5 mM potassium phosphate, pH 7.8, and 0.1 M potassium chloride was equilibrated at a rotor speed of 28,453 rpm at 20°C. C, Fringe displacement; r, distance from the center of rotation.

32,000-33,000 and 17,000-20,000. When the enzyme was denatured at 100°C for 3 min, a single band was found corresponding to a molecular weight of 20,000 and 18,000 in the presence and absence of 2-mercaptoethanol, respectively. Higher subunit molecular weight in the presence of 2-mercaptoethanol has been observed for Cu,Zn-SOD (15). Thus, the Equisetum Cu,Zn-SOD is a homodimer and the subunits are not covalently linked through disulfide bridge.

Absorption spectrum

Fig. 3 shows the absorption spectrum of the Equisetum enzyme. In ultraviolet region the enzyme exhibits absorption peaks at 252, 258 and 264 nm due to phenylalanine residues. The spectrum shows little absorption in a region of 280 and 290 nm, indicating the absence of tyrosine and tryptophan residues as confirmed by amino acid composition (Table II). The molar absorbance coefficient at 258 nm was 11,800 $M^{-1}cm^{-1}$ and its $A_{1\ cm}^{1\%}$ was 3.80, based on a molecular weight of 31,100 of Cu,Zn-SOD. The spectrum in visible region shows a peak at 680 nm, which is characteristic of the Cu-chromophore of Cu,Zn-SOD due to d-d transition (16). The molar absorbance coefficient at 680 nm was 317 $M^{-1}cm^{-1}$.

Metal analysis

The enzyme was dialyzed against 5 mM Tris-HCl, pH 7.8, for three days with several changes of the buffer and its metal contents were determined. The enzyme contained 2.00 g-atoms of Cu and 1.95 g-atoms of Zn per mol of enzyme. Fe and Mn were not detected or lower than the limit of detection. Thus, Equisetum SOD contains one atom of each Cu and Zn per subunit.

Amino acid composition

Table II shows the amino acid composition of Equisetum Cu,Zn-SOD with that of spinach Cu,Zn-SOD for comparison. The Equisetum enzyme lacked tyrosine, and no tryptophan was detected by the method of Edelhoch (17) as expected by the absorption spectrum (Fig. 3). The Equisetum enzyme had higher contents of serine, isoleucine and phenylalanine, and lower contents of aspartic acid, threonine, and valine than

Table II

Amino Acid Composition of Equisetum Cu,Zn-SOD

Amino acid	*Equisetum* Cu,Zn-SOD[a,b]		Spinach Cu,Zn-SOD[g]	
	Residues/mol	Nearest integer	Stromal	Cytosolic
Aspartic acid	31.01	31	38	35
Threonine[c]	19.89	20	32	26
Serine[c]	16.53	17	10	20
Glutamic acid	26.07	26	22	19
Proline	20.42	20	18	17
Glycine	47.81	48	46	60
Alanine	24.43	24	24	20
Half-cystine[d]	4.16	4	4	4
Valine[e]	26.40	26	36	30
Methionine[d]	1.54	2	2	2
Isoleucine[e]	12.85	13	8	13
Leucine	24.40	24	24	19
Tyrosine	0	0	0	2
Phenylalanine	10.76	11	6	6
Lysine	14.96	15	14	9
Histidine	16.29	16	16	16
Arginine	6.95	7	8	8
Tryptophan[f]	0	0	0	0
Total residues		304	308	306

a) The enzyme was hydrolyzed in 6 M HCl for 24, 48, and 72 hours in duplicate

b) Based on a molecular weight of 31,100.

c) Determined by linear extrapolation to zero time.

d) Determined as methionine sulfone and cysteic acid after performic acid oxidation.

e) Determined from 72-hydrolysis values.

f) Determined spectrophotometrically according to Edelhoch (17).

g) See Chapter V.

that of the spinach enzyme. Two half-cystine residues were contained in subunit and would form an intramolecular disulfide bridge, because Cu,Zn-SOD isolated so far has a disulfide bond (18).

Amino acid composition of the Equisetum Cu,Zn-SOD was compared with those of animal, fungi and plant enzymes to estimate the sequence homology by SΔQ values. The SΔQ is a statistical analysis of amino acid

Table III

Comparison of SΔQ Values of Cu,Zn-SOD from Equisetum, Angiosperms, Fungi, and Animals

		(A)	(B)	(C)	(D)	(E)	(F)	(G)
(A) Horsetail		-						
(B) Wheat	(23)	33	-					
(C) Spinach	(22)	33	29	-				
(D) Yeast	(26)	36	70	60	-			
(E) *Neurospora*	(27)	49	52	34	41	-		
(F) *Drosophila*	(28)	34	60	64	21	44	-	
(G) Bovine	(29)	34	45	47	31	30	17	-
(H) Human	(30)	35	77	70	28	53	10	14

SΔQ value was calculated from the amino acid composition of Cu,Zn-SOD using the following equation (19):

$$S\Delta Q = \sum_{j} (X_{i,j} - X_{k,j})^2,$$

where the subscripts i and k indicate the enzymes which were compared and X_j is the mole percent content of a given amino acid of type j. In the present calculation 18 amino acid residues were used. Numbers in the parentheses refer to references from which data were taken.

composition and defined as the sums of the squares of the difference between the mol percent content of individual amino acid residue in the two proteins compared (19). Table III is a matrix of SΔQ values obtained form the pairwise comparision. The SΔQ values between animal enzymes and between angiosperm enzymes were 10-33, suggesting that the enzymes from animals or angiosperms are closely related in the sequence. However, the SΔQ values of the enzymes between animals and angiosperms were higher than those within each group, reflecting the evolutional distance between them. To the contrary, the SΔQ values of the Cu,Zn-SOD between Equisetum and angiosperms, fungi or animals were similar. This may be a reflection that Equisetum had appeared earlier than animals and angiosperms.

Isoelectric point

pI of the purified Equisetum enzyme was determined to be 4.55 by isoelectric focusing. Isoelectric focusing of the extract of Equisetum followed by activity staining revealed pI of 5.2 for the minor Cu,Zn-SOD isozyme and 6.7 for the Mn-SOD in addition to pI of 4.6 for the the major Cu,Zn-SOD isozyme.

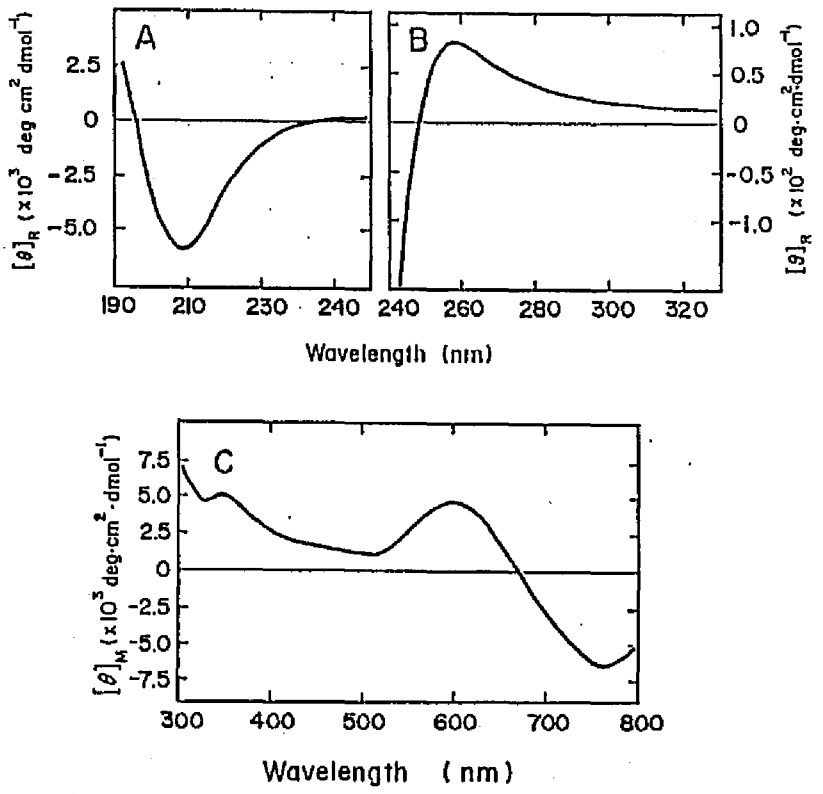

Fig. 5. Circular dichroism spectra of Equisetum Cu,Zn-SOD. The concentration of Equisetum enzyme in 10 mM potassium phosphate, pH 7.8, and light path were 0.248 mg/ml, 1 mm (A); 2.48 mg/ml, 1 mm (B); and 2.48 mg/ml, 10 mm (C), respectively. The spectra were recorded at 25°C.

Circular dichroism spectra

CD spectrum of Equisetum SOD shows a negative band at 208 nm but no double minimum which is characteristic of α-helix conformation (Fig. 5). The mean residue ellipticity, $[\theta]_R$, at 208 nm was -5,880·deg·cm^2 dmol^{-1}. The spectrum resembled that of bovine Cu,Zn-SOD for which the three-dimentional structure has been determined (20). Spinach Cu,Zn-SOD isozymes also show the same spectra (Chapter V). Thus, plant Cu,Zn-SOD are supposed to have β-barrel structure as the bovine enzyme. A positive band at 260 nm could be seen but that around 290 nm not, in agreement with absence of tyrosine and tryptophan (Table II).

The spectrum in visible region of Equisetum SOD resembled that of the bovine enzyme: positive bands at 350, 450, and 600 nm, and a negative band at 750 nm (Fig. 5). The molar ellipticity, $[\theta]_M$, at 600 nm was 4,770 deg·cm^2·dmol^{-1}. The spectrum suggests that the Equisetum

Cu,Zn-SOD has the similar geometry around Cu site as that of the bovine enzyme.

Effect of 2,3-buthanedione

It has been shown that arginine-141 of the bovine Cu,Zn-SOD plays an important role in the catalysis through the attraction of anionic superoxide radical to the Cu site (21). I conducted the chemical modification of arginine residue(s) of the Equisetum enzyme with 2,3-buthanedione.

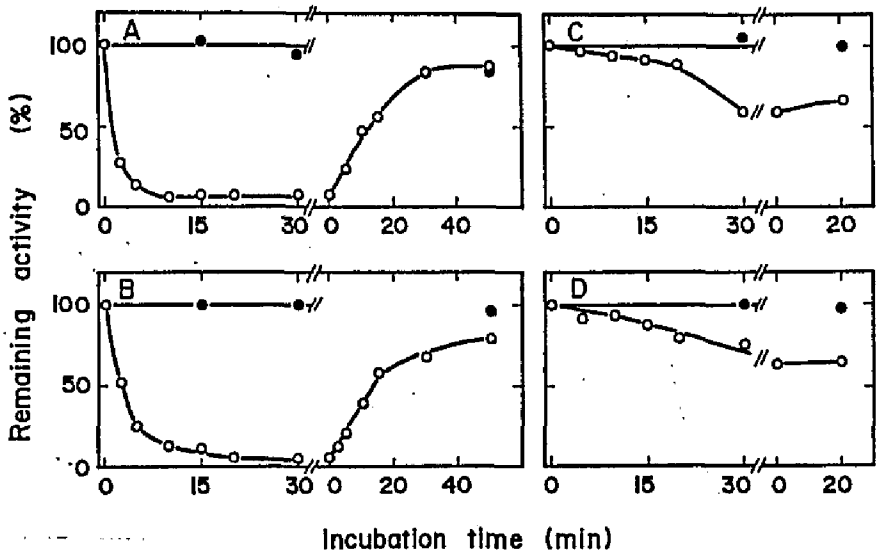

Fig. 6. Effects of 2,3-buthanedione on Equisetum Cu,Zn-SOD (A), spinach Cu,Zn-SOD (B), Azotobacter Fe-SOD (C) and Porphyra Mn-SOD (D). SOD (1.1-1.3 μM) were incubated with 0.41 ml of 30 mM 2,3-buthanedione in 50 mM borate buffer, pH 9.0, at 25°C. The remaining activity was measured at indicated time periods using an aliquot (10 μl) of the incubation mixture in the xanthine-xanthine oxidase-cytochrome c system. The assay mixture of SOD contained 0.1 mM mannitol to trap borate which inhibits xanthine oxidase, and the borate and 2,3-buthanedione contained in the treated enzyme did not interfere the assay. SOD was measured within 30 sec after adding the treated enzyme to the assay mixture, and the restoration of the activity was negligible small. After 30-min incubation with 2,3-buthanedione, an aliquot of the treated enzyme was diluted by the assay mixture containing 0.1 mM mannitol without xanthine oxidase, and the borate was trapped by the mannitol for indicated time periods. The SOD activity was assayed by adding xanthine oxidase to follow the reactivation. Control experiment was done using distilled water instead of 2,3-buthanedione (●——●).

The enzyme was incubated with 2,3-buthanedione in borate buffer, and the remaining activity was determined (Fig. 6). The activity was almost completely lost by the incubation. The treated enzyme was reactivated by removing borate, which stabilized the buthanedione-

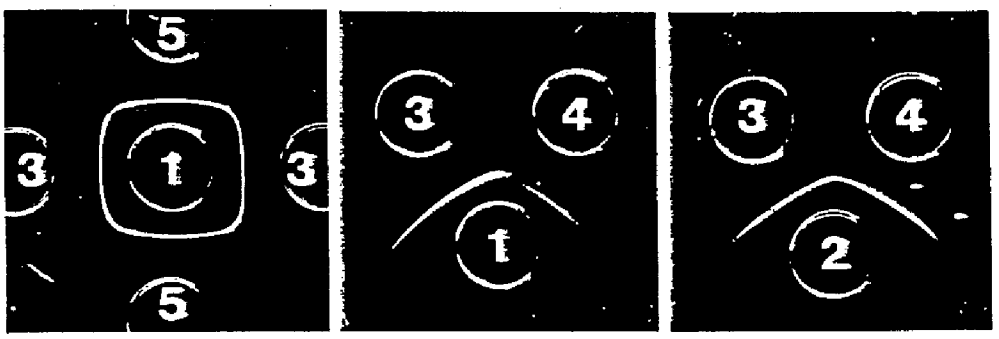

Fig. 7. Ouchterlony double immunodiffusion of anti-Equisetum Cu,Zn-SOD and anti-spinach stromal Cu,Zn-SOD sera with Equisetum and spinach stromal Cu,Zn-SOD. 1, Anti-Equisetum SOD (195 μg); 2, anti-spinach stromal SOD (390 μg); 3, Equisetum SOD (1 μg); 4, spinach stromal SOD (1 μg); 5, cell extract of Equisetum (7 units). Wells contained each 15 ul of sera or Cu,Zn-SOD.

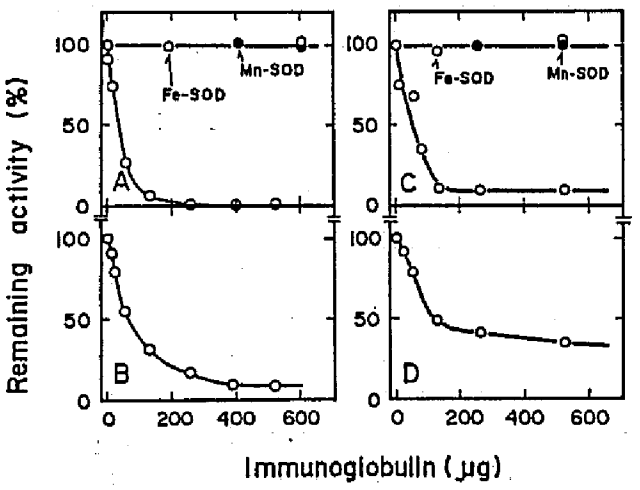

Fig. 8. Effects of anti-Equisetum and anti-spinach stromal Cu,Zn-SOD sera on Equisetum and spinach stromal Cu,Zn-SOD activities. SOD (0.5-0.6 units) were incubated with various amount of anti-sera in 220 μl of 20 mM potassium phosphate, pH 7.8, and 0.15 M NaCl for 1 hour at 4°C. The remaining activity was assayed using the xanthine-xanthine oxidase-cytochrome c system. Effect of anti-Equisetum SOD serum on the Equisetum (A), and spinach stromal (B) Cu,Zn-SOD; effect of anti-spinach stromal SOD serum on the spinach stromal (C) and Equisetum (D) Cu,Zn-SOD. The Azotobacter Fe-SOD (Chapter XV) and Porphyra Mn-SOD (Chapter XIV) were used for control.

arginine complex, from the reaction mixture by incubation in the assay mixture of SOD containing mannitol. After 50-min incubation about 80% of the initial activity was restored. Therefore the Arg residue in both Equisetum and spinach Cu,Zn-SOD appear to participate in facilitating the attraction of the anionic substrate to the active site during the catalysis, as in the bovine Cu,Zn-SOD. In contrast to the Cu,Zn-SOD, Azotobacter Fe-SOD and Porphyra Mn-SOD were affected slightly with a complicated kinetics, and the inactivated activity were not restored after further 20 min-incubation as above. This indicates that arginine residue is not directly involved in the catalysis of Fe-SOD and Mn-SOD.

Immunological properties

The antibody against the Equisetum Cu,Zn-SOD formed a single precipitin line with the purified enzyme and also with the extract of Equisetum in Ouchterlony double immunodiffusion (Fig. 7). Anti-Equisetum Cu,Zn-SOD serum cross-reacted with the spinach stromal Cu,Zn-SOD, but not with the bovine Cu,Zn-SOD (data not shown), and anti-spinach stromal Cu,Zn-SOD serum formed a precipitin line with the Equisetum

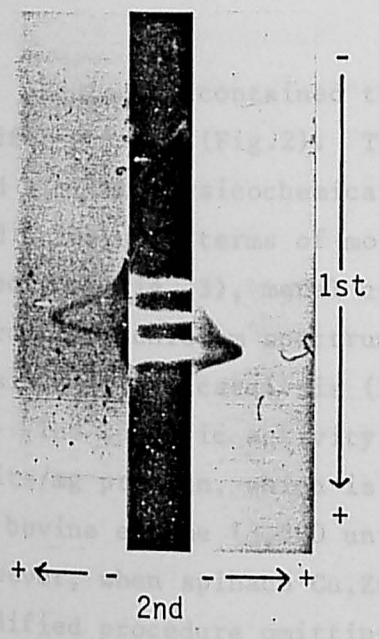

Fig. 9. Crossed-immunoelectrophoresis of Equisetum Cu,Zn-SOD isozymes. SOD in cell-free extract from Equisetum (3 units) were separated on 15% polyacrylamide slab gel (9 cm in length) in the first direction according to Laemmli (10). Top, anode; bottom, cathode. One lane was stained for SOD activity by the method of Beauchamp and Fridovich (31), and unstained lane was subjected to immunoelectrophoresis in the second direction. Electrophoresis was carried out on 1.2% agarose gel (10 cm in width x 5 cm in length) in barbital buffer (ionic strength 0.025), pH 8.6, containing 4% polyethylene glycol 4000 and 25 $\mu g/cm^2$ of anti-spinach cytosolic Cu,Zn-SOD (left gel) or 24 $\mu g/cm^2$ of anti-spinach stromal Cu,Zn-SOD (right gel) at a constant voltage of 20 V for 17 hours. The electrode buffer was barbital buffer (ionic strength 0.05), pH 8.6.

enzyme. However, anti-spinach cytosolic Cu,Zn-SOD serum did not cross-reacted with the Equisetum enzyme (data not shown). Thus, Equisetum Cu,Zn-SOD possesses the antigenic determinat common to the spinacch stromal enzyme. The anti-Equisetum Cu,Zn-SOD serum inhibited completely Equisetum SOD, and by 90% the spinach stromal Cu,Zn-SOD. Anti-spinach stromal Cu,Zn-SOD serum inhibite both spinach stromal and Equisetum enzymes by 90% and 60%, respectively (Fig. 8), in accordance with the reactivity of antiserum with SOD.

Stromal and cytosolic Cu,Zn-SOD isozymes of spinach and rice are distinguishable using anti-spinach stromal and cytosolic Cu,Zn-SOD sera (Chapter V and VI). Crossed-immunoelectrophoresis clearly indicates that the Equisetum extract contained both stromal and cytosolic Cu,Zn-SOD (Fig. 9). The lowermost band (corresponding to the purified enzyme) cross-reacted with anti-spinach stromal Cu,Zn-SOD, but not with anti-spinach cytosolic SOD. To the contrary, the upper two Cu,Zn-SOD isozymes formed precipitins with anti-cytosolic SOD but not with anti-stromal enzyme. Thus, the major Equisetum Cu,Zn-SOD is a stromal enzyme and minor two Cu,Zn-SOD isozymes are cytosolic enzymes.

4. DISCUSSION

Equisetum contained three Cu,Zn-SOD isozymes and one Mn-SOD in the buffer extract (Fig.2). The purified major Equisetum Cu,Zn-SOD isozyme had similar physicochemical properties to those from angiosperms, fungi and animals in terms of molecular weight, subunit structure, absorption spectrum (Fig. 3), metal content, amino acid composition (Table II), circular dichroism spectrum (Fig. 5), and the participation of arginine residue in the catalysis (Fig. 6).

The specific activity of the purified Equisetum Cu,Zn-SOD was 7,000 units/mg protein, which is double as compared with those of the spinach or bovine enzyme (3,500 units/mg protein) reported previously (22). However, when spinach Cu,Zn-SOD was purified from the leaves using a modified procedure omitting acetone fractionation, it showed a specific activity of about 8,000 units/mg protein (Chapter V). This was also the

case for rice Cu,Zn-SOD isozymes (Chapter VI). It seems that the fractionation with organic solvents modifies Cu,Zn-SOD so as to reduce its specific activity.

The three-dimensional structure of Cu,Zn-SOD has been reported for bovine (20). Its structural feature is a β-barrel structure composed of eight anti-pararel β-sheets with two small loops which are connected through disulfide bridge to the cylinder. The content of β-helix structure is only 5% and found in the loop regions. Recent X-ray diffraction study of spinach stromal Cu,Zn-enzyme at 2.8 Å revealed its β-barrel conformation similar to that of bovine enzyme (Kitagawa et al., personal communication). The circular dichroism spectrum of Equisetum enzyme suggests the similar structural feature (Fig. 5).

Comparison of amino acid composition of Equisetum Cu,Zn-SOD with those of animals, fungi, and angiosperms on the basis of SΔQ values indicated that the Equisetum enzyme resembled equally those of animals, fungi, and angiosperms (Table III). The SΔQ values obtained within animals, fungi, or angiosperms were smaller (10-40), while the values between each group were larger (40-70), well reflecting an early divergence of each group in the course of evolution. Therefore, the smaller values of SΔQ between Equisetum Cu,Zn-SOD and animal, fungi, or angiosperm Cu,Zn-SOD may reflect that Equisetum lies near the divergence point in the phylogenetic tree to animal, fungi, and seed plants.

Immunological studies revealed that Equisetum Cu,Zn-SOD was a stromal enzyme, and was more closely related to angiosperm stromal Cu,Zn-SOD than animal or fungi Cu,Zn-SOD. Anti-Equisetum Cu,Zn-SOD serum cross-reacted with spinach stromal Cu,Zn-SOD, and anti-spinach stromal Cu,Zn-SOD serum reacted with the Equisetum enzyme (Fig. 7). In addition, both antisera cross-reacted with the Cu,Zn-SOD from the eukaryotic green alga Spirogyra (Chapter III). However, both antisera failed to react with the purified bovine Cu,Zn-SOD nor the cell extracts of several fungi. Thus, the stromal Cu,Zn-SOD of plants including eukaryotic green algae, ferns and mosses, and angiosperms possess the common antigenic determinant(s), but not with those of animals or fungi.

The immunologically distinguishable stromal and cytosolic Cu,Zn-SOD isozymes have been purified from wheat (23), maize (24,25), spinach

(Chapter V), and rice (Chapter VI). Crossed-immunoelectrophoresis of Equisetum cell extract using anti-spinach stromal and cytosolic Cu,Zn-SOD sera indicated the presence of cytosolic Cu,Zn-SOD in addition to stromal Cu,Zn-SOD (Fig. 9). The occurrence of both types of Cu,Zn-SOD in the fern suggests their divergence and independent evolution prior to the appearance of ferns (see Discussion in Chapter V and VI). In short, the amino acid or DNA sequence information for both types of Cu,Zn-SOD from angiosperms, ferns, mosses and algae may shed light on the time when the two types of Cu,Zn-SOD diverged by extrapolating the degree of the sequence homology between the stromal and cytosolic enzymes to the same level.

5. SUMMARY

The fern Equisetum arvense (horse tail) contained three Cu,Zn-superoxide dismutase (SOD) isozymes and one Mn-SOD. One of the Cu,Zn-SOD was purified to a homogeneous state. The purified Equisetum Cu,Zn-SOD had a molecular weight of 31,100, consisted of two identical subunits and contained two atoms of each Cu and Zn per molecular. Its absorption spectrum showed peaks at 258 and 680 nm, bearing a resemblance to those of angiosperm Cu,Zn-SOD. Circular dichroism spectrum indicated the β-barrel structure which is characteristic of Cu,Zn-SOD. Amino acid composition of Equisetum Cu,Zn-SOD resembled those of angiosperm Cu,Zn-SOD. Ouchterlony double immunodiffusion and crossed-immunoelectrophoresis using anti-spinach stromal and cytosolic Cu,Zn-SOD sera showed the presence of stromal and cytosolic Cu,Zn-SOD isozymes in Equisetum. The purified Cu,Zn-SOD was shown to be a stromal enzyme.

6. REFERENCES

(1) Puget, K., and Michelson, A.M. (1974) Biochem. Biophys. Res. Commun. 58, 830-838.

(2) Martin, J.P., and Fridovich, I. (1981) J. Biol. Chem. 256, 6080-6089.

(3) Steinman, H.M. (1982) J. Biol. Chem. 257, 10283-10292.

(4) Steffens, G.J., Bannister, J.V., Bannister, W.H., Flohe, L., Gunzler, W.A., Kim, S.A., and Otting, F. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364, 675-690.

(5) Rocha, H.A., Bannister, W.H., and Bannister, J.V. (1984) Eur. J. Biochem. 145, 477-484.

(6) Bannister, J.V., and Parker, M.W. (1985) Proc. Natl. Acad. Sci. USA 82, 149-152.

(7) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(8) Henry, L.E.A., and Hall, D.O. (1977) in Photosynthetic Organelles (Miyachi, S., Fujita, Y., and Shibata, K., eds.), pp. 377-382, Japanese Soc. Plant Physiol., Kyoto.

(9) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(10) Laemmli, U.K. (1970) Nature (London) 277, 680-685.

(11) Loomis, W.D. (1974) Methods Enzymol. 31 528-544.

(12) Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y., and Enmanji, K. (1975) J. Biol. Chem. 250, 2801-2807.

(13) Yphantis, D.A. (1964) Biochemistry 3, 297-317.

(14) Cohn, E.J., and Edsall, J.T. (1941) Proteins, Amino Acids and Peptides, p. 375, Reinhold, New York.

(15) Marklund, S.L. (1982) Proc. Natl. Acad. Sci. USA 79, 7634-7638.

(16) Ballhausen, C.J. (1962) Introduction to Ligand Field Theory, pp. 57-98, McGraw-Hill, New York.

(17) Edelhoch, H. (1967) Biochemistry 6, 1948-1954.

(18) Steinman, H.M. (1982) in Superoxide dismutase (Oberley, L.W., ed.), pp. 11-68, CRC Press, Florida.

(19) Marchalonis, J.J., and Weltman, J.K. (1971) Comp. Biochem. Physiol.

38B, 609-625.

(20) Richardson, J.S., Thomas, K.A., Rubin, B.H., and Richardson, D.C. Proc. Natl. Acad. Sci. USA 72, 1349-1353.

(21) Malinowski, D.P., and Fridovich, I. (1979) Biochemistry 18, 5909-5917.

(22) Asada, K., Urano, M., and Takahashi, M. (1973) Eur. J. Biochem. 36, 257-266.

(23) Beauchamp, C.O., and Fridovich, I. (1973) Biochim. Biophys. Acta 317, 50-64.

(24) Baum, J.A., and Scandalios, J.G. (1983) Arch. Biochem. Biophys. 206, 249-264.

(25) Baum, J.A., Chandlee, J.M., and Scandalios, J.G. (1983) Plant Physiol. 73, 31-35.

(26) Steinman, H.M. (1980) J. Biol. Chem. 255, 6758-6765.

(27) Misra, H.P., and Fridovich, I. (1972) J. Biol. Chem. 247, 3410-3414.

(28) Lee, Y.M., Ayala, F.J., and Misra, H.P. (1981) J. Biol. Chem. 256, 8506-8509.

(29) Steinman, H.M., Naik, V.R., Abernethy, J.L., and Hill, R.L. (1974) J. Biol. Chem. 249, 7326-7338.

(30) Jabusch, J.R., Farb, D.L., Kerschensteiner, D.A., and Deutsch, H.F. (1980) Biochemistry 19, 2310-2316.

(31) Beauchamp, C.O., and Fridovich, I. (1971) Anal. Biochem. 44, 276-287.

CHAPTER VIII

Fe-SUPEROXIDE DISMUTASE FROM THE ANAEROBIC PHOTOSYNTHETIC BACTERIUM *CHROMATIUM VINOSUM*

1. INTRODUCTION

Superoxide dismutase (SOD) catalyzes the disproportionation of superoxide to molecular oxygen and hydrogen peroxide (1). This enzyme has been assumed to play a role in scavenging the superoxide radical and, thus, in protecting the cells from the deteriorative effects of superoxide itself and of other active oxygens such as hydrogen peroxide, the hydroxyl radical, and excited singlet oxygen which are formed from superoxide.

Three types of SOD in regard to functional metals have been isolated from aerobic organisms thus far (2). The Cu,Zn-SOD has been isolated form a wide range of eukaryotes including animals, higher plants, and fungi. The Mn-containing enzyme has been obtained from prokaryotes and from mitochondria in eukaryotic cells. The ferric enzyme, which is similar to the Mn-enzyme with respect to the amino acid sequence (3,4), has been isolated only from prokaryotes. From the role of SOD as a component of the defenses against oxygen toxicity, this enzyme was believed to be absent in obligate anaerobes (5). However, Hewitt and Morris (6) reported the occurrence of SOD in anaerobic photosynthetic bacteria, sulfate-reducing bacteria, and clostridia. Lumsden and Hall (7) and I (8,9) also confirmed the occurrence of SOD in anaerobic green and purple sulfur bacteria, *Chlorobium* and *Chromatium*. Recently, SOD from a sulfate-reducing bacterium has been characterized to be the Fe-enzyme (10).

To survey the physiological function of SOD in anaerobic photosynthetic bacteria and to follow the molecular evolution of the enzyme, I have purified and characterized SOD from *Chromatium*. The results in this chapter indicate that *Chromatium* cells contain only an iron-

containing enzyme.

2. MATERIALS AND METHODS

Cytochrome c (type III), xanthine oxidase, and Ampholine (pH 3.5-10) were obtained from Sigma, Boehringer, and LKB, respectively. The antibodies against the Fe- or Mn-SOD from Mycobacterium were the generous gift of Dr. E. Kusunose, Osaka city University. The antibody against the Fe-SOD from a blue-green alga, Plectonema boryanum (22), was prepared by immunization of rabbits as described in Chapter V.

The strain of Chromatium vinosum was kindly provided by Dr. T. Akazawa, Nagoya University. Cells were grown by standing culture in a bottle with a stopper photoautotrophically under illumination from incandescent lamps at 30°C for 4 days in the modified medium of Newton (11). This was composed of the following, per liter of tap water: NaCl, 10 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; NH_4Cl, 1 g; $MgCl_2 \cdot 6H_2O$, 0.5 g; $CaCl_2$, 0.05 g; $NaHCO_3$, 4 g; $FeCl_3 \cdot 6H_2O$, 5 mg; $Na_2S_2O_3 \cdot 5H_2O$, 2 g; $Na_2S \cdot 9H_2O$, 1 g. The concentration of oxygen in the medium was below detection with an oxygen electrode because of the removal of oxygen by sulfide and thiosulfate. The cells grown under strict anaerobic conditions were harvested at the late logarithmic phase and stored at -20°C until use.

SOD was assayed using the procedure of McCord and Fridovich (1) with a slight modification (12). One unit of SOD was defined as that amount of enzyme required to inhibit the reduction of cytochrome c by 50% under the assay conditions, which gave units threefold greater than those of McCord-Fridovich. Activity is presented in McCord-Fridovich units. Protein was determined according to Lowry et al. (13), using bovine serum albumin as standard.

Polyacrylamide gel disc electrophoresis was carried out using the method of Davis (14). For activity staining of SOD, a 10.25% acrylamide was polymerized with riboflavin and light. The activity bands were made visible by the method of Beauchamp and Fridovich (15).

The molecular weight of the enzyme was determined by gel filtration

using Sephadex G-100 equilibrated with 10 mM potassium phosphate, pH 7.8, containing 0.1 M NaCl according to Andrews (16) and by sedimentation equilibrium centrifugation using a Hitachi UCA-1A analytical ultracentrifuge according to Yphantis (17). The subunit molecular weight was determined by sodium dodecyl sulfate-polyacrylamide gel disc electrophoresis according to Weber and Osborn (18). SOD was denatured by incubation with 1% sodium dodecyl sulfate in the presence or absence of 1% 2-mercaptoethanol for 2 h at 50°C. Electrophoresis was performed in a 10% polyacrylamide gel at room temperature in 0.1% sodium dodecyl sulfate-0.1 M sodium phosphate, pH 7.2, with a current of 8 mA per tube for 4.5 h. The following proteins were used as molecular weight standards: bovine serum albumin, ovalbumin, α-chymotrypsinogen-A, myoglobin, and cytochrome c. Protein was stained by Coomassie brilliant blue R-250.

Optical spectra at room temperature and at 77°K were recorded with a Shimadzu UV-200 spectrophotometer and a Shimadzu MPS-5000 spectrophotometer with a low-temperature attachment, respectively.

Metal was analyzed using a Hitachi Perkin-Elmer 303 atomic absorption spectrophotometer. Amino acid composition was determined with a Hitachi KLA-5 amino acid analyzer. Tryptophan and half-cystine were estimated by the methods of Edelhoch (19) and Moore (20), respectively. The isoelectric point was measured by isoelectric focusing with Ampholine (pH 3.5-10) in a thin-layer polyacrylamide gel as described by Vesterberg (21). The dry weight of the enzyme was determined after dialysis against water and then drying in an oven at 105°C to constant weight.

TABLE I

SUMMARY OF THE PURIFICATION OF SUPEROXIDE DISMUTASE FROM *Chromatium vinosum*[a]

Purification step	Total protein (mg)	Total activity (units × 10^{-3})	Specific activity (units/mg of protein)	Yield (%)	Purification (*n*-fold)
Sonicate	140,000	966	6.9	100	1
50 to 90% $(NH_4)_2SO_4$	40,700	1,036	25.5	107	3.7
Stepwise DEAE-Sephadex	2,900	775	267	80	38.7
Linear gradient DEAE-Sephadex	422	425	1,007	44	146
Sephadex G-100	110	345	3,136	36	454
$(NH_4)_2SO_4$ fractionation	50	225	4,500	23	652

[a] Protein was determined by the method of Lowry *et al.* (13) from the sonicate up to Sephadex G-100 steps and in the final step spectrophotometrically using an $A_{1cm}^{1\%}$ at 280 nm of 21.4. The enzymatic activity is presented in McCord-Fridovich units (1).

3. RESULTS

Purification of the Enzyme

All purification procedures were performed at 0-4°C and potassium phosphate buffer was used at pH 7.8 throughout.

Frozen cells of Ch. vinosum (1.6 kg) were suspended in 16 liters of 50 mM phosphate containing 0.5 mM EDTA. Suspended cells were disrupted with an ultrasonic oscillator in 300-ml batches for eight 2-min periods at 2-min intervals. The sonicate was clarified by centrifugation at 11,000 g for 30 min. Ammonium sulfate was added slowly to the supernatant to 50% saturation and the precipitate was removed by centrifugation at 14,000 g for 30 min. The supernatant was brought to 90% saturation with ammonium sulfate. The protein salted out was collected by filtration through Toyo No. 2 filter paper and dissolved in and dialyzed against 40 mM phosphate containing 0.1 mM EDTA for 48 h with four changes. The precipitate formed was removed by centrifugation at 14,000 g for 30 min.

The dialyzed solution was applied to a column of DEAE-Sephadex A-50 (9 x 50 cm), previously equilibrated with 40 mM phosphate containing 0.1 mM EDTA. After washing with 4.5 liters of the equilibrating buffer, the adsorbed proteins were eluted stepwise by 0.1, 0.15, 0.2, and 1.0 M phosphate and then by 1.0 M phosphate containing 1 M NaCl for each 5 liters. The enzyme was eluted by 0.2 M phosphate and the pooled active fraction was concentrated by the addition of ammonium sulfate to 90% saturation. The precipitate collected by centrifugation was dissolved in and dialyzed against 40 mM phosphate containing 0.1 mM EDTA for 48 h with seven changes. No SOD activity was detected in the other fractions, including the unadsorbed fraction.

The dialyzed solution (270 ml) was adsorbed to a column of DEAE-Sephadex A-50 (2.5 x 43 cm) previously equilibrated with 40 mM phosphate containing 0.1 mM EDTA. A linear gradient elution with 4 liters of phosphate (40-200 mM) was then applied. Active fractions were collected and concentrated by ultrafiltration in a collodion bag under reduced pressure.

The concentrated solution (25 ml) was loaded on a column of Sephadex G-100 (4 x 80 cm) equilibrated with 20 mM phosphate. The enzyme was eluted with the same buffer and the active fractions were recovered by centrifugation after adding ammonium sulfate to 100% saturation.

The resulting faint yellow precipitate was suspended in 20 ml of ammonium sulfate solution at 80% saturation containing 10 mM phosphate. Afte 30 min, the precipitate was collected by centrifugation at 12,000 g for 10 min and successively extracted with 10-ml volumes of ammonium sulfate at 77, 74, 71, 68, 65, 62, 59, 56, 53, 50, 47, and 44% saturation, each containing 10 mM phosphate. Each extraction was carried out in an ice bath for about 30 min and the extract was obtained by centrifugation at 12,000 g for 10 min at 0°C. The extract with ammonium sulfate at 50% was electrophoretically pure. Successive extractions with ammonium sulfate were repeated and the best extracts were combined. The purification is summarized in Table I. The yield was 50 mg with specific activity of 3000 units/mg of protein. During purification, no SOD other than the isolated enzyme was detected, indicating that the Chromatium cells contain only the Fe-enzyme, at least in a soluble form.

Properties of the Enzyme

Purity of enzyme

The purified enzyme gave a single protein band on disc electrophoresis, as shown in Fig. 1. The location of the purified enzyme in the gel was identical with the active zone of the crude extract, indicating no modification of the enzyme during the purification procedure. Thin-layer acrylamide gel electrofocusing using Ampholine showed a single band at pH 4.1. The homogeneity of the purified enzyme was also confirmed by equilibrium centrifugation. The slope obtained from log(fringe displacement) plotted as a function of the square of the distance from the center of the rotation showed a straight line (Fig. 2).

Stability

The purified enzyme (3.35 ug/ml) was incubated for 30 min at 25°C in 50 mM buffers in the pH range of 2.7 to 12 (pH 2.7–4.8, citrate–HCl and acetate; pH 6.0–8.5, phosphate and borate; pH 10.2–12.0, carbonate, glycine–NaOH, and phosphate). The activity was then assayed in 50 mM

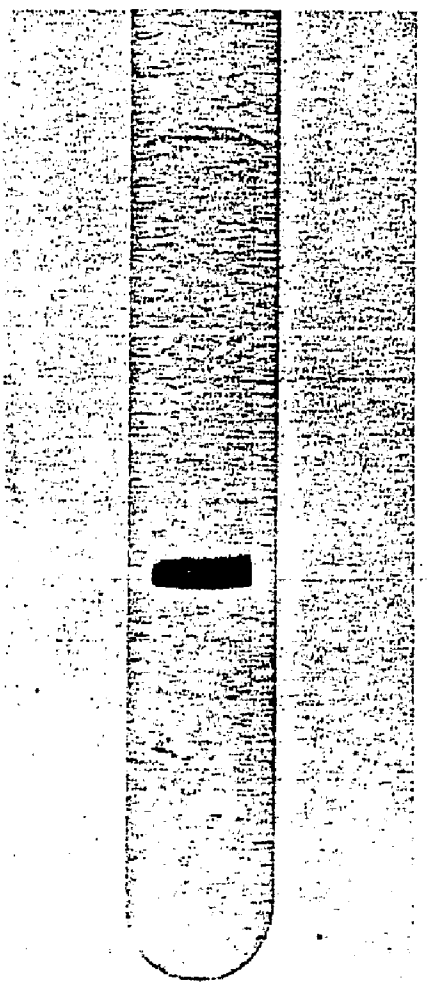

FIG. 1. Polyacrylamide gel disc electrophoresis of *Chromatium* superoxide dismutase. The purified enzyme (40 μg) was subjected to electrophoresis at 4°C with a 7.5% polyacrylamide gel in Tris–glycine, pH 8.9, at 3 mA per gel for about 30 min. Protein was stained with amido black 10B. Cathode, top; anode, bottom.

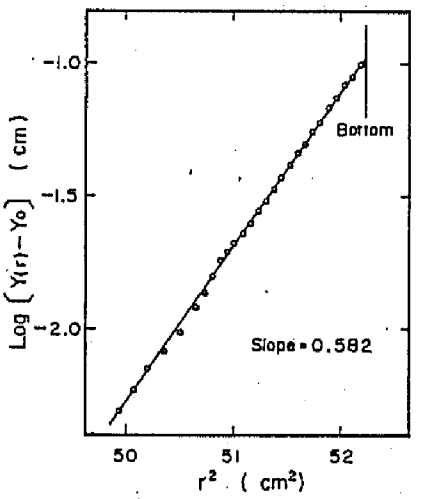

FIG. 2. Sedimentation equilibrium of *Chromatium* superoxide dismutase. Purified enzyme at a concentration of 0.22 mg/ml in 10 mM potassium phosphate, pH 7.8, containing 0.1 M potassium chloride was equilibrated at a rotor speed of 23,320 rpm at 20°C. The ultracentrifuge was equipped with interference optics, and log fringe displacement $[Y(r) - Y_0]$ is plotted as a function of the square of the distance from the center of rotation (r^2).

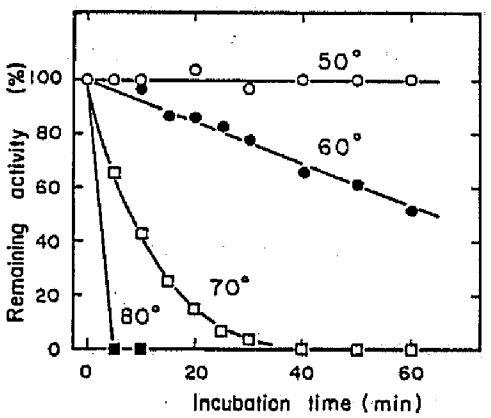

FIG. 3. Temperature stability of *Chromatium* superoxide dismutase. The purified enzyme (13.4 μg/ml) in 10 mM potassium phosphate, pH 7.8, was incubated at the indicated temperatures. Aliquots were withdrawn at intervals and then assayed for the remaining enzyme activity under standard conditions at 25°C as described under Materials and Methods.

potassium phosphate, pH 7.8, under standard conditions. The enzyme was stable from pH 5.0 to 12.0 but was inactivated below pH 5.0. The pH stability curve of Chromatium enzyme is very similar to that of Photobacterium leiognathi Fe-enzyme (23) except that the latter enzyme is unstable above pH 11.

Chromatium enzyme was stable up to 50°C for 1 h. At 60°C half of the activity was lost after 1 h and at 70°C inactivation was apparently first order with respect to the enzyme concentration. The half-life was calculated to be 7 min, compared with 6.5 min for the Spirulina Fe-enzyme (24) (Fig. 3).

Molecular weight and subunit weight

The molecular weight of the purified enzyme was estimated to be 42,000 by gel filtration with a Sephadex G-100 column. The molecular weight of the enzyme was also determined by the sedimentation equilibrium. A molecular weight of 41,000 is estimated from the slope of Fig. 2, and a partial specific volume of 0.73 calculated from the amino acid composition (Table II) according to Cohn and Edsall (25).

The subunit molecular weight was estimated by sodium dodecyl sulfate disc electrophoresis. The enzyme gave one band both in the presence and in the absence of 2-mercaptoethanol, corresponding to a position of molecular weight of 21,000. Thus, it may be concluded that Chromatium SOD is composed of two subunits of equal size without a disulfide bridge.

Absorption spectra

In the ultraviolet region the enzyme exhibits an absorption maximum at 279 nm with shoulders at 260 and 290 nm (Fig. 4). $A^{1\%}_{1\,cm}$ and the absorbance coefficient at 280 nm are 21.4 and 87,900 $M^{-1}cm^{-1}$, respectively. The enzyme also showed a weak and broad absorption from 320 to about 600 nm having a shoulder around 350 nm at room temperature. The absorbance coefficient at 350 nm is 3220 $M^{-1}cm^{-1}$. The spectrum at 77°K in 50% glycerol and 0.1 M Tris-HCl, pH 7.5, revealed an absorption peak at 363 nm.

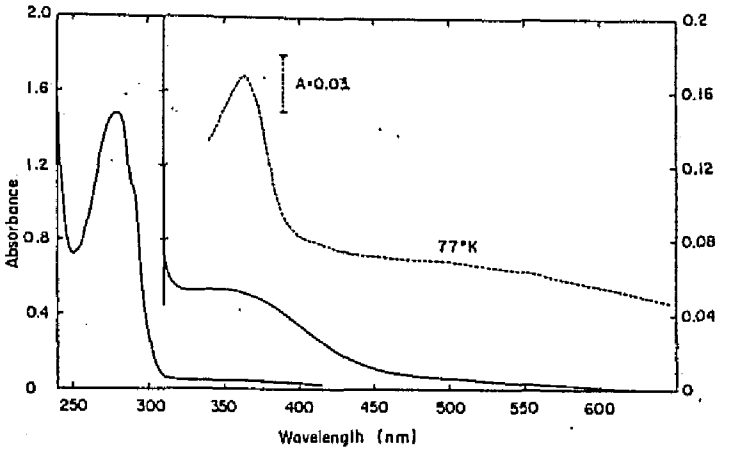

FIG. 4. Absorption spectra of *Chromatium* superoxide dismutase. The ultraviolet and visible spectra at room temperature (solid lines) were obtained with a solution containing 0.67 mg/ml (16.3 μM) of the enzyme in 10 mM potassium phosphate, pH 7.8. The light path was 10 mm. The concentration of the enzyme for the visible spectrum at 77°K (broken line) was 0.30 mg/ml (7.3 μM) in 50% glycerol (v/v) and 0.1 M Tris-HCl, pH 7.5. The light path was 1 mm.

Metal analysis

The purified enzyme (0.67 mg) was dialyzed against 10 mM Tris-HCl, pH 7.5, for 4 days with several changes of buffer and was digested in 0.2 ml of 60% perchloric acid, in two runs. The results were corrected for the blank which contained the same volumes of dialysis buffer and perchloric acid as the sample. The atoms of iron to moles of enzyme ratio was estimated to be 1.9 $\pm$ 0.2 on the basis of a molecular weight of 41,000. Manganese, copper, zinc, and magnesium were below detection or were insignificant. Assay for sulfide after acidification of the enyzme indicated the absence of acid-labile sulfide.

Amino acid analysis

The amino acid composition is summarized in Table II with those of Escherichia coli (26) and Plectonema boryanum (22) Fe-SOD and E. coli Mn-enzyme (27). The Chromatium enzyme has higher contents of glutamic acid, serine, valine, and tyrosine and lower contents of lysine and histidine than those of Fe- or Mn-enzyme from the other sources.

The addition of 5,5'-dithiobis(2-nitrobenzoic acid) to the enzyme in 0.15 M sodium phosphate, pH 8.0, did not cause any increase in absorbance at 412 nm. However, in the presence of 0.5% sodium dodecyl

sulfate, the enzyme caused a slow increase in absorbance at 412 nm, corresponding finally to 1.95 mol of sulfhydryl group per mole of enzyme after 30 h at 25°C. Consequently, the two half-cystine residues of the enzyme (Table II) occur as two sulfhydryl groups buried in the protein and do not form a disulfide linkage.

Isoelectric point

The isoelectric point of the enzyme was 4.1 according to isoelectric focusing with Ampholine. A low pI of Chromatium SOD corresponds to a high content of acidic amino acids.

Effect of antibody against Plectonema Fe-enzyme on chromatium enzyme

Since the amino acid composition of Chromatium SOD resembles that of Plectonema Fe-enzyme, the immunological reaction with the antibody against Plectonema Fe-SOD was tested. Plectonema Fe-enzyme antibody did not affect the activity of the Chromatium enzyme (Fig. 5) and gave no

TABLE II

COMPARISON OF AMINO ACID COMPOSITIONS OF *Chromatium*, *Plectonema*, AND *E. coli* SUPEROXIDE DISMUTASES

Amino acid	*Chromatium*, Fe-SOD[a]	*Plectonema*, Fe-SOD[b]	*E. coli* Fe-SOD[c]	*E. coli* Mn-SOD[d]
Lysine	16	24	20	29
Histidine	8	10	11	12
Arginine	4	3	8	10
Aspartic acid	47	47	45	42
Threonine	23	20	26	19
Serine	32	18	20	22
Glutamic acid	39	32	32	37
Proline	14	20	18	15
Glycine	27	29	32	26
Alanine	42	48	53	47
Valine	28	21	22	20
Methionine	4	3	0	3
Isoleucine	16	5	16	14
Leucine	32	34	29	38
Tyrosine	18	10	13	12
Phenylalanine	17	25	20	18
Half-cystine	2	2	2	—
Tryptophan	12	11	8	—

[a] The number of residues was calculated for 41,000 g of enzyme; SOD, superoxide dismutase.
[b] Reference 22.
[c] Reference 26.
[d] Reference 27.

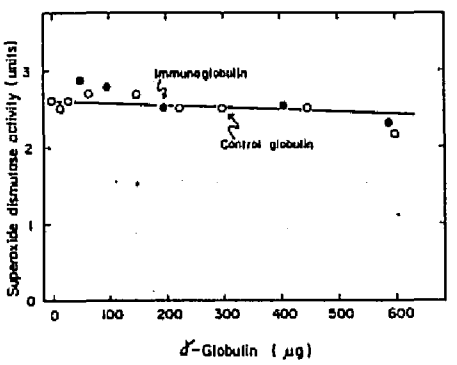

FIG. 5. Effect of antibody against *Plectonema* Fe-superoxide dismutase on *Chromatium* superoxide dismutase. *Chromatium* superoxide dismutase (2.6 units) in a total volume of 140 μl was incubated with the indicated amounts of anti-*Plectonema* Fe-superoxide dismutase at 25°C for 1 h, and then the enzyme activity was measured under standard conditions. O, control globulin; ●, anti-*Plectonema* Fe-superoxide dismutase.

precipitin band with the Chromatium enzyme in the Ouchterlony double immunodiffusion test. The antibody against Mycobacterium Fe- or Mn-SOD (28,29) neither inhibited nor formed a complex with the Chromatium Fe-enzyme. Thus, Chromatium enzyme is immunologically distinguished from the Fe- and Mn-enzymes from the other sources, irrespective of the similar amino acid composition.

Effects of several compounds on Chromatium enzyme

Effects of various chemicals on the enzyme were tested according to the method of Asada et al. (12). Chromatium enzyme was not affected by 1 mM KCN, which is similar to other Fe-SOD. The enzyme was 60% inhibited by 10 mM NaN_3. The inhibition by azide was also observed in the assay system using the reduction of nitroblue tetrazolium monitored at 560 nm by O_2^- generated by either xanthine-xanthine oxidase or infusion of KO_2 dissolved in crown ether-dimethyl sulfoxide. However, Slykhouse and Fee reported that, with assay procedures other than the reduction of cytochrome c, azide did not affect E. coli Fe-SOD (30). Iron chelating agents, o-phenanthroline and α,α'-dipyridyl, at 1 mM, did not show any effect on the enzymatic activity.

Inactivation by methylene blue and light

Chromatium enzyme was inactivated by methylene blue-sensitized photooxidation. As shown in Fig. 6 the loss of enzymatic activity followed first-order kinetics. The enzyme was not affected in the dark or by light in the absence of methylene blue.

Inactivation by hydrogen peroxide

Chromatium enzyme was inactivated by H_2O_2 as shown in Fig. 7. The plot of log(remaining activity) against time gave a straight line and the rate constant for the inactivation at pH 10 was calculated to be 9.1 $M^{-1}s^{-1}$, which was of the same order as 6.7 $M^{-1}s^{-1}$ for Cu,Zn-SOD (31). Hodgson and Fridovich (31,32) have proposed a mechanism of inactivation for bovine Cu,Zn-SOD by H_2O_2 in which a bound oxidant formed with H_2O_2 destroys histidine residues coordinatedly bounded to the copper of the enzyme. Several compounds including xanthine, urate, and azide are

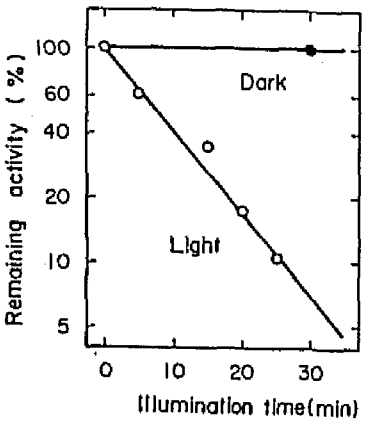

FIG. 6. Inactivation of *Chromatium* superoxide dismutase by photooxidation. The purified enzyme (67 μg/200 μl) in 50 mM potassium phosphate, pH 7.8, was exposed to a tungsten lamp (Light) or kept in the dark (Dark) in the presence of 0.005% methylene blue at 25°C. Light intensity was 460 W/m². Aliquots (20 μl) were removed at intervals and, after a 20-fold dilution with 10 mM potassium phosphate, pH 7.8, the remaining activity was measured using an aliquot (30 μl) of the diluted solution under standard assay conditions. Although methylene blue at a high concentration stimulated the reduction rate of cytochrome c by O_2^-, under the above conditions this effect was negligible.

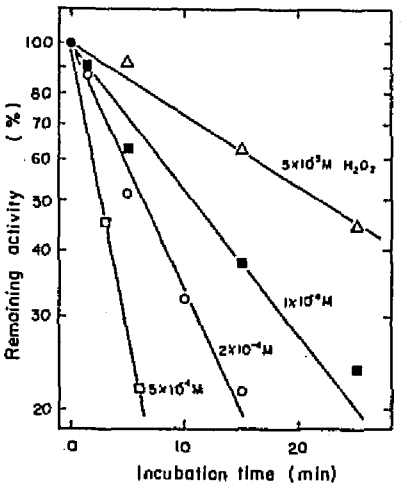

FIG. 7. Inactivation of *Chromatium* superoxide dismutase by hydrogen peroxide. *Chromatium* superoxide dismutase (1.63 μM) in 50 mM sodium carbonate, pH 10.0, was incubated at 25°C with various concentrations of hydrogen peroxide, and aliquots (10 μl) were withdrawn at intervals and assayed for residual enzymatic activity under standard assay conditions.

oxidized by a system of H_2O_2 and bovine Cu,Zn-enzyme and protect the enzyme from inactivation by H_2O_2. In the case of Chromatium Fe-enzyme, however, the addition of these compounds at 1 mM to the reaction system of Fig. 7 did not show any protective effect except that azide slightly depressed the inactivation.

Rate constants between the enzyme and superoxide

The second-order rate constants for the reaction of Chromatium SOD with O_2^- at various pH were determined from the kinetic competition of O_2^- between SOD and cytochrome c according to Forman and Fridovich (33) assuming that the rate constant of the spinach Cu,Zn-SOD is the same as that of erythrocyte Cu,Zn-SOD (34). The spinach Cu,Zn-enzyme used (35) has a specific activity similar to that of the erythrocyte enzyme. The concentrations of Chromatium and spinach enzymes causing 50% inhibition of the reduction of cytochrome c by O_2^- generated by the xanthine-xanthine oxidase system were determined under standard conditions at

TABLE III

RATE CONSTANTS FOR O_2^- OF VARIOUS SUPEROXIDE DISMUTASES[a]

Enzyme	Rate constant (10^{-9} M^{-1} s^{-1})				Reference
	pH 6.0	pH 7.8	pH 8.5	pH 10.2	
Chromatium Fe-SOD	4.4	2.4	1.6	0.8	This work
E. coli Fe-SOD	1.9	1.6	0.3	0.4	33
E. coli Mn-SOD	2.0	1.8	0.7	0.3	33
Bovine Cu,Zn-SOD	3.4	1.9	1.8	1.6	34
Mitochondrial Mn-SOD	7.3	4.7	1.0	1.8	33

[a] The concentrations of *Chromatium* superoxide dismutase that caused 50% inhibition of cytochrome c reduction were measured at various pH values under standard conditions except that the following buffers were used: pH 6.0 and 7.8, 50 mM potassium phosphate; pH 8.5, 50 mM sodium borate; pH 10.2, 50 mM sodium carbonate. Details are given in the text. SOD, superoxide dismutase.

several pH. The rate constant of the Chromatium enzyme was then calculated from th ratio of concentrations of both enzymes. Table III shows the estimated constants of the Chromatium enzyme, with those of other SOD for comparison. The Chromatium enzyme has the highest rate constant at pH 6.0 and shows progressively lower activity as the pH is raised. This effect of pH on the reaction rate resembles the other Fe- and Mn-containing enzymes.

4. DISCUSSION

SOD of a purple sulfur bacterium, Chromatium vinosum, grown under strict anaerobic conditions has been purified to homogeneity as judged from polyacrylamide gel disc electrophoresis and sedimentation analysis. Chromatium cells contain only a single SOD and the present work reveals that this is the Fe-enzyme. The properties of the Chromatium enzyme are very similar to the Fe-SOD of aerobes, so far isolated from Escherichia coli (26), Photoacterium sepia and P. leiognathi (23), Plectonema boryanum (22,36), Spirulina platensis (24), Pseudomonas ovalis (37) and Mycobacterium tuberculosis (28), with respect to molecular weight, subunit structure, amino acid composition, absorption spectra, and reaction rate with superoxide. Among them, only the Mycobacterium enzyme is a tetramer. I have also purified and crytallized the Fe-enzyme from a green sulfur bacterium, Chlorobium thiosulfatophilum. Chlorobium cells contain only this type of SOD, which is very similar to Chromatium Fe-enzyme in physicochemical properties (Chapter IX). Very recently, Fe-SOD has been purified from a sulfate-reducing bacterium, Desulfovibrio desulfuricans (10). Thus, it seems likely that SOD in anaerobes are the Fe-enzyme.

Iron contents of Fe-SOD from various sources have been reported in the range of one to two atoms per mole of enzyme. Plectonema enzyme (22) contains 2 atoms per mole, whereas Spirulina enzyme (24) contains one atom per mole. Photobacterium (23), Psudomonas (37), Escherichia (30), and Desulfovibrio (10) enzymes have an intermediate value or a value near two atoms. The present Chromatium enzyme contains nearly two atoms of Fe per mole.

Amino acid sequence and X-ray crystalographic analysis of bovine Cu,Zn-SOD (38,39) showed that the copper coordinates to four histidine residues and the zinc to three histidine residues and one aspartic acid residue. On the other hand, the metal binding site of the Fe- and Mn-SOD is still unrevealed. Methylene blue-sensitized photoinactivation for the Chromatium enzyme suggests the participation of a histidine residue in the catalytic action of the enzyme. The Chromatium enzyme contains four residues of histidine per subunit (Table II). Similar

photoinactivation has also been observed in bovine Cu,Zn-SOD, but only after removal of the metals (40).

Cu,Zn-SOD from mammals and plants are inactivated by H_2O_2 (22,41,42), and a mechanism for this inactivation has been proposed involving the oxidation by H_2O_2 of histidine residues coordinated to Cu (31). The Chromatium enzyme was sensitive to H_2O_2, like the other Fe-SOD (22,43), but substances protecting the Cu,Zn-enzyme from inactivation have no effect on the Chromatium enzyme.

Although aerobic organisms produce the superoxide radical through many biological processes, SOD scavenges the radical and protects the cells against the harmful action of active oxygen (2). In photosynthetic organisms the photoproduction of superoxide has been demonstrated in chloroplasts (44-46) and in chromatophores of aerobic purple nonsulfur bacterium (47). Recently, the photoproduction of O_2^- under aerobic conditions has been obseved in Chromatium (48). Chromatium cells are characterized by their anaerobic habitat. When the cells are transferred to aerobic conditions no growth is observed, but the cells do survive for a limited period (49). Under such conditions superoxide produced by autooxidation may be scavenged by SOD. This is one of the possible functions of SOD in anaerobic photosynthetic bacteria, because under natural environments the cells may have contact with air. In this respect, the observations of Tally et al. (50) should be noted because they show a correlation between the contents of SOD and oxygen tolerance among anaerobes. In the absence of molecular oxygen the only possible source of superoxide is the decomposition of water by high-energy radiation, such as ultraviolet light and X-ray, and the scavenging of superoxide formed by this anaerobic process is another possible function of SOD in the anaerobes. The contents of SOD in anaerobically grown Ch. vinosum and Chl. thiosulfatophilum (5 and 9 units/mg of protein) were similar to those of aerobic bacteria, E. coli and Plectonema boryanum (19 and 6 units/mg of protein (22,50). This might indicate that the enzyme has a function other than the disproportionation of superoxide. However, no evidence is available for this viewpoint.

Distribution studies on various types of SOD in organisms at different levels of evolution indicate the presence of the Fe- and/or

Mn-enzymes and the absence of the Cu,Zn-enzyme in prokaryotes, eukaryotic algae, and protozoa. Cu,Zn-SOD is found in land plants, most fungi, and animals including vertebrates and invertebrates (8,9,51). Recently Henry and Hall (52) reported the occurrence of Cu,Zn-enzyme in phragmoplast algae, including Spirogyra, Chara, and Nitella, but not in other eukaryotic algae. Anaerobic photosynthetic bacteria and sulfate-reducing bacteria contain only the Fe-enzyme, and the presence of the Mn-enzyme starts from aerobic photosynthetic bacteria and blue-green algae (51). The distribution of the Fe- and Mn-enzymes and the phylogenic relation of these organisms suggest that the Fe-enzyme of the anaerobes is an ancestor of the Fe- and Mn-enzymes of aerobes and that the acquisition of Fe-SOD by the anaerobes preceded the accumulation of molecular oxygen in the atmosphere by blue-green algae. The phylogenic tree deduced from the comparison of cytochromes by Dickerson et al. (53) shows that the ancestors of the organisms containing Mn-SOD are anaerobic photosynthetic bacteria and clostridia. I can only speculate as to why anaerobic bacteria acquired Fe-SOD. Function as a scavenger of the superoxide formed by anaerobic processes and by molecular oxygen at a very low concentration (0.002%) (54) produced with the Urey reaction is presumably one reason, but a function other than the disproportionation of superoxide is not ruled out. Further characterization of the Fe-SOD may provide a key to this interesting question.

5. SUMMARY

Superoxide dismutase of anaerobic purple sulfur bacterium, Chromatium vinosum, was purified to a homogeneous state. The enzyme contains two atoms of iron per mole and has a molecular weight of 41,000. It is composed of two identical subunits. Amino acid composition, absorption spectra, and the reaction rate constant with O_2^- are also similar to those of the Fe-SOD from aerobes. The enzyme is sensitive to hydrogen peroxide and methylene blue-sensitized photooxidation. The functional and evolutional aspects of SOD in anaerobes are discussed.

6. REFERENCES

(1) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(2) Fridovich, I. (1975) Annu. Rev. Biochem. 44, 147-159.

(3) Steinman, H.M., and Hill, R.L. (1973) Proc. Natl. Acad. Sci. USA 70, 3725-3729.

(4) Bridgen, J., Harris, J.I., and Northrop, F. (1975) FEBS Lett. 49, 392-395.

(5) McCord, J.M., Keele, B.B., Jr., and Fridovich, I. (1971) Proc. Natl. Acad. Sci. USA 68, 1024-1027.

(6) Hewitt, J., and Morris, J.G. (1975) FEBS Lett. 50, 315-318.

(7) Lumsden, J., and Hall, D.O. (1975) Nature (London) 257, 670-672.

(8) Asada, K., Kanematsu, S., Takahashi, M., and Kono, Y. (1976) in Iron and Copper Proteins (Yasunobu, K.T., Mower, H.F., and Hayaishi, O., eds.), pp. 551-564, Plenum Press, New York.

(9) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(10) Hatchikian, E.C., and Henry, Y.A. (1977) Biochimie 59, 153-161.

(11) Newton, J.W. (1962) Methods Enzymol. 5, 73-77.

(12) Asada, K., Takahashi, M., and Nagate, M. (1974) Agric. Biol. Chem. 38, 471-473.

(13) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

(14) Davis, B.J. (1964) Ann. New York Acad. Sci. 121, 404-427.

(15) Beauchamp, C.O., and Fridovich, I. (1971) Anal. Biochem. 44, 276-287.

(16) Andrews, P. (1964) Biochem. J. 91, 222-233.

(17) Yphantis, D.A. (1964) Biochemistry 3, 297-317.

(18) Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.

(19) Edelhoch, H. (1967) Biochemistry 6, 1948-1954.

(20) Moore, S. (1963) J. Biol. Chem. 238, 235-237.

(21) Vesterberg, O. (1972) Biochim. Biophys. Acta 257, 11-19.

(22) Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y., and Enmanji, K. (1975) J. Biol. Chem. 250, 2801-2807.

(23) Puget, K., and Michelson, A.M. (1974) Biochimie 56, 1255-1267.
(24) Lumsden, J., Cammack, R., and Hall, D.O. (1976) Biochim. Biophys. Acta 438, 380-392.
(25) Cohn, E.J., and Edsall, J.T. (1941) Proteins, Amino Acids and Peptides, p. 375, Reinhold, New York.
(26) Yost, F.J., Jr., and Fridovich, I. (1973) J. Biol. Chem. 248, 4905-4908.
(27) Keele, B.B., Jr., McCord, J.M., and Fridovich, I. (1970) J. Biol. Chem. 245, 6176-6181.
(28) Kusunose, E., Ichihara, K., Noda, Y., and Kusunose, K. (1976) J. Biochem. 80, 1343-1352.
(29) Kusunose, M., Noda, Y., Ichihara, K., and Kusunose, E. (1976) Arch. Microbiol. 108, 65-73.
(30) Slykhouse, T.O., and Fee, J.A. (1976) J. Biol. Chem. 251, 5472-5477.
(31) Hodgson, E.K., and Fridovich, I. (1975) Biochemistry 14, 5294-5299.
(32) Hodgson, E.K., and Fridovich, I. (1975) Biochemistry 14, 5299-5303.
(33) Forman, H.J., and Fridovich, I. (1973) Arch. Biochem. Biophys. 158, 396-400.
(34) Klug, D., Rabani, J., and Fridovich, I. (1972) J. Biol. Chem. 247, 4839-4842.
(35) Asada, K., Urano, M., and Takahashi, M. (1973) Eur. J. Biochem. 36, 257-266.
(36) Misra, H.P., and Keele, B.B., Jr. (1975) Biochim. Biophys. Acta 379, 418-425.
(37) Yamakura, F. (1976) Biochim. Biophys. Acta 422, 280-294.
(38) Steinman, H.M., Naik, V.R., Abernethy, J.L., and Hill, R.L. (1974) J. Biol. Chem. 249, 7326-7338.
(39) Richardson, J.S., Thomas, K.A., Rubin, B.H., and Richardson, D.C. (1975) Proc. Natl. Acad. Sci. USA 72, 1349-1353.
(40) Forman, H.J., Evans, H.J., Hill, R.L., and Fridovich, I. (1973) Biochemistry 12, 823-827.
(41) Symonyan, M.A., and Nalbandyan, R.M. (1972) FEBS Lett. 28, 22-24.

(42) Beauchamp. C.O., and Fridovich, I. (1973) Biochim. Biophys, Acta 317, 50-64.

(43) Lavelle, F., McAdam, M.E., Fielden, E.M., Roberts, P.B., Puget, K., and Michelson, A.M. (1977) Biochem. J. 161, 3-11.

(44) Asada, K., and Kiso, K. (1973) Eur. J. Biochem. 33, 253-257.

(45) Asada, K., and Kiso, K. (1973) Agric. Biol. Chem. 37, 453-454.

(46) Asada, K., Kiso, K., and Yoshikawa, K. (1974) J. Biol. Chem. 249, 2175-2181.

(47) Boucher, F., and Gingrans, G. (1975) Biochem. Biophys. Res. Commun. 67, 421-426.

(48) Asami, S., and Akazawa, T. (1977) Biochemistry 16, 2202-2207.

(49) Hurlbert, R.E. (1967) J. Bacteriol. 93, 1346-1352.

(50) Tally, F.P., Goldin, B.R., Jacobus, N.V., and Gorbach, S.L. (1977) Infect. Immun. 16, 20-25.

(51) Asada, K., and Kanematsu, S. (1978) in Evolution of Protein Molecules (Matsubara, H., and Yamanaka, T., eds.), pp. 361-372, Japan Sci. Soc. Press, Tokyo.

(52) Henry, L.E.A., and Hall, D.O. (1977) in Photosynthetic Organelles (Miyachi, S., Katoh, S., Fujita, Y., and Shibata, K., eds.), pp. 377-382, Japanese Society of Plant Physiologists, Kyoto.

(53) Dickerson, R.E., Timkovich, R., and Almassy, R.J. (1976) J. Mol. Biol. 100, 473-491.

(54) Dole, M. (1965) J. Gen. Physiol. 49, 5-27.

CHAPTER IX

CRYSTALLINE Fe-SUPEROXIDE DISMUTASE FROM THE ANAEROBIC GREEN SULFUR BACTERIUM *CHLOROBIUM THIOSULFATOPHILUM*

1. INTRODUCTION

Superoxide dismutase (SOD) reduces the steady state concentration of the superoxide radical by catalyzing disproportionation of the radicals, thus protecting cells from oxygen toxicity. Although little superoxide is produced in anaerobes, SOD has been found in several anaerobic bacteria (1-5). The SOD from a purple sulfur photosynthetic bacterium, *Chromatium vinosum* (Chapter VIII,2,3), and from a sulfate-reducing bacterium, *Desulfovibrio desulfuricans* (4) have been characterized as the Fe-enzyme. Aerobic prokaryotes, most algae and protozoa contain Fe- and/or Mn-SOD and lack Cu,Zn-SOD (2,5-7). This chapter reports the isolation of crystalline SOD from an anaerobic, green sulfur photosynthetic bacterium, *Chlorobium thiosulfatophilum*, and I describe its properties including metal contents and amino acid composition. The results herein prove that *Chlorobium* SOD is the Fe-enzyme, confirming further the absence of the Mn-enzyme in anaerobes.

2. MATERIALS AND METHODS

Cytochrome *c* (type III), xanthine oxidase and Ampholine (pH3.5-5.0) were obtained from Sigma, Boehringer and LKB, respectively. The strain of *Chlorobium thiosulfatophilum* was given by Dr. T. Akazawa, Nagoya University (8). Cells were grown anaerobically under illumination from incandescent lamps using the Larsen medium (3). The absence of oxygen in the medium was confirmed using an oxygen-electrode. Cells were harvested at the late logarithmic phase and stored at -20°C until use. SOD was assayded using a modification (9) of the McCord and Fridovich's

procedure (10). Activity is presented in the McCord-Fridovich unit.

Polyacrylamide disc-gel electrophoresis, locating the activity on the disc, determination of the molecular and subunit molecular weights were performed as in Chapter VIII (3). Metal was analyzed with a Hitachi Perkin-Elmer 303 atomic absorption spectrophotometer equipped with a GA-2 graphite atomizer. Amino acid composition was determined as in Chapter VIII. The isoelectric point was measured by isoelectric focusing with Ampholine (pH 3.5-5.0) in a sucrose column. The dry weight of the enzyme was determined after dialysis against water and drying it in an oven at 105°C to constant weight.

3. RESULTS

Purification of the enzyme

Unless specified potassium phosphate buffer was used at pH 7.8. Frozen cells of Chlorobium thiosulfatophilum (900 g) were suspended in 10 liters 50 mM phosphate containing 0.5 mM EDTA and were homogenized using a Dyno-mill with glass beads. The homogenates were centrifuged at 14,000 g for 30 min to remove cell debris. Solid KCl was added to the supernatant to give 0.1 M. The solution (300 ml batch) was heated in an 80°C water bath and kept at 60°C for 3 min after reaching 60°C. After removal of denatured proteins by centrifugation ammonium sulfate was added to make 50% saturation. The precipitate was removed by centrifugation and ammonium sulfate was added to the supernatant to 90% saturation. The precipitate collected by centrifugation was dissolved in and dialyzed against 10 mM phosphate containing 0.1 mM EDTA for 2 days with several changes of buffer. The dialyzed solution was clarified by centrifugation and adsorbed on DEAE-Sephadex A-50 column (9 x 48 cm) pre-equilibtrated with 50 mM phosphate containing 0.1 mM EDTA. After the column had been washed with 22 liters equilibrating buffer (cytochrome c-553 was eluted following cytochrome c-555), a pale yellow band of SOD was eluted ahead of a red band of cytochrome c-551 on elution with 100 mM phosphate. SOD was collected by centrifugation after the addition of ammonium sulfate and was dissolved in and dialzyed against

50 mM phosphate containing 0.1 mM EDTA for 2 days. After removal of insoluble protein by centifugation the dialyzed enzyme was adsorbed on a column of DEAE-Sephadex A-50 (2.5 x 41 cm) and linear gradient elution (50-150 mM phosphate) was conducted. The SOD fractions eluted at 97 mM phosphate were concentrated to 10 ml in a collodion bag under reduced pressure. The concentrated solution was gel-filtered on a column of Sephadex G-100 (4 x 75 cm) equilibrated and eluted with 20 mM phosphate. Active fractions were pooled and dialyzed against 0.5 mM potassium phosphate, pH 6.0. The dialyzed solution was applied to a small column of hydroxylapatite (BDH Chemicals, 2.5 x 6 cm) pre-equilibrated with 0.5 mM phosphate, pH 6.0. The unadsorbed fraction contained homogeneous SOD. Specific activity of the purified enzyme (160 mg) was 2760 units/mg enzyme and the yield was about 40%. Faint brown rectangular crystals (Fig. 1) grew, when the enzyme solution (5 mg/ml) was dialyzed against ammonium sulfate solution at 53% or 56% saturation, at pH 5.1, for several weeks.

Properties of the enzyme

Polyacrylamide disc-gel electrophoresis of the purified enzyme, at pH 8.9, gave a single protein band corresponding to the enzyme-active

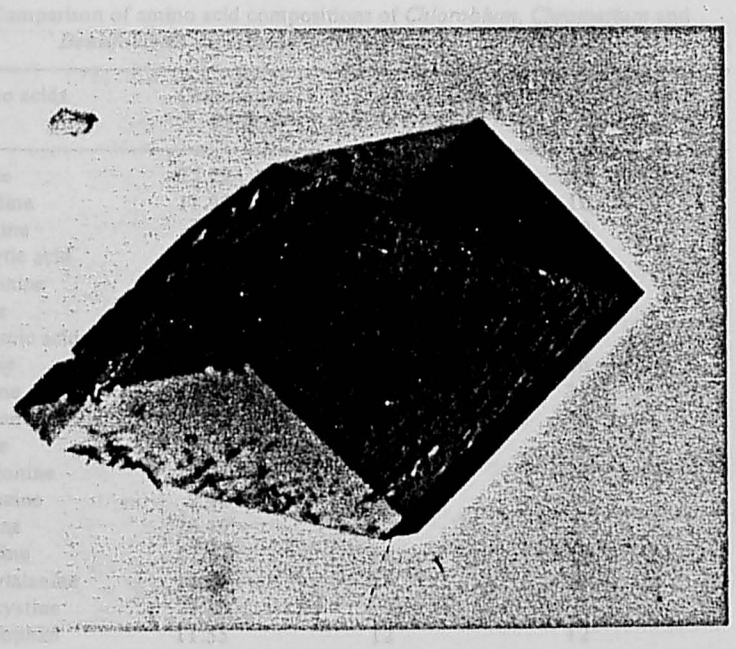

Fig.1. Crystals of *Chlorobium* Fe-superoxide dismutase.

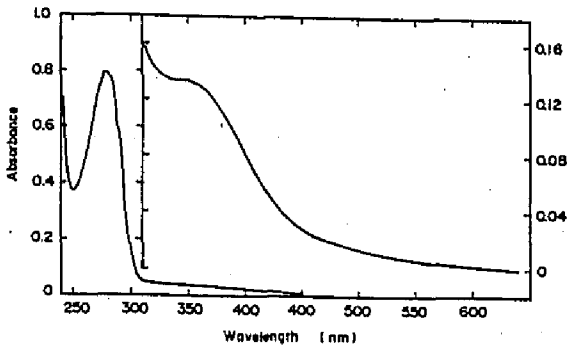

Fig.2. Absorption spectrum of *Chlorobium* superoxide dismutase. The ultraviolet and visible spectra were recorded at 0.415 and 1.659 mg enzyme/ml, respectively, in 10 mM potassium phosphate, pH 7.8, at 25°C. The light path was 10 mm.

zone of the crude extract, indicating that no modification of the enzyme occurred during the purification. The enzyme was estimated to be mol. wt 44,000 by gel filtration with Sephadex G-100. Sedimentation equilibrium centrifugation was conducted at 0.79 mg enzyme/ml in 10 mM phosphate, pH 7.8, containing 0.1 M KCl at 23,276 rev./min at 20°C. A mol. wt 43,000 was obtained from the slope of the log(fringe displace-

Table 1
Comparison of amino acid compositions of *Chlorobium*, *Chromatium* and *Desulfovibrio* superoxide dismutases (SOD) in residues/mol

Amino acids	*Chlorobium* Fe-SOD[a)]	*Chromatium* Fe-SOD[b)]	*Desulfovibrio* Fe-SOD[c)]
Lysine	22.29	16	32
Histidine	11.97	8	10
Arginine	4.87	4	8
Aspartic acid	56.61	47	54
Threonine	19.82	23	24
Serine	19.38	32	14
Glutamic acid	42.06	39	36
Proline	17.80	14	16
Glycine	33.49	27	38
Alanine	45.42	42	44
Valine	25.21	28	16
Methionine	5.04	4	4
Isoleucine	9.42	16	14
Leucine	26.49	32	30
Tyrosine	17.91	18	16
Phenylalanine	16.34	17	24
Half-cystine	3.17	2	4
Tryptophan	11.55	12	12

a) The number of residues was calculated for 43 000 g enzyme
b) From [3]
c) From [4]

ment) plotted against the square of the distance from the center of rotation. A partial specific volume (0.719) was calculated from the amino acid composition (Table I). When exposed to sodium dodecylsulfate (SDS) and subjected to SDS-polyacrylamide disc-gel electrophoresis the enzyme gave one band corresponding to a position of mol. wt 22,000 in the presence and absence of 2-mercaptoethanol. Thus, Chlorobium SOD is a dimer composed of equal subunits without a disulfide bridge.

The ultraviolet spectrum of the enzyme exhibits an absorption maximum at 280 nm with shoulders at 260 nm and 290 nm (Fig. 2). $A^{1\%}_{1\ cm}$ and the absorbance coefficient at 280 nm were estimated to be 19.10 and 82,100 $M^{-1}cm^{-1}$, respectively, based on mol. wt 43,000. The visible spectrum shows a weak and broad absorption from 330-600 nm with a shoulder around 350 nm which is characteristic of Fe-SOD. The absorbance coefficient at 350 nm is 3530 $M^{-1}cm^{-1}$.

The Chlorobium enzyme contained 1.80 $\pm$ 0.05 atoms Fe/mol enzyme on the basis of mol. wt 43,000. The contents of Mn, Cu and Zn were insignificant. No sulfide was detected by acidification of the enzyme. Thus, Chlorobium SOD is the Fe-enzyme.

Isoelectric focusing was performed in 1% Ampholine (pH 3.5-5.0) stabilized by a sucrose gradient using a 30 ml column at 500 V for 24 h followed by 800 V for 48 h at 4°C. After the pH-gradient had been established 1 ml fractions were collected and measured for pH and enzyme activity, which revealed an isoelectric point of 4.17.

Table I shows the amino acid composition of the Chlorobium Fe-SOD, with those of the Fe-enzymes from other anaerobes. The composition is very similar to compositions of the Fe-enzymes from anaerobic and aerobic bacteria. In spite of the similar amino acid compositions, antibodies against the Plectonema Fe-SOD (5) and the Mycobacterium Fe- and Mn-SOD (11) did not affect the activity of the Chlorobium enzyme nor cross-react with the enzyme in Ouchterlony doulbe immunodiffusion. The antibody against the spinach Cu,Zn-enzyme (5) also showed no reaction.

4. DISCUSSION

The absorption spectrum, metal contents, amino acid composition, molecular weight and subunit structure of Chlorobium SOD are very similar to those of the Fe-enzymes isolated from aerobic and anaerobic bacteria. Green sulfur photosynthetic bacteria contain only this form of enzyme as do other anaerobes, i.e., sulfate-reducing and purple sulfur bacteria (2-4). Thus, all three anaerobes including sulfate-reducing bacteria and photosynthetic sulfur bacteria contain only Fe-SOD. To my knowledge Mn-SOD has not been found in anaerobic bacteria but in aerobic bacteria such as purple nonsulfur bacteria and blue-green algae. In this respect it is interesting to note that Escherichia coli contains only the Fe-enzyme when the cells are cultured under an extremely low partial pressure of oxygen while the cells cultured in air or oxygen contain the Mn-enzyme in addition to the Fe-enzyme (12). A comparison of the energy metabolism and the amino acid sequence of c-type cytochromes suggests that aerobic bacteria have evolved from anaerobic photosynthetic bacteria (13). The present results suppport the proposal (3,6) that the Fe-SOD in anaerobic bacteria is the ancestor of the Fe- and Mn-SOD in aerobes. The physiological function of the enzyme in anaerobes is not yet understood, however.

5. SUMMARY

SOD was purified to a homogeneous state from an anaerobic green sulfur bacterium, Chlorobium thiosulfatophilum. The purified enzyme was a homodimer having a molecular weight of 43,000 and contained 1.8 atoms of Fe per mole. Molecular properties of the enzyme resembled those of Fe-SOD from both anaerobes and aerobes. C. thiosulfatophilum lacked Mn-SOD. The present results confirm further the absence of Mn-SOD but presence of Fe-SOD in anaerobes.

6. REFERENCES

(1) Hewitt, J., and Morris, J.G. (1975) FEBS Lett. 50, 315-318.

(2) Asada, K., Kanematsu, S., Takahashi, M., and Kono, Y. (1976) in Iron and Copper Proteins (Yasunobu, K.T., Mower, H.F., and Hayaishi, O., eds.), pp. 551-564, Plenum Press, New York.

(3) Kanematsu, S., and Asada, K. (1978) Arch. Biochem. Biophys. 185, 473-482.

(4) Hatchikian, E.C., and Henry, Y.A. (1977) Biochimie 59, 153-161.

(5) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(6) Asada, K., and Kanematsu, S. (1978) in Evolution of Protein Molecules (Matsubara, H., and Yamanaka, T., eds.), pp. 361-372, Japan Sci. Soc. Press, Tokyo.

(7) Henry, L.E.A., and Hall, D.O. (1977) in Photosynthetic Organelles (Miyachi, S., Katoh, S., Fujita, Y., and Shibata, K., eds.), pp. 377-382, Japanese Soc. Plant Physiol., Kyoto.

(8) Takabe, T., and Akazawa, T. (1977) Plant Cell Physiol. 18, 753-765.

(9) Asada, K., Takahashi, M., and Nagate, M. (1974) Agric. Biol. Chem. 38, 471-473.

(10) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(11) Kusunose, M., Noda, Y., Ichihara, K., and Kusunose, E. (1976) Arch. Microbiol. 108, 65-73.

(12) Hassan, H.M., and Fridovich, I. (1977) J. Bacteriol. 129, 1574-1583.

(13) Dickerson, R.E., Timkovich, R., and Almassy, R.J. (1976) J. Mol. Biol. 100, 473-491.

CHAPTER X

Fe-SUPEROXIDE DISMUTASES IN AEROBIC DIAZOTROPHS: SOYBEAN ROOT-NODULE BACTEROIDS AND *AZOTOBACTER*

1. INTRODUCTION

Although diazotrophs such as *Azotobacter* and soybean root-nodule bacteroids undergo dinitrogen fixation under aerobic conditions, their nitrogenases are very labile to molecular oxygen. This indicates the presence of defense system against molecular oxygen in cells. Several mechanisms of the defense system in aerobic diazotrophs have been proposed (1). Because of higher reactivity of the active oxygen, i.e. the superoxide radical, hydrogen peroxide, the hydroxyl radical and singlet oxygen than that of molecular oxygen, it is very likely that scavengers for the active oxygen play a role in the protection of nitrogenase in aerobic diazotrophs. Especially, the superoxide radical is a source of the other active species of oxygen, therefore, superoxide dismutase is supposed to protect nitrogenase in aerobic diazotrophs.

Superoxide dismutase (SOD) is classified into three types in respect of the prosthetic metals; i.e. the Cu,Zn-, Fe- and Mn-SOD (2). The three types of enzyme exhibit the phylogenetic distribution among organisms. Thus, anaerobic bacteria contain only the Fe-enzyme, whereas aerobic bacteria have both the Fe- and/or Mn-enzymes (Chapter VIII and IX). Mn-SOD in *Escherichia coli* was induced by molecular oxygen (3) or O_2^- (4), suggesting that the biosynthesis of the Mn-enzyme correlates with oxygen concentration inside the cell. Therefore, it is interesting to survey the type of SOD in aerobic diazotrophs whose oxygen concentration inside the cell seems to be extremely low.

In this chapter the content and the type of SOD in aerobic symbiotic and free-living diazotrophs, root-nodule bacteroids and *Azotobacter*, are described.

2. MATERIALS AND METHODS

Cytochrome c (type III), xanthine oxidase and Ampholine (pH 3.5-10.0) were obtained from Sigma, Boehringer and LKB, respectively. Root-nodules were collected from soybean (Glycine max) plants two months after planting. The strains of Azotobacter vinelandii IFO 12018 and A. chroococcum IFO 12994 were obtained from Institute for Fermentation, Osaka, Japan. The strains of Acinetobacter aerogenes IFO3080, Bacillus sphericus IFO 3525 and Pseudomonas aeruginosa IFO 3080 were supplied by Dr. K. Soda of Kyoto University. Anti-spinach Cu,Zn-SOD serum was prepared as described previously (5).

The cells of Azotobacter were grown with 10% inocula at 30°C in the modified Burk's medium (6), which composed of sucrose, 20 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.08 g; $FeCl_3 \cdot 6H_2O$, 0.1 g; $Na_2Mo_4 \cdot 2H_2O$, 0.0025 g; KH_2PO_4, 0.8 g at pH 7.2 per liter of distilled water. Either KNO_3 or NH_4Cl was added to make 8 mM as a nitrogen source where indicated. The other aerobic bacteria were grown in 500 ml of Trypticase soy-yeast extract medium consisted of 3% Trypticase soy broth (BBL) and 0.5% yeast extract at 30°C for 10 hr with 10% of inocula.

Bacteroids and host cells were separated according to Klucas et al. (7). Fresh soybean root-nodules (20 g) were homogenized in 100 ml of 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA using a mortar with sea sand for 10 min. After filtration through 4 layers of gauze to remove large fragments, the filtrate was centrifuged at 7,000 g for 10 min and host cells (supernatant) and bacteroids (pellet) were separated. Ammonium sulfate was added to the supernatant to 80% saturation, and the resulting precipitate was collected by centrifugation, suspended in a minimal volume of 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA, and dialyzed against the same buffer. The dialyzed solution was referred to as the host cell extract. The pellet containing bacteroids was suspended in 40 ml of 50 mM potassium phosphate, pH 7.8, and 0.5 mM EDTA and disrupted with an ultrasonic oscillator for five 4-min periods at 2-min intervals. The sonicate was clarified by centrifugation at 30,000 g for 20 min. The supernatant was concentrated with ammonium sulfate at 80% saturation followed by dialysis against 10 mM potassium

phosphate, pH 7.8, containing 0.1 mM EDTA and used as the bacteroid extract. The extracts of other bacteria were prepared by sonic disruption. About 5 g in fresh weight of cells were suspended in 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA and disrupted by sonication for 10 min alternately with 2-min run and 2-min rest. Sonicates were dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA for 2 days.

SOD was assayed using a modified procedure (Chapter II) of McCord and Fridovich (8). Activity is presented in the McCord-Fridovich unit. Protein was determined according to Lowry et al. (9). Isoelectric focusing was performed with Ampholine (pH 3.5-10.0) in 5.3% polyacrylamide gel polymerized with riboflavin and light in the presence of ammonium persulfate. Cathode and anode solutions were 0.02 M H_3PO_4 and 1 M NaOH, respectively. The focusing was conducted at a constant voltage of 200 V for 4.5 hours. The activity bands were detected by the method of Beauchamp and Fridovich (10) with a slight modification which made the pH in gels neutral. The gel was first immersed in 2.5 mM nitroblue tetrazolium containing 0.5 M potassium phosphate, pH 7.8, for 20 min, and then in 30 μM riboflavin containing 30 mM TEMED, 0.1 M potassium phosphate, pH 7.8, and 0.1 mM EDTA for 15 min.

Fe- and Mn-SOD on a polyacrylamide gel were distinguished by soaking a gel in the solution containing 5 mM KCN, 10 mM H_2O_2, 0.1 mM EDTA and 0.5 M sodium carbonate, pH 10.2, for 20 min before activity staining. Fe-SOD was inactivated by this treatment, but the Mn-enzyme was not (11).

3. RESULTS

Soybean root-nodule bacteroids; symbiotic diazotroph

Table I summarizes the SOD activities in cell-free extracts of the bacteroids (Rhizobium japonicum) from soybean root-nodules and of other tissues of soybean plant. The content of SOD in the bacteroids was similar to that of anaerobically grown Escherichia coli (12). The enzyme activity in host cells of the root-nodules was higher than that in the bacteroids.

Table I

Activities of Superoxide Dismutases and their Cyanide-Insensitivities in Extracts of Soybean Root-Nodule Bacteroids and Soybean Plant Tissues

Tissues	SOD activity (units/mg protein)	Cyanide-insensitive activity[a] (%)
Root-nodules		
Bacteroids	1.7	100
Host cells	11.3	7
Leaves	2.3	9
Roots	12.0	9

a) Activity remaining in the presence of 1 mM KCN.

Three types of SOD in regard to functional metals were characterized using the two criteria that the Cu,Zn-enzyme is sensitive to cyanide, but the Fe- and Mn-enzymes are not, and that antiserum against spinach Cu,Zn-enzyme cross-reacts with the Cu,Zn-enzyme in other plants (13). Cyanide at 1 mM did not affect the SOD activity in the bacteroids, suggesting the absence of the Cu,Zn-enyzme and the presence of the Fe- and/or Mn-enzyme (Table I). The enzyme activities in the host cells, leaves and roots were inactivated by 90% with 1 mM cyanide, thus, the major SOD in soybean plant is the Cu,Zn-enzyme. This was also confirmed by Ouchterlony double immunodiffusion (Fig. 1). Immunoglobulin against spinach Cu,Zn-SOD formed a precipitin line with the enzymes in extracts from the host cells, roots and leaves, but not with the enzyme in the bacteroids.

Isoelectric focusing on gel (pH 3.5-10.0) of the extracts from bacteroids and staining for SOD activity revealed a single activity band corresponding to a pI of 4.7 (data not shown).

SOD in the bacteroids was further characterized using the criterion that the Fe-enzyme is sensitive to H_2O_2 but the Mn-enzyme is not (11). However, because of the high catalase activity in the crude extract, the effect of H_2O_2 on SOD activity could not be tested even in the presence of 1 mM KCN. Therefore, a partial purification of the bacteroid SOD was

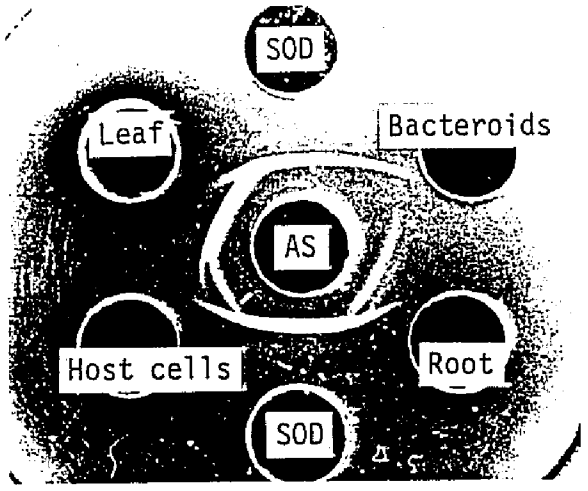

Fig. 1. Ouchterlony double immunodiffusion plate of the spinach Cu,Zn-superoxide dismutase antiserum and superoxide dismutase in cell-free extrcts from soybean tissues and bacteroids in soybean root-nodules. Center well (AS) contained 260 μg of the antiserum to spinach Cu,Zn-enzyme, Outer wells; cell-free extracts containing 3 to 7 units of enzyme from leaves and roots of the soybean and from host cells and bacteroids of the root-nodules, SOD; spinach Cu,Zn-superoxide dismutase (1.1 μg, 3 units).

Note: When this experiment was done, I was not aware of the presence of two immunologically distinguishable Cu,Zn-superoxide dismutase isozymes in spinach (see Chapter V). The anti-spianch Cu,Zn-SOD used (5) was dispecific, since the antigen (stromal Cu,Zn-SOD) contained a small amount of cytosolic Cu,Zn-SOD. These immunological properties of the antiserum are responsible to the spar formation between the leaf and host cells (or root) extract from soybean.

conducted to remove catalase. The crude extract dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA was adsorbed on a column of DEAE-Sephadex equilibrated with 10 mM potassium phosphate, pH 7.8. The enzyme was eluted with a linear gradient of potassium phosphate (10-100 mM). The enzyme fractions eluted at 25 mM were pooled and concentrated.

Incubation of the partially purified enzyme with 5 mM H_2O_2 resulted in inactivation by 45% for an hour, but further inactivation was not observed due to consumption of H_2O_2. Readdition of 5 mM H_2O_2 affected the activity only a little, indicating the presence of catalase even in the partially purified bacteroid enzyme. Thus, the final remaining

activity should be underestimated for the sensitivity of bacteroid SOD against H_2O_2. Nevertheless, taking into consideration that Mn-SOD is resistant against 5 mM H_2O_2 for at least 24 hours (Chapter XVI), and that the bacteroids contain a single SOD, it could be concluded that the SOD in the bacteroids is the Fe-enzyme.

A further support for that the bacteroid SOD is the Fe-enzyme was the effect of azide on the enzymatic activity. According to Misra and Fridovich (14), three types of SOD were distinguishable by their sensitivity against 10 mM azide at pH 7.8; the Fe-enzyme was inhibited by 70% whereas the Mn- and Cu,Zn-enzymes were only slightly affected (30% and 10% inhibitions, respectively). The enzymatic activity in the bacteroid extract was inhibited by 71% in the presence of 10 mM azide at pH 7.8. Thus, all of evidence suggest that the bacteroid SOD is the Fe-SOD.

Azotobacter; free-living diazotroph

Table II shows the typical results of the SOD activity in genous Azotobacter cultured under different conditions, with those of several aerobic bacteria. In comparison with the other aerobic bacteria, A. chroococcum and A. vinelandii contained high amounts of SOD which comprised 2% of total soluble proteins (15). A similar high content has been also reported by Nishie and Yano (16). The enzyme activity was not affected appreciably under dinitrogen fixing conditions, or NH_3-, NO_3^-- or organic form of nitrogen-supplimented conditions. Thus, the high content of the enzyme was not restricted only to the dinitrogen fixing conditions.

Isoelectric focusing on polyacrylamide gel of crude extracts from A. chroococcum and A. vinelandii shows a single activity band corresponding to a pI of 4.1 (data not shown). Nitrogen sources, i.e. N_2, NH_3 or NO_3^- did not affect the pattern of the activity band on gels. These results indicate that Azotobacter contain only a single SOD under any growth conditions. With H_2O_2-treatment prior to activity staining on a gel, the activity band was not visualized, indicating that Azotobacter SOD was H_2O_2-sensitive Fe-enzyme. The purification of SOD from A. vinelandii revealed that this is the Fe-enzyme and no other SOD was

Table II

Superoxide Dismutase Activity in Azotobacter in Comparison with that of Several Aerobic Bacteria

Species	Medium[a)]	Culture time[a)] (hours)	Superoxide dismutase activity (units/mg protein)
Azotobacter vienelandii	N-free	18	48
	N-free	24	42
	N-free	38	40
	NO_3^--N	24	43
	NH_3-N	24	43
	TSY	24	39
	N-free	38[b)]	63
Azotobacter chroococcum	NO_3^--N	18[b)]	61
Acinetobacter aerogenes	TSY	10	20
Bacillus sphericus	TSY	10	5
Pseudomonas aeruginosa	TSY	10	15

a) The cells were cultured with shaking, in 500 ml-medium, with 50 ml of the inoculum which had been cultured for 18-24 hours. N-free; Modified Burk's N-free medium. NO_3^- and NH_3-N; To the Burk's N-free medium 8 mM KNO_3 or NH_4Cl was added. TSY; 3% Trypticase soy broth and 0.5% yeast extract.

b) The cells were cultured in a jar-fermenter under vigorous agitation with air.

detected during the purification processes (Chapter XV).

Oxygen induces the SOD activity in several organisms; Streptococcus faecalis (17), Escherichia coli (3,18,19), Photobacterium leiognathi (20,21), Saccharomyces cerevisiae (23), rat lung (24-26) and guinea pig leucocytes (27). Euglena gracilis Fe-SOD was induced by light (22). Incubation of Azotobacter cells in 100% O_2 for 24 hour increased SOD by 20-50% but the drastic enhancement of the activity due to the induction of new type of the enzyme as shown in E. coli (3,28), was not observed. Disc electrophoresis confirmed that there was not the induction of a new isozyme under high oxygen partial pressure (data not shown). In addition to molecular oxygen, methyl viologen induces SOD in E. coli (4). Methyl viologen is reduced to form the methyl viologen radical by

electron transport chain. This radical, in turn, reduces molecular oxygen to the superoxide radical which is responsible for the induction of SOD. Cells of Azotobacter cultured in Burk's nitrogen-free medium for 18 hours were further incubated in fresh Burk's medium containing various concentrations of methyl viologen for 1 to 6 hours at 30°C. However, methyl viologen up to 1 mM did not affect the enzymatic activity.

4. DISCUSSION

Most diazotrophs (Clostridium, Chromatium, Chlorobium Desulfovibrio, etc.) are anaerobes, but bacteria such as Azotobacter, Rhizobium and blue-green algae are aerobic diazotrophs. Nitrogenase is a highly oxygen labile enzyme; its half life in air is only 4.5 min (29); therefore, for the dinitrogen fixation, the concentration of oxygen inside the cells should be extremely low or near zero even in the aerobic diazotrophs. In fact, a sharp gradient for oxygen concentration has been shown in the "unstirred layer" surrounding of Rhizobium cells, and the oxygen concentration of the surface of the cells have been estimated to be less than 3×10^{-8} to 3×10^{-9} M (30).

The specific activity of SOD in soybean root-nodule bacteroids was similar to that in E. coli (12) cultured under anaerobic conditions. The activity of the bacteroid-free nodule tissues is higher than that of bacteroids but is in comparable level with the other tissues of soybean plant (Table I). This result indicates that the production of the superoxide radical in the bacteroids is low and additional SOD as the defense against active oxygen is not required. It should be noted that host cells in the nodule play a role as physical and chemical barriers, which are derived from leghemoglobin, against oxygen for the bacteroids (1), and that dinitrogen fixation of Rhizobium is undergone only during the symbiosis. To the contrary, the specific activity of SOD in Azotobacter is very high, comprising 2% of soluble protein (Table II). Azotobacter is an aerobe and consumes oxygen at high rate during dinitrogen fixation. This high respiration results in the removal of

molecular oxygen in the cells and is considered to play a role of defense against molecular oxygen. The production of superoxide is supposed to be high due to high respiration rate, therefore SOD might be responsible for its removal.

Among three types of SOD, the Cu,Zn- and Mn- enzymes were induced by oxygen or the superoxide radical (2). In facultative aerobe, Escherichia coli, the cells grown under anaerobic conditions contained only the Fe-enzyme but the cells cultured aerobically possessed the Mn-enzyme in addition to the Fe-enzyme, suggesting that the Mn-enzyme is induced by oxygen but the Fe-enzyme is not (3,4). Thus, the Fe-enzyme is a constitutive enzyme and could be biosynthesized even under low oxygen concentrations.

Thus, anaerobes, anaerobically grown-facultative anaerobes, and aerobic diazotrophs (present results) contain only Fe-SOD and lack the Mn-enzyme. The property common to the three bacterial groups is an extremely low or nearly zero concentration of O_2 or O_2^- inside the cells. Therefore, I propose that the biosynthesis of the Fe-enzyme occurs even when the concentration of O_2 or O_2^- is nearly zero inside the cells, and that the Mn-enzyme is formed only in cells that contain O_2 or O_2^- above a definite concentration. If further evidence supports this proposal, the type of SOD should be useful in prediction the concentration of O_2 or O_2^- inside the cells.

5. SUMMARY

The content and the type of SOD in aerobic symbiotic and free-living diazotrophs are presented. In soybean root-nodules, the bacteroids contain Fe-SOD but lack Mn-SOD, while the host cells have Cu,Zn-SOD. SOD in Azotobacter is also the Fe-enzyme and its content is very high although it is unaffected by culture either in dinitrogen or in ammonium and nitrate. Mn-SOD was not detected under any culture conditions. It is proposed that by the type of SOD in organisms the concentration of O_2 inside the cells can be predicted.

6. REFERENCES

(1) Burns, R.C., and Hardy, R.W.F. (1975) Nitrogen Fixation in Bacteria and Higher Plants, pp. 102-104, Springer-Verlag, Berlin.

(2) Steinman, H.M. (1982) in Superoxide Dismutase (Oberley, L.W., ed.), pp. 11-68, CRC Press, Florida.

(3) Hassan, H.M., and Fridovich, I. (1977) J. Bacteriol. 129, 1574-1583.

(4) Hassan, H.M., and Fridovich, I. (1977) J. Biol. Chem. 252, 7667-7672.

(5) Asada, K., Takahashi, M., and Nagate, M. (1974) Agric. Biol. Chem. 38, 471-473.

(6) Burns, R.C., and Hardy, R.W.F. (1972) Methods Enzymol. 24, 480-476.

(7) Klucas, R.V., Koch, B., Russell, S.A., and Evans, H.J. (1968) 43, 1906-1912.

(8) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(9) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

(10) Beauchamp, C.O., and Fridovich, I. (1971) Anal. Biochem. 44, 276-287.

(11) Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y., and Enmanji, K. (1975) J. Biol. Chem. 250, 2801-2807.

(12) Tally, F.P., Goldin, B.R., Jacobus, N.V., and Gorbach, S.L. (1977) Infect. Immun. 16, 20-25.

(13) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(14) Misra, H.P., and Fridovich, I. (1978) Arch. Biochem. Biophys. 189, 317-322.

(15) Kanematsu, S., and Asada, K. (1977) Proc. Agric. Chem. Soc. Japan, p. 266.

(16) Nishie, H., and Yano, K. (1977) Proc. Agric. Chem. Soc. Japan, p. 266.

(17) Gregory, E.M., and Fridovich, I. (1973) J. Bacteriol. 114, 543-548.

(18) Gregory, E.M., and Fridovich, I. (1973) J. Bacteriol. 114,

1193-1197.

(19) Gregory, E.M., Yost, F.J., Jr., and Fridovich, I. (1973) J. Bacteriol. 115, 987-991.

(20) Puget, K., and Michelson, A.M. (1974) Biochem. Biophys. Res. Commun. 58, 830-838.

(21) Puget, K., and Michelson, A.M. (1974) Biochemie 65, 1255-1267.

(22) Kanematsu, S.,and Asada, K. (1978) Arch. Biochem. Biophys. 185, 473-482.

(23) Gregory, E.M., Goscin, S.A., and Fridovich, I. (1974) J. Bacteriol. 117, 456-460.

(24) Crapo, J.D., and Tierney, D.F. (1974) Am. J. Physiol. 226, 1401-1407.

(25) Kimball, R.E., Reddy, K., Piera, T.H., Schwarz, L.W., Mustafa, M.G., and Cross, C.E. (1976) Am. J. Physiol. 230, 1425-1431.

(26) Stevens, J.B., and Autor, A.P. (1677) J. Biol. Chem, 252, 3509-3514.

(27) Rister, M. and Baehner, R.L. (1976) J. Clin. Invest. 58, 1174-1184.

(28) Dougherty, H.W., Sadowski, S.J., and Baker, E.E. (1978) J. Biol. Chem. 253, 5220-5223.

(29) Israel, D.W., Howard, R.L., Evans, H.J., and Russel, S.A. (1974) J. Biol. Chem. 249, 500-508.

(30) Wittenberg, J.B., Bergersen, F.J., Appleby, C.A., and Turner, C.L. (1974) J. Biol. Chem. 249, 4057-4066.

CHAPTER XI

Fe-SUPEROXIDE DISMUTASES IN EUGLENA GRACILIS: LOCALIZATION OF THE Fe-ENZYME IN CHLOROPLAST STROMA

1. INTRODUCTION

Superoxide dismutase (SOD) is classified by its prosthetic metal into Cu,Zn-, Fe-, and Mn-containing enzymes (1,2). The evolutionary or phylogenetic position of the organisms determines the distribution of the three forms of SOD; prokaryotes, protozoa, and most eukaryotic algae lack Cu,Zn-SOD but contain Fe- and/or Mn-SOD (6,7, Chapter II-IV). Anaerobic bacteria contain only Fe-SOD (3-5, Chapter VIII and IX). To my knowledge there is no Fe-SOD in vertebrates, fungi, or land plants which contain the Cu,Zn- and Mn-enzymes (8) (see following chapters).

The absence of Cu,Zn-SOD has been confirmed (6,9) in several protozoa, but no further characterization of the enzyme has been made. In the eukaryotic algae only the enzyme of a red alga has been shown to be Mn-SOD (10); however, no SOD has been characterized in other eukarytotic algae; including the green algae. I here describe the isolation of SOD from a geeen alga, the protozoan Euglena gracilis. This isolated Euglena SOD is the Fe-enzyme, the first proof of ferric SOD in a eukaryote.

2. MATERIALS AND METHODS

Cytochrome c (type III), xanthine oxidase, and Ampholine were products of Sigma, Boeheringer, and LKB, respectively. Euglena gracilis, strain Z, was a gift from Dr. S. Kitaoka, and its cells were cultured at 25°C under photoautotrophic conditions with the bubbling of 5% CO_2 in the medium of Cramer and Myers (11). Cells were harvested in the late logarithmic phase of growth and stored at -20°C until use.

Chromatium vinosum (4), Chlorobium thiosulfatophilum (5), and Plectonema boryanum (12) Fe-SOD, P. boryanum Mn-SOD (12), and spinach Cu,Zn-SOD (13) were obtained as before. Fe-SOD from Azotobacter vinelandii was purified to homogeneity and Rhizobium Fe-SOD was partially purified from bacteroids of soybean root nodules by DEAE-Sephadex column (Chatper X and XV). Antibody to Plectonema boryanum Fe-SOD was prepared as described in Chapter II (6). SOD was assayed by a modification of the procedure (14) of McCord and Fridovich (15) with a Hitachi 356 two-wavelength double-beam spectrophotometer at 550 nm with a fixed reference at 540 nm. Enzymatic activity is shown in McCord-Fridovich units (15).

Polyacrylamide gel disc electrophoresis, location of activity on the disc, amino acid analysis, measurement of the dry weight of the enzyme, determination of the molecular and subunit molecular weights, and metal analysis were performed as before (4,5). The isoelectric point was measured by isoelectric focusing with Ampholine on a 5% polyacrylamide gel disc (16).

3. RESULTS

Fe-Superoxide Dismutases from Soluble Extracts of Euglena - Purification

Unless specified, all operations were carried out at 0-4°C, and potassium phosphate buffer was used at pH 7.8. The cells of Euglena gracilis (300 g) were suspended in 2 liters of 50 mM phosphate containing 0.5 mM EDTA, then disrupted by sonication at 20 kHz in an ice bath for two 4-min runs at 2-min intervals. After removal of cell debris by centrifugation at 14,000 g for 30 min, KCl was added to the supernatant to make a final concentration of 0.1 M. A 200-ml batch of the supernatant was heated to 60°C for 3 min, then cooled in an ice bath. The green precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant up to 35% saturation, and the resulting precipitate was removed by centrifugation. The supernatant was further brought to 80% saturation with ammonium sulfate and centrifuged. The precipitate collected was dissolved in and dialyzed against 5 mM

phosphate containing 0.1 mM EDTA.

After clarification by centrifugation, the dialyzed solution was adsorbed on a column of DEAE-Sephadex (10 x 29 cm) previously equilibrated with 5 mM phosphate containing 0.1 mM EDTA, and the column was washed with 4 liters of the equilibrating buffer. The washings contained about 15% of the total activity. Further purification of the unadsorbed enzyme by CM-Sephadex column chromatography failed due to unstability of the enzyme. The adsorbed enzyme was eluted with 50 mM phosphate and concentrated by ammonium sulfate. No SOD was detected on further elution of the column with 1 M KCl. The 50 mM phosphate eluate was dialyzed against 5 mM phosphate and again applied to a column of DEAE-Sephadex (2.5 x 40 cm) equilibrated with 5 mM phosphate. Linear gradient elution with 1.5 liters of phosphate (5-60 mM) gave two peaks of SOD at 26 (SOD-II) and 32 mM phosphate (SOD-I) (Fig. 1). Each peak was pooled and concentrated to 10 ml in a collodion bag under reduced pressure. The concentrated enzyme was gel-filtered with 20 mM phosphate as the eluent on a column of Sephadex G-100 (4 x 80 cm) equilibrated with the same buffer. Active fractions were dialyzed against 5 mM phosphate, pH 6.8, then applied to a hydroxylapatite column (1.5 x 8 cm)

TABLE I

PURIFICATION OF Fe-SUPEROXIDE DISMUTASE-I AND -II FROM SOLUBLE FRACTION OF *Euglena*

Purification step	Total protein[a] (mg)	Total activity (10^{-3} unit)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Sonicate	13,585	106	8	100	1
Heating	5,248	84	16	79	2.0
35 to 80% $(NH_4)_2SO_4$	1,830	67	37	63	4.6
Stepwise DEAE Sephadex					
Unadsorbed fraction	127	10	79	9	10
50 mM Phosphate eluate	456	54	118	51	15
Linear gradient DEAE Sephadex					
SOD-I[b]	101	22	218	21	27
SOD-II	62	15	242	14	30
Sephadex G-100					
SOD-I	60	22	367	21	46
SOD-II	20	15	750	14	94
Hydroxylapatite					
SOD-I	5.5	15.7	2,855	15	357
SOD-II	4.8	13.1	2,729	12	341

[a] Protein was measured by the methods of Lowry *et al.* (17) and in the final step the enzyme was determined assuming an $A|^{1\%}_{1cm}$ at 280 nm of 22.63 and of 21.52 for superoxide dismutase-I and -II, respectively.

[b] SOD-I, superoxide dismutase-I; SOD-II, superoxide dismutase-II.

equilibrated with 5 mM phosphate, pH 6.8. SOD was separated because it was not adsorbed although flavoprotein was. The unadsorbed fraction was collected and frozen for storage. The purification procedure is summarized in Table I.

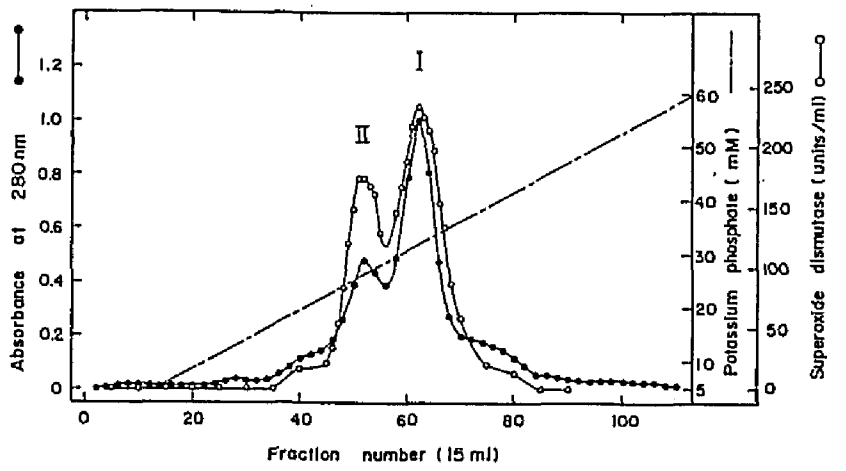

FIG. 1. Linear gradient chromatography of the soluble superoxide dismutase isozymes from *Euglena* on DEAE Sephadex. Details are given in the text.

Properties of Euglena Fe-Superoxide Dismutases

SOD-I and -II were homogeneous as based on polyacrylamdie gel disc electrophoresis (Fig. 2) and on isoelectric focusing on a polyacrylamide gel disc (pH 3.5-10.0). For both isozymes, the location of the protein bands on the gel disc corresponded to the activity bands of the crude extract. Even if the sonicated of the Euglena cells was incubated in 0.1 mM phenylmethyl sulfonyl fluoride, 1 mM monoiodoacetamide, and 1 mM EDTA for 2 days at 5°C, and after dialysis was subjected to polyacrylamide gel disc electrophoresis, these protease inhibitors did not affect the location of activity on the gel. These observations, thus, exclude modification of the enzyme by proteolysis during purification. Isoelectric focusing of the crude extracts on a polyacrylamide gel disc revealed several activity bands at high pI values (pI 5.7, 6.8, 7.8) in addition to the positions found for the two isolated isozymes (pI 4.2, 4.3). These minor SOD isozymes may be enzymes which were not adsorbed on the DEAE-Sephadex column during purification. Cyanide at 1 mM did not affect the pattern of the activity bands of the purified enzymes or

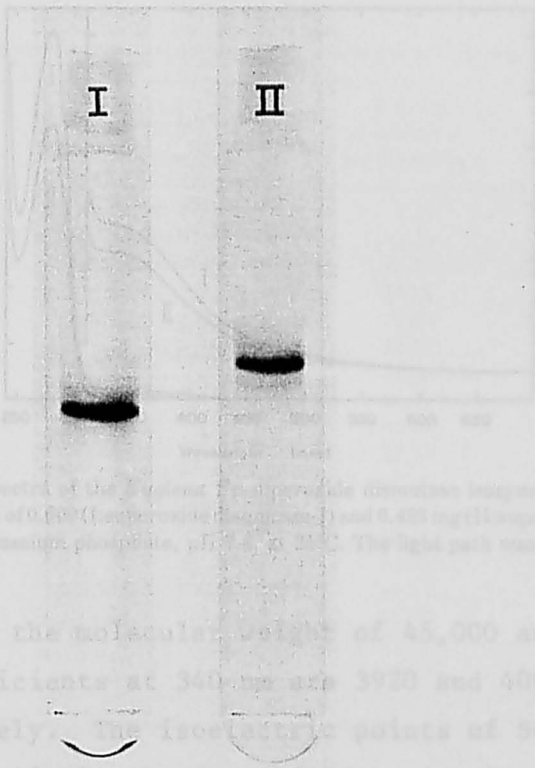

FIG. 2. Polyacrylamide gel disc electrophoresis of *Euglena* Fe-superoxide dismutase isozymes. I, Fe-superoxide dismutase-I; II, Fe-superoxide dismutase-II. Cathode, top; anode, bottom. Protein (24 μg) was stained with amido black 10 B.

that of the crude extract. These observations confirm the absence of cyanide-sensitive Cu,Zn-SOD in Euglena, even as a minor isozyme component, as described in Chaper II (6).

The molecular weights of both SOD-I and -II were 45,000 as determined by gel-filtration with a Sephadex G-100 column. The subunit molecular weight determined by sodium dodecyl sulfate-disc electrophoresis was 22,000 for both isozymes. Both isozymes gave one band in the presence and the absence of 2-mercaptoethanol. Thus, Euglena SOD are composed of two subunits of equal size without a disulfide bridge.

SOD-I and -II showed the same ultraviolet and visible spectra (Fig. 3). An absorption maximum at 280 nm with shoulders at 260 and 290 nm and a weak absorption from 320 to 600 nm with a shoulder at 340 nm are characteristic of Fe-SOD. $A_{1\,cm}^{1\%}$ and the absorbance coefficient at 280 nm are 22.63 and 101,800 $M^{-1}cm^{-1}$ for SOD-I and 21.52 and 96,000 $M^{-1}cm^{-1}$

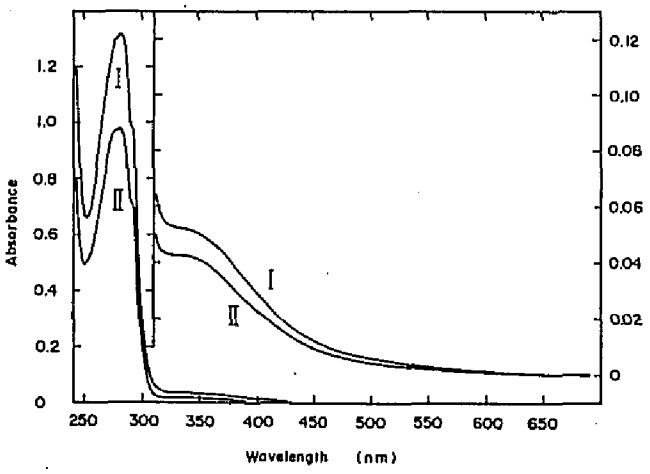

FIG. 3. Absorption spectra of the *Euglena* Fe-superoxide dismutase isozymes. Spectra were recorded at a concentration of 0.609 (I:superoxide dismutase-I) and 0.483 mg (II:superoxide dismutase-II) enzyme/ml in 10 mM potassium phosphate, pH 7.8, at 25°C. The light path was 10 mm.

for SOD-II, based on the molecular weight of 45,000 and the dry weight. The absorbance coefficients at 340 nm are 3920 and 4000 $M^{-1}cm^{-1}$ for SOD-I and -II, respectively. The isoelectric points of SOD-I and -II are 4.18 and 4.30, respectively, as determined by isoelectric focusing with Ampholine (pH 3.5-5.0). Antibody to Plectonema Fe-SOD (12) did not affect the activity of the purified Euglena enzymes nor cross-react with the enzymes in Ouchterlony double immunodiffusion as described previously (6). This is not surprising because the antigenic determinants of the Fe-enzyme are highly specific to each organism as obserbed in several Fe-enzymes (6,18).

Determination of the metal with atomic absorption spectrometry indicates that Euglena SOD-I and -II contained 1.8 $\pm$ 0.1 and 1.9 $\pm$ 0.1 g-atoms of Fe per mole of the enzyme, respectively, based on a molecular weight of 45,000. The contents of manganese, copper, and zinc were below detection or were insignificant. No acid-labile sulfide was detectable.

Comparison of Fe-Superoxide Dismutases from Euglena and from Prokaryotes

The properties of the soluble Euglena SOD described above resemble those of the Fe-SOD isolated from prokaryotes. This then, is the first isolation of Fe-SOD from eukaryotes, and a comparison of Fe-SOD from Euglena and from prokaryotes was made.

TABLE II

AMINO ACID COMPOSITION OF Fe-SUPEROXIDE DISMUTASES FROM *Euglena* AND FROM *Chlorobium*

Amino acids	*Euglena* Fe-SOD-I[a] (residues/mol)	*Euglena* Fe-SOD-II[a] (residues/mol)	*Chlorobium* Fe-SOD[b] (residues/mol)
Lysine	7.74	7.53	22
Histidine	13.42	12.56	12
Arginine	19.36	18.71	6
Aspartic acid	38.71	39.03	57
Threonine	22.92[c]	22.37[c]	20
Serine	10.74[c]	10.11[c]	19
Glutamic acid	44.02	44.10	42
Proline	25.19	26.04	18
Glycine	39.86	40.48	33
Alanine	51.86	53.54	45
Valine	22.07[d]	22.59[d]	25
Methionine	5.38[e]	7.01[e]	5
Isoleucine	12.83[d]	12.77[d]	9
Leucine	32.14	33.05	26
Tyrosine	14.02	13.00	18
Phenylalanine	20.74	21.00	16
Half-cystine	9.12[e]	11.22[e]	3
Tryptophan	15.62[f]	14.28[f]	12

[a] The number of residues was calculated for 45,000 g of enzyme.
[b] Ref. (5).
[c] The values extrapolated to zero hydrolysis time.
[d] The values at 72 h hydrolysis.
[e] Determined as methionine sulfone and cysteic acid after performic acid oxidation (19).
[f] Spectrophotometric determination according to Edelhoch (20).

TABLE III

COMPARISON OF $S\Delta Q$ VALUES OF Fe-SUPEROXIDE DISMUTASES FROM PROCARYOTES AND *Euglena*[a]

	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)
(a) *Desulfovibrio* (3)[b]										
(b) *Chlorobium* (5)	30									
(c) *Chromatium* (4)	70	36								
(d) *Spirulina* (22)	43	41	45							
(e) *Plectonema* (12)	31	34	56	19						
(f) *Photobacterium* (23)	24	36	43	25	24					
(g) *Escherichia* (24)	33	36	39	29	25	15				
(h) *Pseudomonas* (25)	50	57	40	60	49	35	41			
(i) *Mycobacterium* (26)	51	56	76	36	31	37	36	84		
(j) *Euglena*-I (this work)	86	76	95	101	77	63	52	82	85	
(k) *Euglena*-II (this work)	90	79	99	101	76	65	55	88	86	1

[a] $S\Delta Q$ value was calculated from the amino acid composition of Fe-superoxide dismutases reported here as well as published previously using the following equation (21):

$$S\Delta Q = \sum_j (X_{i,j} - X_{k,j})^2,$$

where the subscripts i and k indicate the enzymes which were compared and X_j is the mole percent content of a given amino acid of type j. In the present calculation 16 amino acids were included omitting half-cystine and tryptophan.

[b] Numbers in parentheses refer to references from which data were taken.

Table II shows the amino acid composition of the Euglena enzymes. The composition of the two isozymes is the same within the experimental error. The principal differences between the Euglena and prokaryote Fe-SOD are the higher content of arginine and the lower content of lysine in the Euglena enzyme. The Euglena enzymes are also distinguished from the prokaryote enzymes by their serine, proline, alanine, and half-cystine contents.

To assess the degree of sequence homology between the Euglena and prokaryote Fe-SOD, a statistical analysis of their amino acid compositions was made by the method of Marchalonis and Weltman (12); this is summarized in Table III. The SΔQ values are the sums of the squares of the difference between the mole percent content of individual amino acid

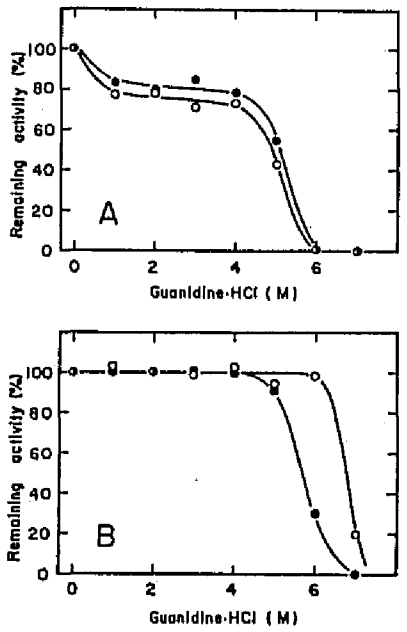

FIG. 4. Effect of guanidine on Fe-superoxide dismutases from *Euglena* and from procaryotes. Fe-superoxide dismutases from *Euglena*, *Chlorobium*, and *Chroamtium*, each about 5 μg, were incubated for 1 h at 25°C with 50 mM potassium phosphate, 0.5 mM EDTA, and guanidine hydrochloride as indicated, in a total volume of 80 μl. The pH of the mixture had been adjusted to 7.8 with KOH before incubation. The remaining activity was determined using a sample (about 4 μl) under standard assay conditions. Introduction of guanidine to the assay mixture under the present conditions did not disturb the assay. (A) ○, *Euglena* isozyme-I; ●, *Euglena* isozyme-II. (B) ○, *Chlorobium*; ●, *Chromatium*.

residues on the two proteins compared. The SΔQ values among prokaryote Fe-SOD, including those from anaerobes, are below 50 in most cases. In contrast, the values between Euglena and the prokaryote enzymes range from 50 to 100. This suggests that the amino acid sequence in Euglena Fe-SOD differs considerably from that in prokaryote enzymes: a reflection of the phylogenetic distance between prokaryotes and eukaryotic Euglena.

In addition the Euglena enzymes are distinguished from the prokaryote Fe-enzyme by the higher structure of the molecule. Euglena SOD were stable against heat at 40°C for at least 1 h, but above 50°C were inactivated with a time course showing first-order kinetics in respect to enzyme concentration. At 60°C, the half-lives of SOD-I and -II were 17 and 9 min, respectively. In contrast, at 60°C, Chlorobium Fe-SOD was not inactivated for at least 1 h and only half of the activity of the Chromatium Fe-enzyme (4) was lost after 1 h. Spirulina Fe-SOD also lost only half activity at 60°C for 1 h (22). Thus, the Euglena enzymes are more thermolabile than are the prokaryote Fe-enzymes.

Another difference between the Euglena and prokaryote Fe-SOD was

TABLE IV

HALF-LIVES OF Fe-SUPEROXIDE DISMUTASES IN HYDROGEN PEROXIDE

Superoxide dismutases[a]	Half-life (min)
Euglena Fe-SOD-I	6.5
Fe-SOD-II	6.5
Plectonema Fe-SOD (12)[b]	40
Chromatium Fe-SOD	15
Chlorobium Fe-SOD	17
Escherichia Fe-SOD (28)[b]	12
Escherichia hybrid Fe-SOD (28)[b]	25
Spinach Cu,Zn-SOD (12, 13)[b]	28

[a] Superoxide dismutases (SOD) were incubated with 0.5 mM H_2O_2, 10 mM potassium phosphate, pH 7.8, and 0.1 mM EDTA at 25°C. Activity was determined using a small aliquot of the treated enzyme under the standard assay conditions at suitable intervals and half-life of the activity was determined.

[b] The results from the references in parentheses which had been determined under the same conditions as the present ones.

the effect of the denaturants, guanidine and dimethylsulfoxide, on hydrogen bonds and hydrophobic interaction. The Euglena SOD were labile to guanidine in comparison to the prokaryote enzymes (Fig. 4). The Euglena enzymes were inactivated 20% in 1 M guanidine and were progressively destroyed above 4 M showing a sigmoidal denaturation profile, whereas the Chlorobium and Chromatium enzymes were stable up to 6 and 5 M guanidine, respectively. The guanidine-treated Euglena enzymes shown in Fig. 4 were diluted fivefold with 10 mM phosphate, pH 7.8, then incubated for 2 days at 5°C. However, no recovery of activity was observed. In contrast, incubation of the Euglena in 7 M urea to pH 7.8 for 1 h at 25°C did not affect enzymatic activity. The enzyme was incubated in various concentrations of dimethylsulfoxide for 30 min, then assayed for remaining activity. Both Euglena enzymes lost 50% of their activities with 45% dimethylsulfoxide, but the Chlorobium enzyme showed the same inactivation with 60% dimethylsulfoxide. Thus, the Euglena Fe-SOD seems to be more labile to denaturating stresses than are the prokaryote enzymes.

Fe-SOD from prokaryotes is inactivated by H_2O_2 (4,12,22,27,28). This is also the case of the Euglena enzymes. The time course of inactivation showed apparent first-order kinetics in respect to enzyme concentration. The half-inactivation by 0.5 mM H_2O_2 at pH 7.8 was calculated to be 6.5 min for SOD-I and -II. Table IV compares this data

TABLE V

INHIBITION OF SUPEROXIDE DISMUTASES FROM VARIOUS ORGANISMS BY AZIDE

Superoxide dismutases	% Inhibition by 10 mM azide at pH 7.8
Chlorobium Fe-SOD	70
Chromatium Fe-SOD	67
Azotobacter Fe-SOD	71
Rhizobium Fe-SOD	71
Plectonema Fe-SOD	73
Mn-SOD	28
Euglena Fe-SOD-I	50
Fe-SOD-II	49

[a] The soluble fraction in Table VI.

[b] Thylakoids was prepared from the chloroplast fraction (1000*g* pellet in Table VI) as described in text. Assayed in the presence of 1% Triton X-100.

with data for other SOD; it shows that of all the enzymes the Euglena enzymes are the most labile to H_2O_2.

Further, Euglena Fe-SOD are different from the prokaryote enzymes in respect of the effect of azide. In accordance with Misra and Fridovich (29), Fe-SOD from bacteria including blue-green alga and aerobic diazotrophs were inhibited about 70% by 10 mM azide. However, the Euglena Fe-enzymes were less sensitive to azide: about half-inhibition by 10 mM azide (Table V).

Localization of Fe-Superoxide Dismutase in Chloroplast Stroma

The above results show that Fe-SOD exists in soluble fraction of Euglena cells. In addition, I detected low but distinct SOD activity in the green sediments after centrifugation of the sonicate of Euglena cells, indicating the occurrence of the enzyme in chloroplasts. To confirm the localization of SOD, I fractionated the cells by differential centrifugation.

The cells (2 g fresh wt) were washed three times with water, then once with the isolation medium; 0.5 M sucrose and 50 mM Tricine-KOH, pH 7.8. The washed cells suspended in 40 ml of the isolation medium were disrupted by Yeda press at 120 kg/cm^2. After removal of unbroken cells by centrifugation at 500 g for 1 min, the supernatant was again centrifuged at 1000 g for 5 min; the pellet is referred to as the chloroplast fraction. The supernatant was further centrifuged at 15,000 g for 15 min; the resultant pellet contained broken chloroplasts and mitochondria. Then the supernatant was centrifuged at 100,000 g for 60 min. The supernatant from this step is referred to as the soluble fraction and the pellet contained mitochondria and microsomes. SOD activity in each fraction was assayed in the presence of Triton X-100 and is summarized in Table VI with chlorophyll contents and cytochrome c oxidase activity.

The bulk of SOD is found in the soluble fraction. Part of this might be released form the organelles possibly from chloroplasts and mitochondria, during the cell fractionation. The SOD associated with the organelles was low, but the association was always observed. From the distribution of chlorophyll and cytochrome c oxidase activity in

TABLE VI

SUBCELLULAR LOCALIZATION OF SUPEROXIDE DISMUTASES IN *Euglena*[a]

Fraction	Superoxide dismutase		Chlorophyll (mg)	Cytochrome *c* oxidase (%)
	Total activity (units)	Specific activity (units/mg of protein)		
1000*g* Pellet (chloroplasts)	22	2.0	1.16	14
15,000*g* Pellet (broken chloroplasts and mitochondria)	9	1.1	0.76	47
100,000*g* Pellet (mitochondria and microsomes)	7	1.7	0.13	39
100,000*g* Supernatant (soluble fraction)	390	12.9	0.23	0

[a] Superoxide dismutase was assayed in the presence of 1% Triton X-100. Cytochrome *c* oxidase was determined following the oxidation of reduced cytochrome *c* at 550 nm with a fixed reference at 540 nm after 1-min incubation of sample with 2% digitonin according to Simon (30). Chlorophyll was determined spectrophotometrically (31).

each fraction, it is evident that chloroplasts and mitochondria contain SOD. I have observed the association of SOD with mitochondria which were separated from the protoplasts from heterotrophically grown Euglena cells by sucrose density centrifugation. Mitochodrial SOD may be the Mn-enzyme, since Mn-SOD localizes in mitochondria of various organisms. No SOD has been detected in peroxisomes from the same cells.

Further fractionation of the chloroplast fraction showed the localization of Fe-SOD in stroma. The chloroplast fraction suspended in 50 mM Tricine-KOH, pH 7.8, 10 mM NaCl, and 1 mM $MgCl_2$ was sonicated for 10 s at 20 kHz in an ice bath, then centrifuged at 50,000 g for 1 h. About 80% of SOD in the chloroplast fraction was recovered in the stromal fraction.

4. DISCUSSION

Three types of SOD categorized by their prosthetic metals show characteristic distributions in organisms according to the phylogenetic position of evolutionary stage. Fe-SOD has been isolated from prokaryotes, including the anaerobes. Anaerobic sulfate reducing bacteria and photosynthetic sulfur bacteria contain only the Fe-enzyme (3-5), although several aerobic bacteria, including E. coli and a blue-green algae, contain both the Fe- and Mn-enzymes (12,28,32). No Fe-SOD has been isolated from vertebrates, fungi, or land plants which contain the Cu,Zn-enzyme in addition to the Mn-enzyme in their mitochondria (8). The absence of the Cu,Zn-enzyme has been confirmed in protozoa; only cyanide-insensitive enzymes have been detected in Euglena, Tritrichromonas foetus, Monocercomonas sp., Crithidia fasciculata, and Tetrahymena pyriformis (6,9). This type of distribution is similar to eukaryotic algae except for the phragmoplasts (6,7).

The results presented in this study show that Euglena cells have Fe-SOD in soluble fraction and chloroplast stroma fraction. This indicates that Fe-SOD is not restricted to prokaryotes only. The Mn-enzyme has been isolated from mitochondria of eukaryotes (33,34), from prokaryotes, including a blue-green alga (12), and from a red alga (10). However, no Mn-enzyme has been found in anaerobic bacteria (3-5).

The stroma of Euglena chloroplasts contains Fe-SOD. The cytosol of blue-green algae contain the Fe-enzyme (35,36). Distribution of Fe-SOD in the cells of prokaryotic algae and in the chloroplasts of eukaryotic algae sustains the endosymbiotic hypothesis (37) on the origin of the chloroplasts of the eukaryotic algae. It is interesting to note that SOD in the stroma of higher plants is replaced by the Cu,Zn-enzyme (13,38).

Fe-SOD-I and -II from Euglena were similar in their absorption spectra, molecular weights, subunit structures, metal contents and amino acid composition; only their pI values differed. These charge isomers were not artifacts of the purification procedure because the location of the purified enzyme on a polyacrylamide gel disc after electrophoresis or isoelectric focusing coincided with that of the crude extract from

the cells.

Fe-SOD from Euglena resembles the prokaryote enzymes in its molecular weight, subunit structures, and absorption spectra. However, the Euglena enzyme is more sensitive to denaturing stresses including heat, denaturants, and H_2O_2 and less sensitive to azide than are the prokaryote enzymes. These differences in higher structure of the enzyme molecules suggest a difference in the primary structures of the Euglena and prokaryote enzymes. In fact, a statistical analysis of the amino acid composition (Table III) suggested that the degree of difference in the amino acid sequence between the eukaryotic Euglena enzyme and the prokaryotic enzymes is higher than that found among the prokaryote enzymes, a reflection of the phylogenetic distance between prokaryotes and eukaryotes.

5. SUMMARY

Iron-containing superoxide dismutase (SOD) was found in the soluble and chloroplast stroma fractions from Euglena gracilis. Two major Fe-SOD were isolated in the homogeneous state. Their absorption spectra, molecular weights, subunit structures, and metal contents resemble those of the Fe-enzymes from prokaryotes. However, the Euglena enzymes are more sensitive to heating, to denaturants, and to H_2O_2 and less sensitive to azide than are the prokaryote enzymes. The amino acid composition of the Euglena enzyme differs substantially from the compositions of the enzymes from prokaryotes.

6. REFERENCES

(1) Fridovich, I. (1975) Annu. Rev. Biochem. 44, 147-159.

(2) Asada, K. (1976) J. Japan. Biochem. Soc. 48, 226-257.

(3) Hatchikian, E.C., and Henry, Y.A. (1977) Biochimie 59, 153-161.

(4) Kanematsu, S., and Asada, K. (1978) Arch. Biochem. Biophys. 185, 473-482.

(5) Kanematsu, S., and Asada, K. (1978) FEBS Lett. 91, 94-98.

(6) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(7) Henry, L.E.A., and Hall, D.O. (1977) in Photosynthetic Organelles (Miyachi, S., Katoh, S., Fujita, Y., and Shibata, K., eds.), pp. 377-382, Japanese Society of Plant Physiologists, Kyoto.

(8) Asada, K., and Kanematsu, S. (1978) in Evolution of Protein Molecules (Matsubara, H., and Yamanaka, T., eds.), pp. 361-372, Japan Sci. Soc. Press, Tokyo.

(9) Lindmark, D.G., and Muller, M. (1974) J. Biol. Chem. 249, 4634-4637.

(10) Misra, H.P., and Fridovich, I. (1977) J. Biol. Chem. 252, 6421-6423.

(11) Cramer, M., and Myers, J. (1952) Arch. Mikrobiol. 17, 384-402.

(12) Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y., and Enmanji, K. (1975) J. Biol. Chem. 250, 2801-2807.

(13) Asada, K., Urano, M., and Takahashi, M. (1973) Eur. J. Biochem. 36, 257-266.

(14) Asada, K., Takahashi, M., and Nagate, M. (1974) Agric. Biol. Chem. 38, 471-473.

(15) McCord, J. M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(16) Wrigley, C.W. (1971) Methods Enzymol. 22, 559-564.

(17) Lowry, O.H., Rosebrough, N. J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

(18) Kusunose, M., Noda, Y., Ichihara, K., and Kusunose, E. (1976) Arch. Microbiol. 108, 65-73.

(19) Moore, S. (1963) J. Biol. Chem. 238, 235-237.

(20) Edelhoch, H. (1967) Biochemistry 6, 1948-1954.

(21) Marchalonis, J.J., and Weltman, J. K. (1971) Comp. Biochem. Physiol.

38B, 609-625.

(22) Lumsden, J., Cammack, R., and Hall, D.O. (1976) Biochim. Biophys. Acta 438, 380-392.

(23) Puget, K., and Michelson, A.M. (1974) Biochimie 56, 1255-1267.

(24) Yost, F.J., Jr., and Fridovich, I. (1973) J. Biol. Chem. 248, 4905-4908.

(25) Yamakura, F. (1976) Biochim. Biophys. Acta 422, 280-294.

(26) Kusunose, E., Ichihara, K., Noda, Y., and Kusunose, M. (1976) J. Biochem. 80, 1343-1352.

(27) Lavelle, F., McAdam, M.E., Fielden, E.M., Roberts, P.B., Puget, K., and Michelson, A.M. (1977) Biochem. J. 161, 3-11.

(28) Dougherty, H. W., Sadowski, S.J., and Baker, E.E. (1978) J. Biol. Chem. 253, 5220-5223.

(29) Misra, H.P., and Fridovich, I. (1978) Arch. Biochem. Biophys. 189, 317-322.

(30) Simon, E.W. (1958) Biochem. J. 69, 67-74.

(31) Arnon, D.I (1949) Plant Physiol. 24, 1-15.

(32) Hassan, H.M., and Fridovich, I. (1977) J. Biol. Chem. 252, 7667-7672.

(33) Weisiger, R.A., and Fridovich, I. (1973) J. Biol. Chem. 248, 3582-3592.

(34) Ravindranath, S.D., and Fridovich, I. (1975) J. Biol. Chem. 250, 6107-6112.

(35) Asada, K., Kanematsu, S., Takahashi, M., and Kono, Y. (1976) in Iron and Copper Proteins (Yasunobu, K.T., Mower, H.F., and Hayaishi, O., eds.), pp. 551-564, Plenum, New York.

(36) Okada, S., Kanematsu, S., and Asada, K. (1979) FEBS Lett. 103, 106-110.

(37) Raven, P.H. (1970) Science 169, 641-646.

(38) Jackson, C., Dench, J., Moore, A.L., Halliwell, B., Foyer, C.H., and Hall, D.O. (1978) Eur. J. Biochem. 91, 339-344.

CHAPTER XII

OCCURRENCE OF Fe-SUPEROXIDE DISMUTASE IN THALLI AND CULTURED CELLS OF THE MOSS MARCHANTIA POLYMORPHA

1. INTRODUCTION

Three forms of superoxide dismutase (SOD) having different prosthetic metals have been found; Cu,Zn-SOD, Fe-SOD and Mn-SOD (1,2). Cu,Zn-SOD has little homology in amino acid sequence with the other two enzymes (2) and has been found in eukaryotes including animals, fungi and plants with a few exceptions (Chapter II-IV). Mn-SOD is present in prokaryotes and mitochondria (1,3). Fe-SOD has a high homology in animo acid sequence with Mn-SOD (2) and its occurrence was belived to be limited to prokaryotes.

Recently, however, Fe-SOD has been found in eukaryotes. The SOD purified from Euglena gracilis is the first Fe-enzyme from eukaryotes, and it is localized in the chloroplast stroma (4, Chapter XI). Thereafter, Salin and Bridges have isolated Fe-SOD from angiosperms; Brassica campestris (5) and Nuphar luteum (6) and shown its localization in the stroma (7). Fe-SOD has been found also in Lycoperiscon esculentum (8) and Citrus limonum (9). I have purified and characterized Fe-SOD from Spinacia oleracia (Chapter XIII). Thus, Fe-SOD appears to distribute in eukaryotic algae and angiosperms in addition to prokaryotes.

Previously I showed that major SOD in ferns and mosses is the cyanide-sensitive Cu,Zn-enzyme (10, Chapter II). These organisms contain also the cyanide-insensitive SOD; either Fe- or Mn-containing enzyme. I report here the occurrence of Fe-SOD in thalli and cultured cells of the moss Marchantia polymorpha based on its sensitivity to hydrogen peroxide and insensitivity to cyanide. The thalli contained both Cu,Zn-SOD and Fe-SOD, but the cultured cells only the Fe-enzyme.

2. MATERIALS AND METHODS

Thalli of Marchantia polymorpha were collected in the campus and used immediatly for the preparation of extract. Cultured cell line of Marchantia was donated by Dr. K. Ohyama of Kyoto University, and the cells were photomixotrophically suspension-cultured in 1-M 51 C medium (11). The cultured cells were green in color and contained chloroplasts. Anti-spinach Cu,Zn-SOD-I and Cu,Zn-SOD-II sera were prepared as described previously (Chapter V).

The cell-free extract was prepared by homogenizing the thalli or cultured cells with 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA, and with Polyclar AT (0.3 g per g of materials) using a Polytron blender for total 4 min at maximum power with 30-sec run and 1-min rest at 0°C. The homogenate was squeezed through four layers of gauze and centrifuged at 27,000 g for 20 min. The supernatant was dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA for a day with several changes of buffer. The dialyzed extract was concentrated using an Amicon YM 5 membrane, if necessary, and subjected to SOD activitiy assay or to electrophoresis.

SOD was assayed by a modified procedure of the xanthine-xanthine oxidase-cytochrome c system (Chapter II). Enzymatic activity is presented by the McCord and Fridovich unit (12). Polyacrylamide gel electrophoresis, location of SOD on a gel, and distinction of three types of SOD were performed as described previously (Chapter V).

Ouchterlony double immunodiffusion was conducted as before (Chapter II). Protein was determined by the method of Lowry et al. (13) using bovine serum albumin as a standard.

3. RESULTS AND DISCUSSION

The extract from the suspension-cultured cells of Marchantia showed only a single SOD band on polyacrylamide slab gel after electrophoresis (Fig. 1). The activity band was not affected by the treatment of the gel with cyanide, but disappeared after the treatment of the gel with

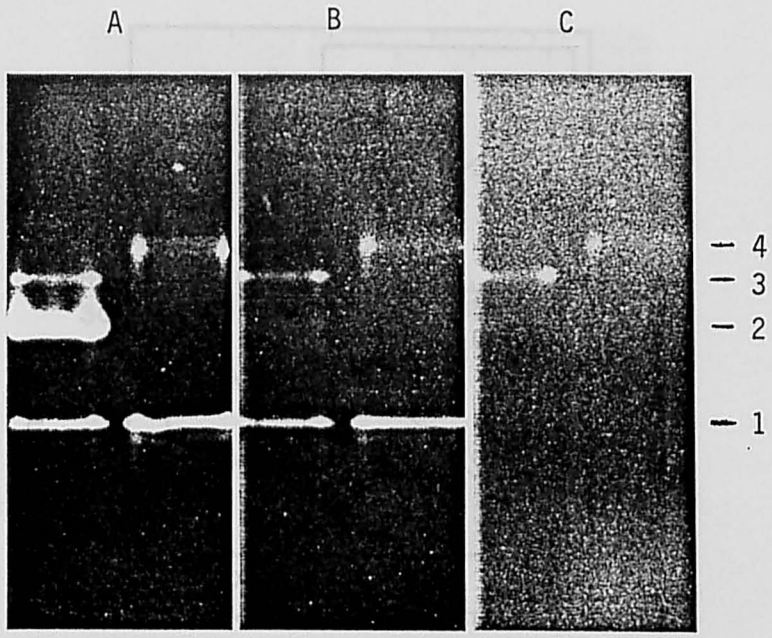

Fig.1 Native polyacrylamide gel electrophoresis of SOD from thalli and suspension-cultured cells of Marchantia polymorpha. (A) No treatment; (B), stained in the presence of 2 mM KCN; (C), stained after the treatment wiht 3 mM H_2O_2. Left of each panel, thalli (3.4 units); Right, cultured cells (1.9 units). 1, Fe-SOD; 2, Cu,Zn-SOD; 3 and 4, Mn-SOD. Details are described in Materials and Methods.

hydrogen peroxide. Thus, the SOD in the cultured cells was inactivated by hydrogen peroxide, but not by cyanide and is supposed to be Fe-containing enzyme. Little Cu,Zn-SOD was found in the cultured cells.

When the SOD activity in the extract from the cultured cells was assayed by the xanthine-xanthine oxidase-cytochrome c system, the activity was not inhibited by 1 mM cyanide. By incubation of the extract with 0.5-3.0 mM hydrogen peroxide, the SOD activity was lost with time following a first-order kinetics, with an apparent second-order rate constant of 2.6 $M^{-1}s^{-1}$ at 25°C (Fig. 2). After 20 min SOD activity was completely inactivated by 3 mM H_2O_2 which was used for distinguishing of SOD type on a gel. These results confirm the results of Fig. 1; occurrence of only Fe-SOD in the cultured cells of Marchantia. SOD in the extract was heat-labile; inactivation at 60°C was followed by first-order kinetics with a half-life time of 4.5 min^{-1}.

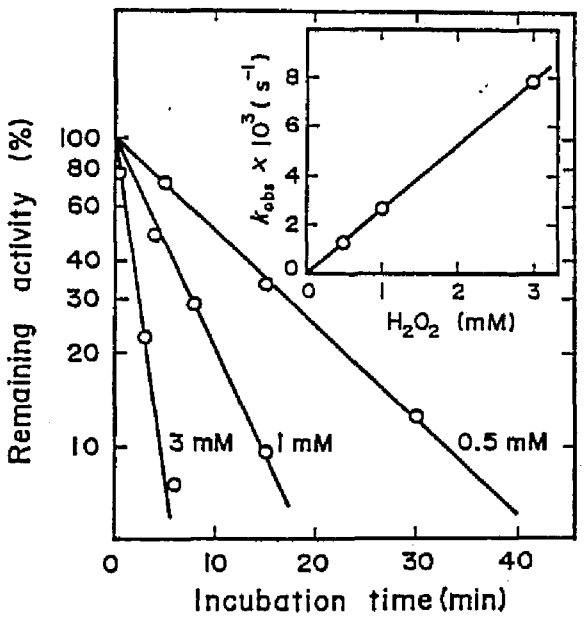

Fig.2 Inactivation of SOD from the cultured cells by H_2O_2. The extract in 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA was incubated at 25°C with H_2O_2 at various concentrations, and aliquots were withdrawn at intervals and assayed for residual enzymatic activity in the standard assay system containing 500 units catalase.

Fe-SOD so far isolated from higher plants were homodimers having a molecular weight of 40,000-45,000 (5,6,8). Using partially purified Fe-SOD from the cultured cells of Marchantia, the molecular weight was determined by gel filtration. After being chromatographed on DEAE-Sephacel and hydroxylapatite, the enzyme was subjected to Sephadex G-100 column previously equilibrated with 10 mM potassium phosphate, pH 7.8, and 0.1 M KCl and eluted with the same buffer. The enzyme activity was found at a position corresponding to a molecular weight of 45,000.

In contrast to the cultured cells, SOD in the thalli extract form Marchantia was inhibited by 76% with 2 mM cyanide in the xanthine-xanthine oxidase-cytochrome c assay system, in agreement with the previous results (10). The extract from the thalli showed two SOD bands (Fig. 1). The major, slow moving band was sensitive to both cyanide and hydrogen peroxide, thus being Cu,Zn-SOD. The minor band which mobility was the same as the SOD of the cultured cells disappeared after the

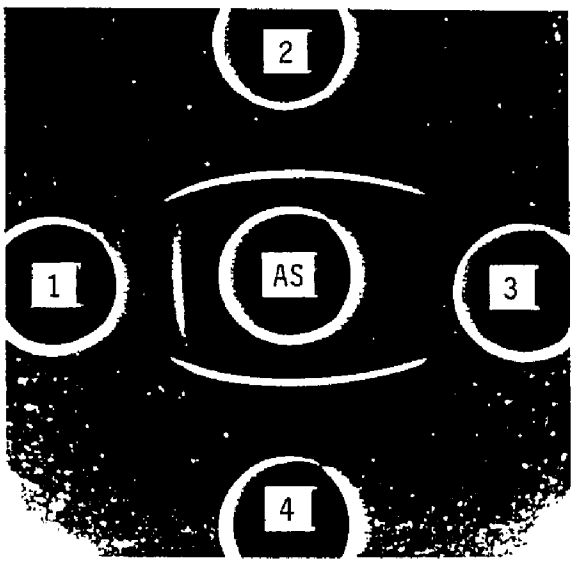

Fig.3 Ouchterlony double immunodiffusion test of the extracts from Marchantia thalli and cultured cells. 1, extract of thalli (5 units); 2 and 4, spinach stromal Cu,Zn-SOD (5 units); 3, extract of cultured cells (5 units); AS, anti-spinach stromal Cu,Zn-SOD (132 ug).

treatment with hydrogen peroxide, but not with cyanide, suggesting the Marchantia thalli contained Fe-SOD in addition to major Cu,Zn-SOD.

Fe-SOD activity in the thalli accounted for about 10% that of Cu,Zn-SOD as determined from the densitometric scanning of the gel of Fig. 1, which was similar to the cyanide-insensitive SOD activity in the extract. For detection of Fe-SOD in the thalli, the preparation of the extract from the fresh thalli in the presence of Polyclar AT and an immediate application after the preparation of extract to the gel were prerequisite, otherwise no Fe-SOD band was found.

The extract from thalli of Marchantia formed a precipitin line with anti-spinach Cu,Zn-SOD-II (stromal) serum but not with anti-spinach Cu,Zn-SOD-I (cytosolic) serum. Thus, Marchantia appears to contain stromal Cu,Zn-SOD but lacked cytosolic Cu,Zn-SOD isozyme. On the other hand, the extract from the cultured cells did not form any precipitin line with the both antisera, confirming the absence of Cu,Zn-SOD in the cultured cells (Fig. 3).

The total SOD activity in the thalli and the cultured cells of

Marchantia was almost same; 11 and 22 units/mg soluble protein, respectively. Therefore, the content of Fe-SOD in the cultured cells is similar to that of Cu,Zn-SOD plus Fe-SOD in the thalli. Fe-SOD in the cultured cells appears to replace the functions of the Cu,Zn-SOD in the thalli because the cultured cells have chloroplasts. I do not know the step where, and the reasons why the gene expression of Cu,Zn-SOD is repressed and that of Fe-SOD is enhanced in the cultured cells.

The present results show the occurrence of Fe-SOD in a moss, in addition to prokaryotes, eukaryotic algae and angiosperms. Occurrence of Fe-SOD in a moss suggest that the gene for Fe-SOD in angiosperms was descended from prokaryote through eukaryotic algae and moss, rather than the gene transfer from prokaryotes to angiosperms or mutation of the gene for Mn-SOD to that for Fe-SOD. In angiosperms the content of Fe-SOD is generally low as compared with that of Cu,Zn-SOD, and Fe-SOD has been found in limited species. However, water lily contains only Fe-SOD, and no Cu,Zn-SOD (14), as in the case of Marchantia cultured cells. Understanding of the regulation for the expression of the Fe-SOD gene may infer the distribution of Fe-SOD in angiosperms.

4. SUMMARY

Superoxide dismutase (SOD) in thalli and cultured cells of the moss Marchantia polymorpha was separated by polyacrylamide gel electrophoresis, and characterized by its responses to cyanide and hydorgen peroxide. The cultured cells showed two SOD bands on the gel one of which was inactivated by hydrogen peroxide but not by cyanide, and the other was insensitive to both reagents. The former molecular weight was 45,000. The thalli showed three bands. One of them occupied about 70% of total activity and was sensitive to both cyanide and hydrogen peroxide. Another SOD band showed the same mobility on the gel and responses to cyanide and hydrogen peroxide as those of the cultured cells. These results indicate that the thalli contained Fe-SOD in addition to Cu,Zn-SOD and Mn-SOD, and the cultured cells Fe-SOD and Mn-SOD.

5. REFERENCES

(1) Fridovich, I. (1975) Annu. Rev. Biochem. 44, 147-159.

(2) Steinman, H.M. (1982) in Superoxide Dismutase (Oberley, L.W., ed.), pp. 11-68, CRC Press, Florida.

(3) Weisiger, R.A., and Fridovich, I. (1973) J. Biol. Chem. 248, 3582-3592.

(4) Kanematsu, S., and Asada, K. (1979) Arch. Biochem. Biophys. 195, 535-545.

(5) Salin, M.L., and Bridges, S.M. (1980) Arch. Biochem. Biophys. 201, 369-374.

(6) Salin, M.L., and Bridges, S.M. (1982) Plant Physiol. 69, 161-165.

(7) Salin, M.L., and Bridges, S.M. (1980) Z. Pflanzenphysiol. 99, 37-45.

(8) Kwiatowski, J., Safianowska, A., and Kaniuga, Z. (1985) Eur. J. Biochem. 146, 459-466.

(9) Sevilla, F., Del Rio, L.A., and Hellin, E. (1984) J. Plant Physiol. 116, 381-387.

(10) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(11) Ono, K., Ohyama, K., and Gamborg, O.L. (1979) Plant Sci. Lett. 14, 225-229.

(12) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(13) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

(14) Bridges, S.M., and Salin, M.L. (1981) Plant Physiol. 68, 275-278.

CHAPTER XIII

PURIFICATION AND CHARACTERIZATION OF AN Fe-SUPEROXIDE DISMUTASE FROM SPINACH SEEDS

1. INTRODUCTION

Fe-superoxide dismutase (SOD) was belived to restrict its occurrence in prokaryotes, however,it has been isolated from eukaryotes; Euglena gracilis (1, Chapter XI), and three angiosperms,i.e. mustard (2), water lily (3), and tomato (4) leaves. Furthermore, the occurrence of Fe-SOD in a moss, Marchantia polymorpha has been demonstrated (Chapter XII), although the distribution of Fe-SOD in vascular plants is limited to several families (5). In the course of the purification of Cu,Zn-SOD isozymes from spinach seeds (Chapter V), I noted the occurrence of cyanide-insensitive and H_2O_2-sensitive Fe-SOD. In this chapter, I describe the purification and some properties of Fe-SOD from spinach seeds and its subcellular distribution in cells.

2. MATERIALS AND METHODS

Cytochrome c (horse heart, type III) and xanthine oxidase (milk) were obtained from Sigma and Boehringer, respectively. Chelating Sepharose, Percoll, and marker proteins for isoelectric point and molecular weight were obtained from Pharmacia, and Ampholine from LKB. Bio-Gel HTP was a product of Bio-Rad. Mercaptoethanesulfonic acid was obtained from Pierce. Spinach seeds (Spinacia oleracea L. cv. King of Denmark) were obtained from Takii Seeds Co. Ltd, Kyoto, Japan. Spinach was water-cultured using a medium of Walker (6) in a green house for a month.

Intact spinach chloroplasts were prepared by discontinuous Percoll

density gradient centrifugation after differential centrifugatoin as described in Chapter V. Stromal fraction was obtained by rupturing the intact chloroplasts in 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA for 1 hour, and by centrifugation at 27,000 g for 30 min. The stroma was dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA and concentrated through an Amicon YM 5 membrane.

SOD was assayed using the xanthine-xanthine oxidase-cytochrome c system as Chapter II. The enzymatic activity is shown in the McCord and Fridovich unit (7). Since my reaction volume was 1 ml as compared with the original (3 ml), the activity given in my system was divided by 3. Protein concentration was estimated according to Lowry et al. (8) using bovine serum albumin as a standard.

Native and SDS-polyacrylamide gel electrophoresis, location of the SOD activity on the native gel, and characterization of three types of SOD (Cu,Zn-, Fe- and Mn-SOD) by cyanide and hydrogen peroxide were done as described in Chapter V. Isoelectric focusing was conducted as before (1). Molecular weight determination, amino acid analysis, metal analysis were carried out as in Chapter V.

2. RESULTS

Purification of Fe-SOD

Cyanide-insensitive SOD and Cu,Zn-SOD were simultaneously purified from spinach seeds. The purification of Cu,Zn-SOD isozymes were reported elsewhere (Chapter V). The early steps of purification upto first DEAE-Sephacel chromatography was the same as that for Cu,Zn-SOD.

The 200 mM KCl fraction obtained from the DEAE-Sephacel step, in which two thirds of the SOD activity was cyanide-insensitive (see Chapter V), was concentrated using an Amicon PM 10 membrane and dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. The clarified solution was applied onto a DEAE-Sephacel column (2.5 x 42 cm) equilibrated with the above buffer, and then the enzyme was eluted by a linear gradient of KCl (0-200 mM, 3 liters) in 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. Most of the Cu,Zn-SOD was eluted

just ahead of cyanide-insensitive SOD. The fractions containing cyanide-insensitive SOD were pooled, concentrated by ultrafiltration and dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA.

The dialyzed enzyme was then applied onto a Bio-Gel HTP column (5 x 23 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. The column was washed with 3 liters of the above buffer, and a small amount of Cu,Zn-SOD appeared in the washing fraction. Cyanide-insensitive SOD was eluted by a linear gradient of potassium phosphate, pH 7.8 (10-100 mM, 3 liters). The enzyme was eluted at 40 mM, and the active fractions were pooled and concentrated by ultrafiltration.

Ammonium sulfate was added to the enzyme to 35% saturation, and the enzyme was adsorbed onto a Phenyl-Sepharose column (4 x 14 cm) equilibrated with 35% ammonium sulfate in 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA. After washing the column with 2.2 liters of the equilibrating solution, the column was eluted by a simultaneous cross linear gradient (3 liters) of ammonium sulfate (35-0%) and ethylene glycol (0-20%). The SOD was found at 10% ammonium sulfate and 14% of ethylene glycol (Fig. 1). The active fractions were concentrated by ultrafiltration.

The concentrated enzyme was applied to a Sephadex G-100 column (4 x 86 cm) equilibrated 10 mM potassium phosphate, pH 7.8, and 0.1 M KCl, and eluted by the same buffer. The active fractions were pooled and concentrated by ultrafiltration.

The enzyme to which KCl and potassium phosphate, pH 7.8, were added to make 0.5 M and 20 mM, respectively, was applied onto a column (1.5 x 9 cm) of metal-chelating Sepharose 4B. The upper one third of the column was previously loaded with $CuSO_4$, and equilibrated with 20 mM potassium phosphate, pH 7.8, containing 0.5 M KCl. After washing with the equilibrating buffer, the adsorbed enzyme was eluted with a linear gradient of ammonium chloride (0-200 mM, 400 ml) in 20 mM potassium phosphate containing 0.5 M KCl. A single active peak which corresponded to the absorbance at 280 nm appeared at 50 mM ammonium chloride. The active fractions were pooled, concentrated using a PM 10 membrane, and

Table I

Summary of the Purification of Fe-Superoxide Dismutase from Spinach Seeds (10 kg)

Purification step	Total protein[a] (mg)	Total activity[b] (10^{-3} units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Homogenate	105,000	613	6	100	1
30 to 80% $(NH_4)_2SO_4$	38,700	570	15	93	2.5
1st DEAE-Sephacel					
(50 mM KCl eluate)[c]	(3,400)	(457)	(134)	(75)	(22)
200 mM KCl eluate	10,300	54	5	8.9	0.8
2nd DEAE-Sephacel	6,290	32	5	5.2	0.8
1st Bio-Gel HTP	856.9	13	15	2.1	2.5
Phenyl-Sepharose	33.2	12	361	2.0	60
Sephadex G-100	22.4	12	536	2.0	89
Cu-Chelating-Sepharose	5.3	5.6	1057	0.9	176
2nd Bio-Gel HTP	2.2	4.9	2227	0.8	371

a) Protein was measured by the method of Lowry et al. (8).

b) Represented by McCord and Fridovich unit (7).

c) This fraction was used for the purification of Cu,Zn-SOD (see Chapter V).

dialyzed against first 10 mM potassium phosphate, pH 7.8, containing 1 mM EDTA and then the buffer only.

Finally, the enzyme was loaded on a column of Bio-Gel HTP (1.5 x 3.5 cm) which was previously equilibrated with 10 mM potassium phosphate, pH 7.8. After washing the column with the equilibrating buffer, the enzyme was eluted by a linear gradient of potassium phosphate, pH 7.8 (10-100 mM, 200 ml). The enzyme appeared at 35 mM potassium phosphate. The active fractions were pooled, concentrated, and dialyzed against 10 mM potassium phosphate, pH 7.8. Table I summarizes the purification of Fe-SOD from 10 kg of spinach seeds.

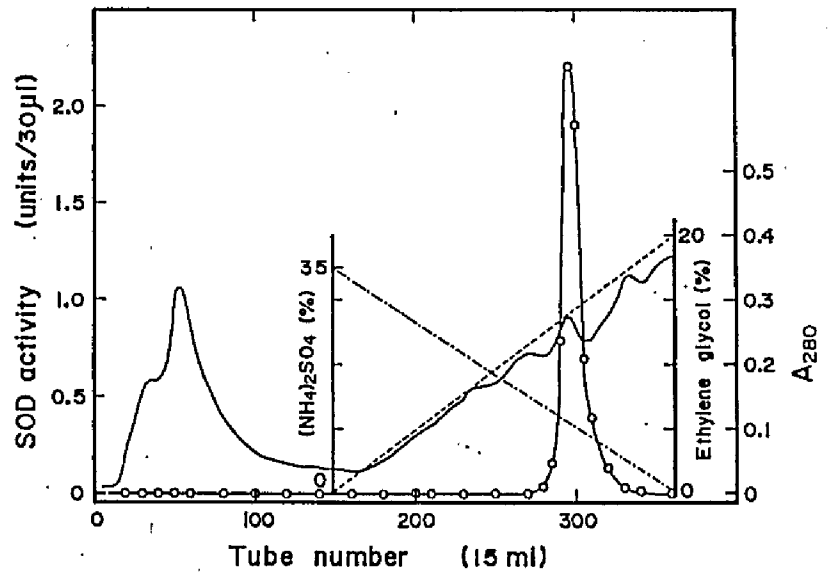

Fig. 1. Hydrophobic chromatography of spinach Fe-SOD on Phenyl-Sepharose. Details are given in the text.

Purity of the enzyme

The purified enzyme showed a single protein band corresponding to the enzymatic activity on polyacrylamide disc gel after electrophoresis at pH 8.9 (Fig. 2). The purified enzyme was localized on the gel corresponding to the cyanide-insensitive and H_2O_2-sensitive SOD band of the buffer extract, indicating little modification of the enzyme during purification (data not shown). Isoelectric focusing (Fig. 3) and SDS-polyacrylamide gel electrophoresis (data not shown) of the enzyme gave also a single protein band. Ultracentrifugation analysis for sedimentation equilibrium of the enzyme indicated a single component with respect to the sedimentation properties (Fig. 4). Thus, the purified enzyme is homogeneous.

Molecular weight

The molecular weight of the enzyme was estimated by gel filtration with a Sephadex G-100 column (2 x 85 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, and 0.1 M KCl. The column was calibrated with the following proteins: bovine serum albumin (67,000), ovalbumin

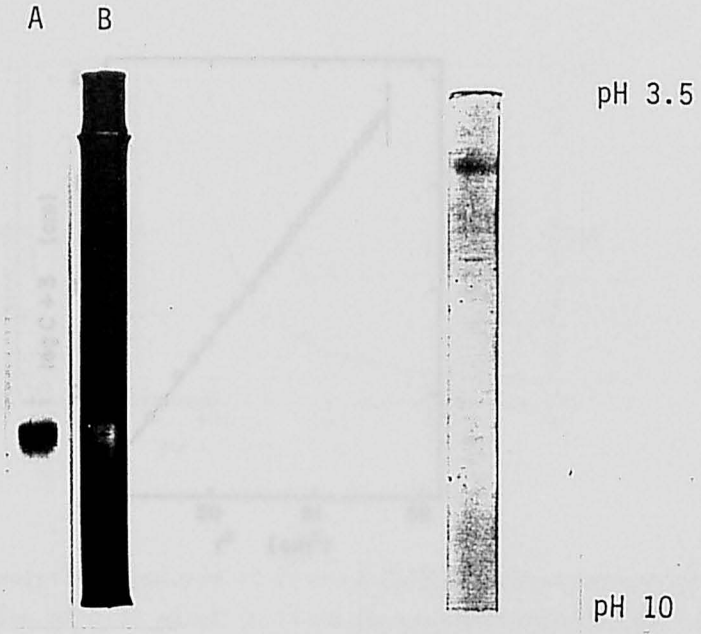

Fig. 2 (left). Polyacrylamide gel electrophoresis of spinach Fe-SOD. 1, Protein staining (20 μg); 2, activity staining (3 units). Protein was stained with Commassie Brilliant Blue R-250. Cathode, top; anode, bottom.

Fig. 3 (right). Isoelectric focusing of spinach Fe-SOD. The enzyme (20 μg) was focused with Ampholine (pH 3.5-10) on 5% polyacrylamide gel disc. pI of the enzyme was determined by the paralled run of gel with pI marker proteins. Protein was stained with Commassie Brilliant blue G-250 after fixing with 10% trichloracetic acid-5% sulfosalicylic acid.

(45,000), α-chymotrypsinogen-A (25,000), myoglobin (17,800), and cytochrome c (12,400). The enzyme was eluted at a position corresponding to a molecular weight of 52,000.

The molecular weight of the enzyme was also estimated by analytical ultracentrifugation. Sedimentation quilibrium analysis gave a straight line in a plot of log(fringe displacement) vs. square of the distance from the center of rotation (Fig. 4). From its slope and a partial specific volume of 0.733 obtained from amino acid composition (Table II) according to Cohn and Edsall (9), a molecular weight of 51,400 was calculated in accordance with the value obtained by gel filtration.

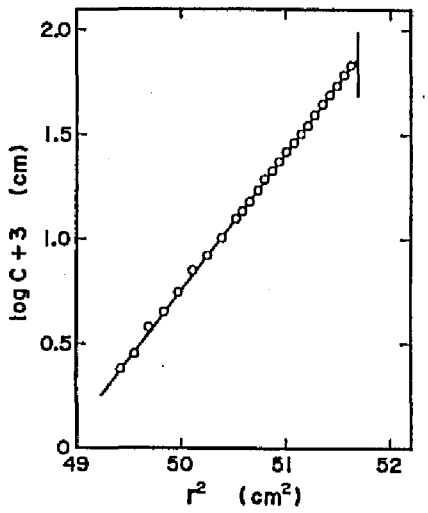

Fig. 4. Sedimentation equilibrium of spinach Fe-SOD. Purified enzyme at concentration of 0.30 mg/ml in 10 mM potassium phosphate, pH 7.8, containing 0.1 M potassium chloride was equilibrated at a rotor speed of 22,411 rpm at 20°C. C, Fringe displacement; r, distance from the center of rotation.

Subunit structure

The purified enzyme was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis after the denaturation with 2% SDS in the presence and absence of 5% 2-mercaptoethanol at 100°C for 3 min. The enzyme gave a single band both in the presence and absence of 2-mercaptoethanol and the subunit molecular weight of 26,600 was estimated by comparison of its mobility to those of the following molecular weight standards: phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400). Thus, the enzyme is composed of two, non-covalently joined subunits of equal size.

Metal analysis

The metal content of the enzyme was determined by atomic absorption spectroscopy. The enzyme was dialyzed against 10 mM potassium phosphate, pH 7.8, and 10 μl of the sample was directly analyzed in a

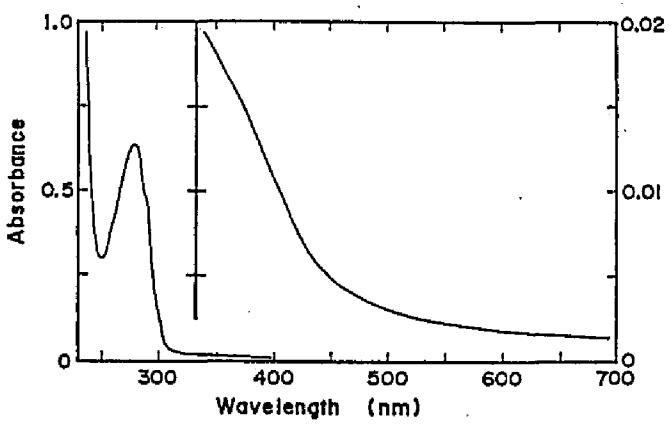

Fig. 5. Absorption spectrum of Spinach Fe-SOD. Spectrum was recorded at a concentration of 0.30 mg/ml in 10 mM potassium phosphate, pH 7.8, at 25°C. The light path was 10 mm.

graphite atomizer. Same volume of dialysis buffer was used for a small blank correction. The enzyme contained 1.3 g-atom of Fe per mol of enzyme, based on a molecular weight of 51,400. The contents of manganese, copper, and zinc were insignificant.

Absorption spectrum

The enzyme exhibited an absorption maximum at 278 nm with shoulders at 260 and 290 nm in the ultraviolet region (Fig. 5). The molar absorbance coefficient and $A_{1\ cm}^{1\%}$ at 280 nm were 108,800 $M^{-1}cm^{-1}$ and 21.17, respectively. In the visible region, the enzyme showed a broad absorption from 320 to 600 nm with a shoulder at around 350 nm. The molar absorbance coefficient at 350 nm was 3,080 $M^{-1}cm^{-1}$. The spectrophotometric properties of spinach Fe-SOD were similar to those of Fe-SOD from Euglena (1) and angiosperms (2-4). The lower absorbance coefficient at 350 nm of spinach Fe-SOD might be ascribed to the lower content of Fe as compared with that of Euglena Fe-SOD which contained nearly 2 g-atoms of Fe per mol of enzyme.

Amino acid analysis

The enzyme was dialyzed against distilled water and hydrolyzed in 6 N HCl at 110°C for 24, 48, and 72 hours in evacuated, sealed tubes in

duplicate. Half-cystine and methionine were determined as cysteic acid and methionine sulfone after performic acid oxidation followed by hydrolysis in 6 N HCl for 18 hours (10). Tryptophan was determined

Table II

Amino Acid Compositions of Spinach Fe-SOD and Mn-SOD, and Fe-SOD and Mn-SOD form Several Organisms (residues per mol subunit)[a)]

	Spinach Fe-SOD[b)]							
Amino acid	Residues/ subunit	Nearest integer	S.o Mn	B.c Fe	N.l Fe	L.e Fe	E.g Fe	S.c Mn
Aspartic acid	27.99	28	25	18	23	19	19	28
Threonine[c)]	9.46	9	11	9	6	9	11	11
Serine[c)]	9.63	10	24	11	15	8	5	8
Glutamic acid	22.40	22	31	16	19	14	22	28
Proline	13.37	13	12	10	9	12	13	10
Glycine	14.14	14	28	16	22	10	20	19
Alanine	23.98	24	18	17	20	16	26	21
half-cystine[d)]	0.73	1	4	2	2	0	2	1
Valine[e)]	14.95	15	13	11	5	10	11	14
Methionine[d)]	4.70	5	2	1	5	4	3	1
Isoleucine[e)]	6.25	6	12	6	4	8	6	12
Leucine	17.42	17	21	14	16	18	16	20
Tyrosine	9.71	10	4	6	6	6	7	9
Phenylalanine	13.23	13	7	7	9	9	10	11
Lysine	15.97	16	18	11	12	10	4	19
Histidine	5.81	6	6	4	5	4	7	7
Arginine	5.78	6	5	5	6	4	10	4
Tryptophan[f)]	9.95	10	3	4	7	7	8	6
Total residues(subunit)		225	244	168	191	168	200	229

a) S.o, Spinacia oleracea (12); B.c, Brassica campestris (2); N.l, Nuphar luteum (3); L.e, Lycopersicon esulentum (4); E.g, Euglena gracilis (1); S.c, Saccharomyces cerevisiae (13); Fe, Fe-SOD; Mn, Mn-SOD

b) The number of residues were calculated for 25,700 g of enzyme.

c) Calculated by linear extrapolation to zero time.

d) Determined as methionine sulfone and cysteic acid after performic acid oxidation (10).

e) Values at 72 hours.

f) The enzyme was hydrolyzed in 3 N mercaptoethane sulfonic acid for 22 hours according to Penke et al. (11).

after hydrolysis in 3 N mercaptoethanesulfonic acid under vacuum for 22 hours (11). The loss or increase in amino acids during hydrolysis was corrected by extrapolation to zero time or taking the values at 72 hours.

The amino acid composition of spinach Fe-SOD is compared with those of Fe-SOD from Brassica campestris (2), Nuphar luteum (3), Lycopersicon esulentum (4), and Euglena gracilis (1), and of Mn-SOD from spinach (12) and yeast (13) (Table II). The amino acid composition of spinach Fe-SOD resemble those of Fe-SOD and Mn-SOD, especially that of yeast Mn-SOD. The composition of spinach Fe-SOD is also similar to that of spinach Mn-SOD, but a large dissimilarity is noted in serine, glutamic acid and glycine contents.

Isoelectric point

Isoelectric point of the enzyme was determined by isoelectric focusing with Ampholine (pH 3.5-10) on polyacrylamide disc gel using pI marker proteins. The enyzme as focused as a single band at a position corresponding to pI of 4.6 (Fig. 3). Similar pI has been reported for angiosperm Fe-SOD (2-4).

Isozymes and subcellular localization

Fig. 6 shows SOD zymogram of spinach leaf and seed. As previously described (Chapter V), spinach contained two major and one minor Cu,Zn-SOD. Cu,Zn-SOD-I is a cytosolic enzyme, while Cu,Zn-SOD-II is a stromal enzyme. In addition to these cyanide-sensitive SOD, cyanide-insensitive SOD were found (Fig. 6). The uppermost band was insensitive to cyanide and H_2O_2, thus being Mn-SOD. Lower cyanide-insensitive band which location was coincided with Cu,Zn-SOD-I was sensitive to H_2O_2 (Fig. 1). I tried to separate the cyanide-insensitive band from Cu,Zn-SOD-I band by changing gel concentration (7.5 to 15%) and buffer system (pH 9.5 to 8.0), but, the separation was insufficient. The activity band observed in the presence of both cyanide and H_2O_2 was due to the insensitive SOD and not to remaining activity of Cu,Zn-SOD-I, because chloroplast stromal fraction which lacked Cu,Zn-SOD-I showed the same activity band in the presence and absence of cyanide (Fig. 6, lane 3). Insensitivity to cyanide and sensitivity to H_2O_2 of this band suggest that it was Fe-

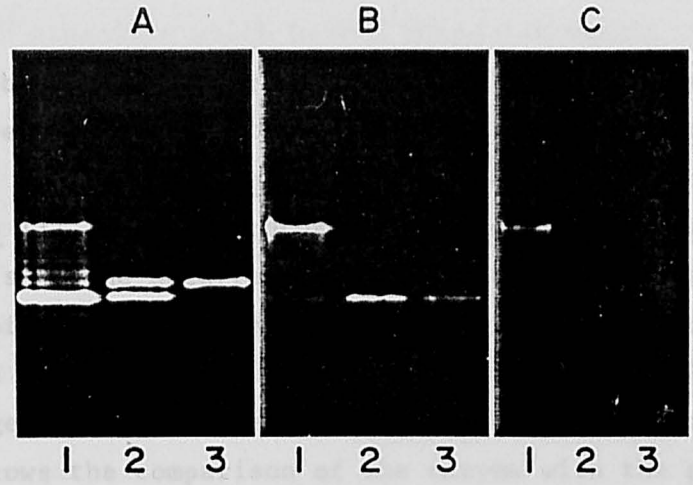

Fig. 6. Zymogram of spinach superoxide dismutases. Spinach seeds and leaves were homogenized using a Polytron blender with 50 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA. The buffer-extracts were fractionated by ammonium sulfate (30-80% saturation) and dialyzed against 10 mM potassium phosphate, pH 7.8, containing 0.1 mM EDTA to improve the sharpness of bands. Chloroplast stroma was prepared as described in Materials and Methods. 1, seeds (2 units); 2, leaves (1 unit in A, 2 units in B and C); 3, chloroplast stroma (1 unit in A, 2 units in B and C). (A), No treatment; (B), stained in the presence of 2 mM KCN; (C) stained after the treatment with 3 mM H_2O_2.

SOD. The purified Fe-SOD coincided with this band on electrophoresis (data not shown). The Mn-SOD purified from spinach leaves (12) had the same electrophoretic mobility as with the uppermost band of the seeds in Fig. 6.

The localization of Fe-SOD in leaf cells was investigated by activity staining after electrophoresis. The chloroplast stroma contained Fe-SOD in addition to Cu,Zn-SOD-II (Fig. 6). Thus, Fe-SOD is a stromal enzyme.

4. DISCUSSION

Fe-SOD has been found in prokaryotes, but its occurrence in eukaryotes has been limited to the angiosperms Brassica campestris (2), Nuphar lutem (3) and Lycopersicon esculentum (4), the eukaryotic alga Euglena gracilis (1), and the protozoan Crithidia fasciculata (14). Present results show the occurrence of Fe-SOD in the angiosperm Spinacia oleracia. One of the reasons for the delayed finding of Fe-SOD in spinach might be due to its comigration with cytosolic Cu,Zn-SOD-II (Chapter V) in gel electrophoresis (Fig. 6). The isolation of Fe-SOD from spinach allows the comparison of the enzyme with the previously purified Mn-SOD (12) from the same plant as well as Fe- and Mn-SOD from other organisms.

Native-polyacrylamide gel electrophoresis revealed that the previously reported "thylakoid-bound" Mn-SOD from spinach leaves (12) had the same mobility (data not shown) as that of soluble Mn-SOD in seeds (uppermost band in Fig. 6). This enzyme was found in larger amount in seeds and roots than leaves. The thylakoid fraction obtained from intact chloroplasts by Percoll density gradient centrifugation contained less than 1% of the cyanide-insensitive SOD activity derived from the leaf extract. In Plectonema boryanum "thylakoid-bound" Mn-SOD has been also shown (15). Although a small amount of Fe- and Mn-SOD was found in the thylakoid fraction of this algae, the electrophoretic mobilities of Fe- and Mn-SOD in the cytosol fraction were the same as those of Fe- and Mn-SOD in thylakoids, suggesting a binding of the enzymes to the membranes (data not shown). Thus, the cyanide- and hydrogen peroxide-insensitive SOD activity in the thylakoids is seemed to be due to adhered soluble Mn-SOD in addition to possible endogeneous activity. Although the subcellular localization of the spinach Mn-SOD is not known yet, the enzyme might associate with mitochondria (16,17) or peroxisomes (glyoxysomes) (18). Occurrence of both Fe-SOD and Mn-SOD and their different subcellular localization in mustard leaves have been reported (19,20).

The purified spinach Fe-SOD have a molecular weight of 51,400 and is consisted of two non-covalently linked subunits of equal size. The

molecular weight of spinach Fe-SOD is higher compared with dimeric Fe-SOD from other organisms which have a molecular weight between 40,000 and 46,000. Spinach Mn-SOD also has a molecular weight of 52,000 (12). Except the molecular weight, spinach Fe-SOD resembled Fe-SOD so far isolated from various sources in physicochemical properties. Specific activity of spinach Fe-SOD is comparable to that of water lily Fe-SOD (3). The optical spectrum of spinach Fe-SOD resembles that of tomato Fe-SOD (4), exhibiting visible broad absorption band with a shoulder at about 350 nm. This absorption is characteristic of the ligand-to-iron charge transfer band. Spinach Fe-SOD is a acidic protein having a pI of 4.6, which is similar to those of Euglena (pI=4.5), mustard (4.5), water lily (4.8), and tomato (4.6). Hydrogen peroxide inactivates Fe-SOD but not Mn-SOD (21). As expected spinach Fe-SOD was inactivated completely within 30 min by incubation with 3 mM H_2O_2. Recently it has been reported that a part of active site of Mn-SOD and Fe-SOD was occupied by Fe, Mn, Cu, and Zn (22,23). The present results clearly indicate that this is not the case for spinach Fe-SOD.

The feature of the amino acid composition of spinach Fe-SOD is higher contents of aspartic acid, glutamic acid and lysine than the other angiosperm Fe-SOD as presented in Table II. Since the molecular weight of spinach Fe-SOD is about 5,000 higher than those of the other angiosperm Fe-SOD, the high content of charged amino acids may be reflected by the difference of molecular weight, indicating the occurrence of additional sequence in spinach Fe-SOD. Similar feature was also found in the composition of spinach Mn-SOD which has the same molecular weight as spinach Fe-SOD. Thus, if the large difference of charged amino acid is neglected, spinach Fe-SOD appears more closely related to Fe-SOD from the angiosperms Brassica, Nuphar and Lycoperison than those of bacterial Fe-SOD. When total compositions are compared, spinach Fe-SOD resembles well yeast Mn-SOD which has the similar subunit molecular weight as that of spinach Fe-SOD and higher charged amino acid contents. The resemblance of angiosperm Fe-SOD to eukaryotic Mn-SOD has been reported (2-4).

Amino acid compositions of spinach Fe-SOD and Mn-SOD are similar, especially with respect to the content of charged amino acid contents

(Table II). However, the two enzymes are distinguished in serine, glutamic acid and glycine contents. Thus, the two SOD are products of different genes, which excludes a possibility of the acceptance of Mn or Fe to identical apoprotein.

It should be noted that most eukaryotic Mn-SOD are tetramer, while the eukaryotic Fe-SOD are dimer. The difference in primary structure involving subunit interaction between the dimer and teramer SOD has been suggested (24). Tetrameric Thermus thermophilus Mn-SOD is constructed with only two interfaces. One set of the contacts of the Mn-SOD closely resembles the dimer interface of Fe-SOD, but the other interface utilizes an inserted polypeptide segment that are not equivalent in Fe-SOD (24). Therefore, with respect of higher molecular weight than those of usual dimeric enzymes, spinach dimeric Fe-SOD and Mn-SOD seem to resemble tetrameric Mn-SOD, although both enzymes do not form a tetramer. Mn-SOD from Pisum sativum is a tetramer having a molecular weight of 94,000 (25,26).

Spinach Fe-SOD was shown to be a stromal enzyme, but Mn-SOD was not detected in stromal fraction. Similar result has been reported for Brassica where Fe-SOD is localized in stroma but not in mitochondrial matrix, while Mn-SOD was found in mitochondria but not in stroma (19,20) Mn-SOD of mammals and fungi are mitochondrial matrix enzymes (16,27). To the contrary, Pisum Mn-SOD is localized in peroxisomes but not in mitochondria (18). Non-green tissues of spinach such as seeds, roots and hypocotyls of etiolated seedlings also contained Fe-SOD which seems to associate with proplastids of non-green tissues. Spinach stromal Cu,Zn-SOD (SOD-II) is also indicated to be localized in proplastids of non-green tissues.

The occurrence of Mn-SOD in prokaryotes and in mitochondria of eukaryotes has been thought to support the endosymbiotic theory for the origin of mitochondria. Fe-SOD has been shown to be localized in cytosol of cyanobacteria (15), and also chloroplasts of green algae (Chapter XI) and angiosperms (19). Thus, the origin of chloroplast is inferred also by the endosymbiotic hypothesis.

Spinach chloroplasts contain Cu,Zn-SOD isozyme (SOD-II) as well as Fe-SOD. Cu,Zn-SOD-II exhibited developmental change of expression, and

related to the biogenesis of chloroplast (Chapter V), while Fe-SOD level seems to be constant upon greening processes of etiolated seedlings. That is, Cu,Zn-SOD-II was induced by light, but Fe-SOD was constitutive. This observation was agreeable with the presence of relatively much amount of Fe-SOD in proplastid of non-green tissues. It is interesting to note that Cu,Zn-SOD level in tomato was affected by environmental conditions, while Fe-SOD was not (28,29). The content of Fe-SOD was 20% as compared with stromal Cu,Zn-SOD-II. It is not clear Fe-SOD in stroma is prerequisite for the chloroplast funciton. Nevertheless, occurrence of distinct SOD in stroma will be advantageous since regulation of both enzymes are different. Furthermore, the absence of Cu,Zn-SOD and the presence of Fe-SOD in water lily (3), and in the suspension-cultured cells of the moss Marchantia polymorpha (Chapter XII) indicates the importance of Fe-SOD in these Cu,Zn-SOD-lacked plants.

The limited distribution of Fe-SOD in vascular plants has been explained either by 1) gene transfer from prokaryotes, 2) Fe-SOD and Mn-SOD were descended from primitive plants, but in most higher plants Fe-SOD gene is silent, or 3) reverse evolution of Fe-SOD from Mn-SOD. The present results suggest that both Fe-SOD and Mn-SOD were derived from primitive plants during evolution, but most plants lack Fe-SOD in chloroplasts where Cu,Zn-SOD occurs. However, since spinach Fe-SOD and Mn-SOD have the same molecular weight, a possibility cannot be excluded that only one gene for most probably Mn-SOD and least probably Fe-SOD was descended from more primitive plants and from this gene a protein which could accomodate Fe was derived by mutation. In this connection, the elucidation of amino acid and DNA sequences of Fe-SOD and Mn-SOD of plants including algae are needed.

5. SUMMARY

Cyanide-insensitive superoxide dismutase (SOD) was purified to a homogeneous state from spinach seeds by DEAE-Sephacel, hydroxylapatite, Phenyl-Sepharose, Sephadex G-100, and Cu-chelating Sepharose chromatographies. The enzyme was a homodimer having a molecular weight of

51,400, and contained 1.3 g-atom of Fe per mol. Its pI was 4.6. The effects of cyanide and hydrogen peroxide on the enzymatic activity were those characteristic of Fe-SOD. Amino acid composition of spinach Fe-SOD more resembled those of the Fe-SOD from angiosperms and Mn-SOD from mammalians, yeast, and angiosperms than those of the Fe-SOD from bacteria, but bore less resemblance to that of spinach Mn-SOD. In the leaves, Fe-SOD was localized in chloroplast stroma.

6. REFERENCES

(1) Kanematsu, S., and Asada, K. (1979) Arch. Biochem. Biophys. 195, 535-545.

(2) Salin, M.L., and Bridges, S.M. (1980) Arch. Biochem. Biophys. 201, 369-374.

(3) Salin, M.L., and Bridges, S.M. (1980) Plant Physiol. 69, 161-165.

(4) Kwiatowshi, J., Safianowska, A., and Kaniuga, Z. (1985) Eur. J. Biochem. 146, 459-466.

(5) Bridges, S.M., and Salin, M.L. (1981) Plant Physiol. 68, 275-278.

(6) Walker, D.A. (1980) Methods Enzymol. 69, 94-104.

(7) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(8) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

(9) Cohn, E.J., and Edsall, J.T. (1941) Proteins, Amino Acids and Peptides, p. 375, Reinhold, New York.

(10) Moore, S. (1963) J. Biol. Chem. 238, 235-237.

(11) Penke, B., Ferenczi, R., and Kovacs, K. (1974) Anal. Biochem. 60, 45-50.

(12) Hayakawa, T., Kanematsu, S., and Asada, K. (1985) Planta, 166, 111-116.

(13) Ravindranath, S.D., and Fridovich, I. (1975) J. Biol. Chem. 250, 6107-6112.

(14) Trant, N.L., Meshnick, S.R., Kitchener, K, Eaton, J.W., and Cerami, A. (1983) 258, 125-130.

(15) Okada, S., Kanematsu, S., Asada, K. (1979) FEBS Lett. 103, 106-110.
(16) Weisiger, R.A., and Fridovich, I. (1973) J. Biol. Chem. 248, 3582-3592.
(17) Jackson, C., Dench, J., Moore, A.L., Halliwell, B., Foyer, C.H., and Hall, D.O. (1978) Eur. J. Biochem. 91, 339-344.
(18) Del Rio, L.A., Lyon, D.S., Olah, I., Glick, B., Salin, M.L. (1983) Planta 158, 216-224.
(19) Salin, M.L., and Bridges, S.M. (1980) Z. Pflanzenphysiol. 99, 37-45.
(20) Salin, M.L., and Bridges, S.M. (1981) Biochem. J. 195, 229-233.
(21) Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y., and Enmanji, K. (1975) J. Biol. Chem. 250, 2801-2807.
(22) Beaman, B.L., Scates, S.M., Moring, S.E., Deem, R., and Misra, H.P. (1983) J. Biol. Chem. 258, 91-96.
(23) Meier, B., Barra, D., Bossa, F., Calabrese, L., and Rotilio, G. (1982) J. Biol. Chem. 257, 13977-13980.
(24) Stallings, W.C., Pattridge, K.A., Strong, R.K., and Ludwig, M.L. (1984) J. Biol. Chem. 259, 10695-10699.
(25) Sevilla, F., Lopez-Gorge, J., Gomez, M., Del Rio, L.A. (1980) Planta 150, 153-157.
(26) Sevilla, F., Lopez-Gorge, J., Del Rio, L.A. (1982) Plant Physiol. 70, 1321-1326.
(26) Steinman, H.M. (1982) in Superoxide Dismutase (Oberley, L.W., ed.), pp. 11-68, CRC Press, Florida.
(27) Michalski, W.P., and Kaniuga, Z. (1982) Biochim. Biophys. Acta 680, 250-257.
(28) Kaniuga, Z., Zabek, J., and Michalski, W.P. (1979) Planta 145, 145-150.

CHAPTER XIV

DIMERIC Mn-SUPEROXIDE DISMUTASE FROM THE RED ALGA PORPHYRA YEZOENSIS: IMPLICATION FOR STROMAL ENZYME

1. INTRODUCTION

According to the distribution studies of three types of SOD, eukaryotic algae can be divided into two groups in respect of Cu,Zn-SOD; most eukaryotic algae including red, brown, and green algae, diatoms, and Euglena lack the cyanide-sensitive Cu,Zn-SOD, and possess the cyanide-insensitive Fe- and/or Mn-SOD (Chapter II), but phragmoplast algae such as Nitella, Chara, and Spirogyra (1) contain the cyanide-sensitive SOD in addition to the cyanide-insensitive SOD (2). I have purified the cyanide-sensitive SOD from Spirogyra and have shown that the Spirogyra SOD is a typical Cu,Zn-SOD found in animals, fungi, and land plants in terms of molecular properties (Chapter III). From the former group of algae, Fe-SOD (a green algal protozoan, Euglena gracilis) (Chapter XI), and Mn-SOD (a single cell red alga, Porphyridium cruentum) (3) has been isolated and characterized, but little is known for SOD in eukaryotic algae, as compared to Cu,Zn-SOD in higher organisms. In addition to this, subcellular localization of SOD in eukaryotic alage is not clarified yet except that Euglena Fe-SOD is localized in chloroplast stroma (4).

In order to elucidate the type of SOD and its subcellular localization in eukayrotic algae, I isolated SOD from the red alga, multicellular Porphyra yezoensis. Porphyra contained only two Mn-SOD and lacked Fe-SOD, suggesting that the Porphyra stroma contains Mn-SOD.

2. MATERIALS AND METHODS

Sources of reagents and proteins used here were described in the

previous and following chapters. Cultured, fresh thalli of Porphyra yezoensis were obtained near Akashi, Hyogo. The contaminated algae were not found by visible observation. The thalli were stored at -20°C until use.

SOD assay and the definition of the activity were same as the previous chapter except that 0.1 mM diethylenetriaminepentaacetic acid (DETAPAC) was used in the assay mixture instead of 0.1 mM EDTA. Polyacrylamide gel disc electrophoresis with or without sodium dodecyl sulfate, isoelectric focusing, location of the activity on the gel, molecular weight determination by gel filtration and sedimentation equilibrium, metal and amino acid analysis, measurement of the dry weight of the enzyme were performed as described in the previous chapter. Circular dicroism spectra were recorded using a JASCO J-20 spectropolarimeter.

3. RESULTS

Purification of the enzyme

Unless noted, all steps were performed at 0-4°C, and potassium phosphate buffer was used at pH 7.8. Frozen thalli (about 20 kg) of Porphyra yezoensis were suspended in 40 liters of 5 mM phosphate containing 0.1 mM EDTA and were allowed to stand for extraction for 3 days with occasional stirring. The pH of the suspension was ajusted to 7.8 by ammonia. The pink colored extract was obtained by filtration through cotton cloth using a basket centrifuge. To ensure the extraction of the enzyme, the debris was further suspended in the same buffer and the extraction was repeated. The extract thus obtained was combined and subjected to the ammonium sulfate fractionation (40-90%). The precipitate formed was dissolved in and dialyzed against 10 mM phosphate containing 0.1 mM EDTA. After removal of insoluble materials by centrifugation, the dialyzed enzyme was applied onto a column of DEAE-Sephadex (9 x 50 cm) previously equilibrated with 10 mM phosphate containing 0.1 mM EDTA. The column was washed with 13.5 liters of the equilibrating buffer, then eluted by 22.5 liters of 50 mM phosphate, 7.5

liters of 100 mM phosphate, 7.5 liters of 200 mM phosphate, and 7.5 liters of 200 mM phosphate plus 1 M KCl, each containing 0.1 mM EDTA. SOD was eluted at 50 mM phosphate. Active fractions were pooled and collected after the addition of ammonium sulfate to 90% saturation. After dialysis against 10 mM phosphate containing 0.1 mM EDTA and removal of insoluble materials by centrifugation, the dialyzed enzyme was adsorbed on a DEAE-Sephadex column (2.5 x 42 cm) equilibrated with 10 mM phosphate containing 0.1 mM EDTA. The enzyme was eluted with a linear gradient of phosphate (10 to 100 mM, 3 liters) containing 0.1 mM EDTA. SOD was eluted as a single peak at 45 mM phosphate. The active fractions were pooled and concentrated with an Amicon PM 10 membrane during which the buffer was changed to 10 mM phosphate containing 0.1 M KCl. The concentrated enzyme (ca. 10 ml) was loaded on a column of Sephadex G-100 (4 x 80 cm) equilibrated with 10 mM phosphate containing 0.1 M KCl. The enzyme was eluted with the same buffer and the active fractions were pooled and concentrated. At this step, the enzyme still

Table I

Summary of the Purification of Mn-SOD from Porphyra yezoensis

Purification step	Total[a] protein (mg)	Total[b] activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
		x10^4			
Crude extract	136,011	261	19	100	1
40 to 90% $(NH_4)_2SO_4$	31,721	233	73	89	4
Stepwise DEAE-Sephadex	5,170	204	395	78	21
Linear gradient DEAE-Sephadex	1,274	195	1531	75	81
Sephadex G-100	735	167	2272	64	120
Phenyl-Sepharose	402	165	4105	63	216

a) Protein was determined by the Lowry method from crude extract to Sephadex G-100 steps and in the final step spectrophotometrically using an $A^{1\%}_{1\ cm}$ at 280 nm of 20.35.

b) The enzymatic activity is presented in the McCord-Fridovich unit.

showed a fluoresence due to phycoerythrin which was removed by hydrophobic chromatography. The enzyme solution was equilibrated with 10 mM phosphate, pH 6.8, containing 30% (W/V) ammonium sulfate, then adsorbed on a Phenyl-Sepharose column (1.2 x 9 cm) equilibrated with the same solution. The elution was conducted by a simultaneous cross-gradient (150 ml) of ammonium sulfate from 30% to 0%, and ethylene glycol from 0% to 20% (V/V) in 10 mM phosphate, pH 6.8. SOD was eluted as a single peak but phycoerythrin was not. SOD thus obtained was homogeneous judging from polyacrylamide gel disc electrophoresis. The enzyme was dialyzed against 10 mM potassium phosphate, pH 7.8, and stored in ammonium sulfate suspension. The purification is summarized in Table I. The yield was 400 mg with a specific activity of 4,100 units/mg protein.

Purity

The purified enzyme (40 μg) was subjected to electrophoresis with a 7.5% polyacrylamide gel in Tris-glycine, pH 8.9 and stained with amido

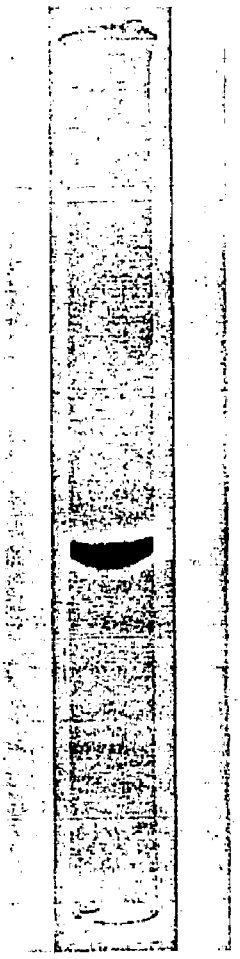

Fig. 1. Polyacrylamide gel disc electrophoresis of Porphyra Mn-SOD. Cathode, top; anode, bottom. Protein (40 μg) was stained with amido black 10 B.

black 10B. This gave a single protein band (Fig. 1). The protein band corresponded to a band stained for the activity. Polyacrylamide gel isoelectric focusing using Ampholine showed also a single protein band. In equilibrium centrifugation, the slope obtained from log(fringe displacement) plotted as a function of the square of the distance from the center of rotation showed a straight line.

Molecular weight and subunit structure

The molecular weight of the enzyme was estimated to be 45,000 by gel filtration with a Sephadex G-100 column, and 45,100 by sedimentation equilibrium centrifugation. A partial specific volume of 0.735 calcu-

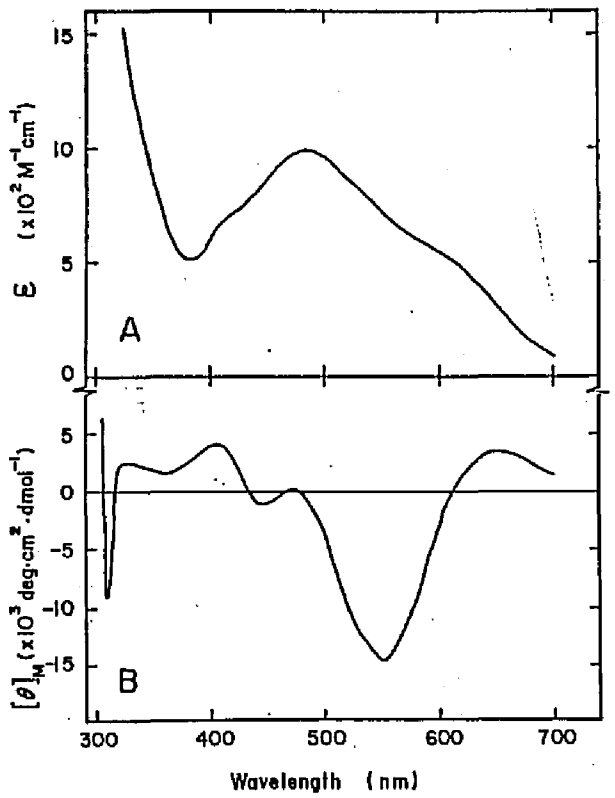

Fig. 2. Absorption and circular dichroism spectra in visible region of Porphyra Mn-SOD. The absorption spectrum (A) was recorded at 2.28 mg protein/ml in 10 mM potassium phosphate, pH 7.8, at 25°C. The light path was 10 mm. Absorbance was converted into molar absorbance coefficient using a molecular weight of 45,000. The CD spetrum (B) was taken at the same concentration in the same buffer at 25°C using a 10 mm light-path cell. The ellipticity was expressed as a molar ellipticity, $[\theta]_M$, based on the molecular weight.

lated from the amino acid composition (Table II) was used.

The subunit molecular weight was determined by sodium dodecyl sulfate polyacrylamide gel disc electrophoresis after denaturating the enzyme with 1% sodium dodecyl sulfate in the presence or the absence of 1% 2-mercaptoethanol. The enzyme gave one band corresponding to a molecular weight of 23,000 in the presence and absence of 2-mercaptoethanol. Thus, Porphyra SOD is a dimer composed of identical subunits without a disulfide bridge.

Table II

Amino Acid Compositions of Porphyra yezoensis and Porphyridium cruentum Mn-SOD[f], and Brassica campestris Fe-SOD[g].

Amino acid	*Porphyra* Mn-SOD		*Porphyridium*[f] Mn-SOD	*Brassica*[g] Fe-SOD
	residue/mol[a]	nearest integer		
Lysine	25.88	26	16	22
Histidine	13.21	13	16	8
Arginine	8.84	9	12	10
Aspartic acid	42.67	43	40	36
Threonine[b]	27.53	28	27	18
Serine[b]	35.49	35	18	22
Glutamic acid	28.78	29	23	32
Proline	24.49	24	17	20
Glycine	36.87	37	33	32
Alanine	32.98	33	36	34
Half-cystine[d]	0.52	1	0	4
Valine[c]	25.41	25	22	22
Methionine[d]	7.11	7	4	2
Isoleucine[c]	20.12	20	15	12
Leucine	33.45	33	25	28
Tyrosine	15.73	16	11	12
Phenylalanine	16.35	16	12	14
Tryptophan[e]	13.88	14	6	8

a) The number of residues was calculated for 45,000 g enzyme.

b) The values extrapolated to zero hydrolysis time.

c) The values at 72 hours hydrolysis.

d) Determined as methionine sulfone and cysteic acid after performic acid oxidation.

e) Determined by the method of Edelhoch (24).

f) Reference 3.

g) Reference 5.

Absorption spectra

In the ultraviolet region the enzyme exhibited an absorption maximum at 280 nm with shoulders at 260 and 290 nm (data not shown). $A^{1\%}_{1\ cm}$ and the molar absorbance coefficient at 280 nm was 20.35 and 91,500 $M^{-1}cm^{-1}$, respectively, based on a molecular weight of 45,000 and dry weight. The enzyme revealed a characteristic absorption for Mn-SOD in visible region exhibiting an absorption centered at 480 nm (Fig. 2A). The molar absorbance coefficient at 479 nm was 990 $M^{-1}cm^{-1}$. The ratio of absorption at 280 nm to that at 479 nm was 92.6.

Metal analysis

Determination of the metal by atomic absorption spectrometry indicated that Porphyra SOD contained 1.41 $\pm$ 0.02 g-atoms of Mn per mole

Table III

SΔQ Values between Porphyra yezoensis Mn-SOD and SOD from Photosynthetic Organisms at Different Level of Evolution

Species	SOD	SΔQ
Sulfur photosynthetic bacteria		
Chromatium vinosum	Fe	43
Chlorobium thosulfatophilum	Fe	74
Non-sulfur photosynthetic bacterium		
Rhodopseudomonas spheroides	Mn	63
Blue-green algae		
Plectonema boryanum	Fe	86
Spirulina platensis	Fe	92
Red alga		
Porphyridium cruentum	Mn	35
Seed plants		
Pisum sativum	Mn	39
Nuphar luteum	Fe	63
Brassica campestris	Fe	24
Green pea	Cu,Zn	184
wheat germ	Cu,Zn	150-169[a)]
Spinach (stromal)	Cu,Zn	142

a) Values for two isozymes.

of enzyme, based on a molecular weight of 45,000. The contents of iron, copper, and zinc were below detection or insignificant. No acid-labile sulfur was detectable.

Amino acid composition

The amino acid composition of Porphyra Mn-SOD is shown in Table II, with those of Porphyridium Mn-SOD (3) and Brassica Fe-SOD (5). The degree of sequence homology between Porphyra Mn-SOD, and Mn- and Fe-SOD from other organisms was assessed using SΔQ values by the method of Marchalonis and Weltman (6), a statistical analysis of amino acid compositions (Table III and IV). The lower the value, the higher the sequence homology. The analysis indicated that the Porphyra Mn-SOD most resembles the angiosperm Brassica Fe-SOD which is localized in chloroplast stroma (7).

Table IV

SΔQ Values of Porphyra Mn-SOD with Bacterial and Mitochondrial Mn-SOD and Bacterial Fe-SOD

Species	SΔQ
Mn-enzyme (bacterial)	
Bacillus stearothermophilus	43
Parracoccus denitrificans	59
Escherichia coli	72
Thermas thermophilus	99
Mycobacterium smegmatis	99
Thermas aquaticus	113
Streptococcus faecalis	126
Mn-enzyme (mitochondrial)	
Chicken liver	32
Rat liver	38
Human liver	78
Fe-enzyme (bacterial)	
Pseudomonas ovalis	27
Azotobacter vinelandii	33
Desulfovibrio desulfuricans	71
Thermoplasma acidophilum	80
Propionibacterium shermanii	80
Methanobacterium bryantii	82

Isoelectric point

The isoelectric point of the enzyme was 4.10 according to isoelectric focusing with Ampholine (pH 3.5-10.0). A low pI of Phorphyra SOD corresponds to a high content of acidic amino acid residues.

Circular dichroism spectra

The ultraviolet CD spectra of Porphyra Mn-SOD are shown in Fig. 3. The ellipticity was expressed in terms of mean residue ellipticity, $[\theta]_R$, which was based on the mean amino acid residue weight of 110.0 calculated from the amino acid composition (Table II). In the 200-250 nm region, two negative bands were observed at 211 and 229 nm indicating the presence of α-helical structure in the enzyme. The mean residue ellipticities at 211 and 229 nm were -10,810 and -11,420 $deg \cdot cm^2 \cdot dmol^{-1}$ respectively. The content of α-helix was estimated using the method of Chen and Yang (8). The mean residue ellipticity of -9,675 $deg \cdot cm^2 \cdot dmol^{-1}$ at 222 nm indicates 24% α-helical content of the enzyme, which is almost same as that of Fe-SOD from Pseudomonas being 32% (9). Higher α-helical

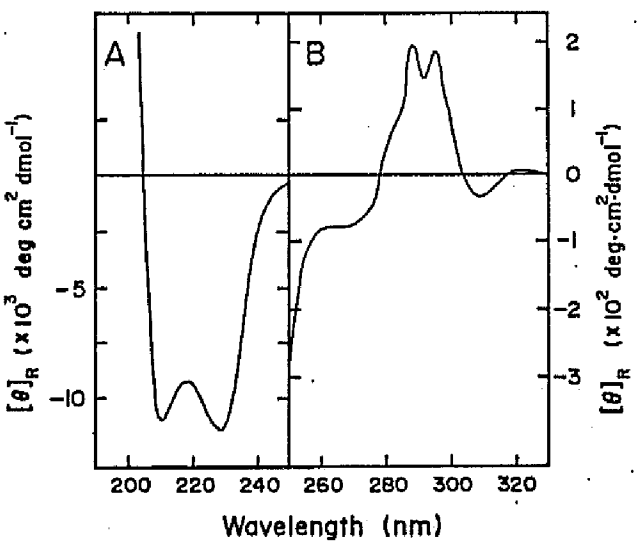

Fig. 3. Ultraviolet circular dichroism spectra of Porphyra Mn-SOD. The spectra were obtained at 0.23 mg/ml from 200 to 250 nm (A), and 2.28 mg/ml from 250 to 330 nm (B), respectively, in a 1 mm light-path cell. Both measurements were carried out in 10 mM potassium phosphate, pH 7.8, at 25°C. The ellipticity was expressed in terms of mean residue ellipticity, $[\theta]_R$, using a mean residue weight of 110.

content of Mn-SOD from a thermophilic bacterium, Thermus thermophilus, has been reported (10). The CD specturm in the 250-330 nm region showed two positive bands at 288 nm ($[\theta]_R$ = 191) and at 295 nm ($[\theta]_R$ = 184 deg·cm^2·dmol^{-1}) with shoulders at 282 and 300 nm, and negative band at 309 nm and a broad shoulder centered at 270 nm (Fig. 3).

Fig. 2B shows the visible CD spectrum of Porphyra Mn-SOD with the absorption spectrum. The Porphyra enzyme exhibited a strong negative band at 552 nm ($[\theta]_M$ = -14,580 deg·cm^2·dmol^{-1}, the value is expressed as molar ellipticity), which is characteristic to Mn-SOD. Moreover, positive bands at 325, 405 and 650 nm, and negative bands at 309 (see also Fig. 3) and 445 nm were observed. Above 400 nm, the spectrum very resembles those of Mn-SOD from Escherichia coli (11) and Thermus thermophilus (10), except for a band at 460 nm of the Thermus enzyme. Four bands of CD spectrum above 400 nm correspond quite well to the absorption spectrum (Fig. 2A) exhibiting absorption maximum at 480 nm with shoulders at 410, 500 and 620 nm. The bands of CD spectrum and the peaks of absorption spectrum can be accounted for by four spin-allowed d-d transitions of a high-spin Mn(III) ion in octahedral ligand field with rhombic distortion (12,13).

Effects of several compounds on the activity

The Porphyra Mn-SOD activity was not affected by neither 1 mM KCN nor 10 mM H_2O_2 at pH 7.8. The enzyme was inhibited by 50% with 10 mM NaN_3 at pH 7.8 (14). These properties are similar to the other Mn-SOD.

Isozymes

The crude extract from Porphyra showed two isozymes on polyacrylamide gel electrophoresis and isoelectric focusing after activity staining. The major isozyme (pI = 4.10) was corresponded to the purified enzyme, but the minor one could not be isolated due to its low content. The minor SOD is seemed to be the Mn-enzyme, because the SOD activity in crude extract was not inhibited by the incubation with 10 mM H_2O_2 or 1 mM KCN.

4. DISCUSSION

Mn-SOD has been purified from many bacteria, and from mitochondria of animals and fungi (15). However, the isolation of the enzyme from plants has been restricted to a few species; the red alga Porphyridium cruentum (3) and the angiosperms Pisum sativum (16) and Spinacia oleracia (17). Pisum Mn-SOD was localized in peroxisomes but not in mitochondria (18). Porphyra yezoensis contained two SOD and one of them was characterized to be the Mn-containing enzyme by purification. The other was also suggested to be the Mn-enzyme on the basis of its H_2O_2-insensitivity. The purified Porphyra Mn-SOD was a dimer having a molecular weight of 45,000, and contained 1.4 g-atoms of manganese per mol of enzyme. The gross properties of Porphyra Mn-SOD are similar to those of other Mn- and Fe-SOD.

Amino acid composition of Porphyra Mn-SOD resembles those of the Mn-enzyme from bacteria, animals and fungi (Table II). The comparison of the amino acid compositions based on $S\Delta Q$ values between Porphyra Mn-SOD and other Mn- and Fe-SOD clearly indicates that the Porphyra Mn-enyzme is most related in amino acid sequence to Brassica Fe-SOD, which is localized in chloroplast stroma (7).

Mn-SOD can be classified into two groups in terms of subunit structure; i.e. a dimer or tetramer. In general, prokaryotic Mn-SOD is a dimer, but the Mn-SOD from mitochondria of animals and fungi is a tetramer with a few exceptions (15). Higher plants, Pisum (16) and rice (Chapter VI), also possess tetrameric Mn-SOD. Exceptionally, spinach Mn-SOD was a dimer, although its subunit molecular weight was higher than those of typical Mn-SOD (17). On the contrary, Mn-SOD from two red algae, Porphyridium and Porphyra, are dimers. In this respect, Porphyra Mn-SOD also resembles Brassica dimeric Fe-SOD as well as bacterial dimeric Mn- and Fe-SOD. So far, chloroplast stromal Fe-SOD is a dimer without exception (7,Chapter XIII).

In plants, the major site of superoxide production is chloroplast. Thus, chloroplast stroma of Euglena (4) and of some angiosperms (7, Chapter XIII) contain Fe-SOD, whereas most angiosperms possess Cu,Zn-SOD in the stroma. The stromal Cu,Zn-SOD of spinach structurally differs

from the cytosolic Cu,Zn-SOD (Chapter V). Chloroplast stroma of red algae should also contain SOD to scavenge superoxide radicals which are inevitably formed inside chloroplasts under illumination (19), otherwise chloroplast components would be oxidized. Thus, the chloroplast stromal SOD of Porphyra seems to be Mn-SOD, because Porphyra contains two Mn-SOD isozymes but lack Fe-SOD.

It had been believed a strict metal specificity between Fe- and Mn-containing SOD, although both enzymes are evolutionary related (15). Recently, however, the enzymes having both Fe and Mn have been isolated from several bacteria (20,21). Furthermore, metal replacement has been successful from Fe-SOD to Mn-SOD, and vice versa with the restoration of the enzymatic activity (22,23). These facts indicate that Mn/Fe-SOD apoprotein has a potentiality to accommodate either Fe or Mn by a small change of configuration around the active site. Thus, it is tempting to postulate that the conversions of Fe-SOD to Mn-SOD, and of Mn-SOD to Fe-SOD might have occurred randomly by mutation during the course of evolution after acquisition of Fe-SOD by anaerobes. Random distribution of Fe- and Mn-SOD in aerobic bacteria rather than phylogenetic could be accounted for by the above hypothesis. In the phylogenetic line of photosynthetic organisms, Fe-SOD is localized in chloroplast stroma of Euglena (Chapter XI), moss (Chapter XII), and some angiosperms (7, Chapter XIII). The case of Porphyra chloroplast stroma being localized by Mn-SOD is also interpreted by random conversion of Fe-SOD to Mn-SOD. It should be noted that stromal SOD could be distinguished from cytosolic SOD through protein synthesis. Thus, the elucidation of Mn-SOD precursor sequence will be a clue to evaluate the above hypothesis.

5. SUMMARY

The red alga Porphyra yezoensis contained major and minor Mn-superoxide dismutase (SOD) isozymes and lacked Fe-SOD. The major SOD was purified to a homogeneous state. The enzyme was a dimer having a molecular weight of 45,000 consisted of two subunits of equal size, and contained 1.4 atoms of manganase per molecule but no iron. Optical and circular dichroism spectra were presented. Amino acid composition of Porphyra Mn-SOD most resembled that of the angiosperm Brassica campestris chloroplast stromal Fe-SOD. Occurrence of only Mn-SOD in the red alga suggests its localization in chloroplast stroma.

6. REFERENCES

(1) Henry, L.E.A, and Hall, D.O. (1977) in Photosynthetic Organelles (Miyachi, S., Fujita, Y., and Shibata, K., eds.), pp. 377-382, Japanese Soc., Plant Physiol., Kyoto.

(2) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(3) Misra, H.P., and Fridovich, I. (1977) J. Biol. Chem. 252, 6421-6423.

(4) Kanematsu, S., and Asada, K. (1979) Arch. Biochem. Biophys. 195, 535-545.

(5) Salin, M.L., and Bridges, S.M. (1980) Arch. Biochem. Biophys. 201, 369-374.

(6) Marchalonis, J.J., and Weltman, J.K. (1971) Comp. Biochem. Physiol. 38B, 609-625.

(7) Salin, M.L., and Bridges, S.M. (1980) Z. Pflanzenphysiol. 99, 37-45.

(8) Chen, Y.H., and Yang, J.T. (1971) Biochem. Biophys. Res. Commun. 44, 1285-1291.

(9) Yamakura, F. (1976) Biochim. Biophys. Acta 422, 280-294.

(10) Sato, S., and Nakazawa, K. (1978) J. Biochem. 83, 1165- 1171.

(11) Keele, B.B, Giovagnoli, C., and Rotilio, G. (1975) Physiol. Chem. and Phys. 7, 1-6.

(12) Fee, J.A., Shapiro, E.R., and Moss, T.H. (1976) J. Biol. Chem. 251, 6157-6159.

(13) Ballhausen, C.J. (1962) Introduction to Ligand Field Theory, pp.57-98, McGraw-Hill, New York.

(14) Misra, H.P., and Fridovich, I. (1978) Arch. Biochem. Biophys. 189, 317-322.

(15) Steinman, H.M. (1982) in Superoxide Dismutase (Oberley, L.W., ed.), pp. 11-68, CRC Press, Florida.

(16) Sevilla, F., Lopez-Gorge, J., and Del Rio, L.A. (1982) Plant Physiol. 70, 1321-1326.

(17) Hayakawa, T., Kanematsu, S., and Asada, K. (1985) Planta 166, 111-116.

(18) Del Rio, L.A., Lyon, D.S., Olah, I., Glick, B., and Salin, M.L. (1983) 158, 216-224.

(19) Asada, K., Kiso, K., and Yoshikawa, K. (1974) J. Biol. Chem. 249, 2175-2181.

(20) Beaman, B.L., Scates, S.M., Moring, S.E., Deem, R., and Misra, H.P. (1983) J. Biol. Chem. 258, 91-96.

(21) Meier, B., Barra, D., Bossa, F., Calabrese, L., and Rotilio, G. (1982) J. Biol. Chem. 257, 13977-13980.

(22) Gregory, E.M., and Dapper, C.H. (1983) Arch. Biochem. Biophys. 220, 293-300.

(23) Gregory, E.M. (1985) Arch. Biochem. Biophys. 238, 83-89.

(24) Edelhoch, H. (1967) Biochemistry 6, 1948-1954.

CHAPTER XV

Fe-SUPEROXIDE DISMUTASE FROM AZOTOBACTER VINELANDII: PURIFICATION, MOLECULAR SHAPE AND DIMENSION, AND MÖSSBAUER SPECTRUM

1. INTRODUCTION

Among three types of superoxide dismutase (SOD), Fe- and Mn-SOD are evolutionally related: their amino acid sequences have a high homology, but Cu,Zn-SOD is different from the formers in the sequence (1). According to the distribution studies of SOD, Fe-SOD was the first SOD which was acquired by organisms, and Mn-SOD might be derived from ancestral Fe-SOD (Chapter VIII and IX). Although the reason of the appearance of new enzyme from ancestral enzyme which catalyzes the same reaction is quite obscure, the elucidation of structural difference between Fe- and Mn-SOD would be one of the clues to solve this problem.

Several attempts have been done to perform the interconversion from Fe-SOD to Mn-SOD and from the Mn-enzyme to the Fe-enzyme but all failed with a few exceptions (2). This indicates that microenvironment at metal binding site is different each other. The complete amino acid sequence has been reported of Mn-SOD from Escherichia coli and Bacilus stearothermophilus (3,4), but that of Fe-SOD is not accomplished yet. As to the subunit structure, Fe-SOD is most commonly dimer having a molecular weight of 38,000-45,000, with a exception, e.g. the Mycobacterium tuberculosis enzyme (tetramer, MW 90,000) (5), whereas Mn-SOD is rather complicated: prokaryotic Mn-enzyme is a dimer or tetramer, but eukaryotic Mn-enzyme which were isolated from mitochondria seems to be a homotetramer (MW 80,000-90,000). The X-ray crystallographic studies on Fe- and Mn-SOD have been continuing (6,7), but no three-dimensional structure has been reported yet. Under these cercumstances, it should be worthwhile to obtain their gross structure, i.e. molecular dimension and shape by hydrodynamic methods.

The molecular mechanism of protein evolution is accounted for by

insertion or deletion of some portion of DNA gene or by one-point mutation (8). The former two cases would result in the change in moleucular weight. Thus, precise determination of the molecular weight may give the estimation whether such a insertion or delation of DNA fragment in the gene for SOD took place. This can be tested by the several molecular weight determinations using hydrodynamic parameters.

It has been suggested that a diazotroph, Azotobacter, contained relatively high amount of SOD, and this enzyme seemed to be the Fe-enzyme judging from its reactivity against hydrogen peroxide (Chapter X). Thus, this organism is a reasonable source for Fe-SOD. In this chapter, a large-scale purification of Azotobacter SOD and its physicochemical properties are described. Furthermore, the electronic state of the iron in active site is analyzed by electron paramagnetic resonance and Mössbauer spectroscopies.

2. MATERIALS AND METHODS

Cytochrome c (type III) and xanthine oxidase were purchased from Sigma and Boehringer, respectively. The strain of Azotobacter vinelandii IFO 12018 was obtained from the Institute for Fermentation, Osaka, Japan. Cells were grown aerobically in 140-180 liters of the modified Burk's medium (9) using a New Brunswich Scientific's or Marubishi's jar fermenter at 20°C for 2 days, harvested in the late logarithmic phase of growth and stored at -20°C until use. The kind helps of Drs. H. Misono and H. Oe in the cell culture are acknowledged.

SOD was assayed by a modification of the procedure of McCord and Fridovich, and the enzymatic activity is shown in the McCord-Fridovich unit (Chapter II). Native and SDS-polyacrylamide gel electrophoresis, isoelectric focusing with Ampholine on polyacrylamide gel, location of activity on the gel, amino acid analysis, measurement of dry weight of the enzyme, metal analysis, protein determination were performed as described in previous chapters.

Sedimentation equilibrium of the enyzme was carried out in a Hitachi UCA-1A analytical ultracentrifuge equipped with interference

optics using the high speed meniscus-depletion method of Yaphantis (10). A multichannel cell was used for the equilibrium run. The sedimentation coefficient, s, and diffusion coefficient, D, of the enzyme were determined using the analytical centrifuge equipped with schlieren optics. A double sector cell and a double-sector synthetic boundary cell were employed for the determination of s and D. The $s^{\circ}_{20,w}$ was obtained by extrapolating $s_{20,w}$ at three different protein concentrations to infinite concentration. The $D_{20,w}$ was calculated from the schlieren patterns by the height-area method (11) and used as $D^{\circ}_{20,w}$. SOD was used throughout in 0.1 M KCl and 5 mM potassium phosphate, pH 7.8, for the ultracentrifugal analysis and other hydrodynamic measurements.

Partial specific volume, $\bar{v}$, of the enzyme was determined with a 4-ml pycnometer at 20.00 ± 0.01°C, using the following equation:

$$\bar{v} = \frac{1}{\rho_o}\left(1 - \frac{\rho - \rho_o}{c}\right) \quad ,$$

where ρ_o is the density of solvent (g/ml), ρ is the density of solution and c is the cencentration of protein (g/ml). The $\bar{v}$ was also calculated from the amino acid composition according to Cohn and Edsall (12).

Viscosity of the enyzme was determined using an Ubbelohde semi-microdilution viscometer with a flow time of more than 120 sec for 4 ml of distilled water at 20.00 ± 0.01°C. The intrinsic viscosity, $[\eta]$, of the enzyme was calculated by extrapolation of the reduced viscosity plotted against the protein concentration to zero.

Molecular weight, M, of the enzyme was estimated by sedimentation equilibrium according to the following equation (13):

$$M = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{2.303(d\log c)}{d(r^2)} \quad ,$$

where R is the gas constant, T is the temperature (K), ω is the angular velocity (radian/sec), c is fringe displacement, and r is the distance from the center of rotation. Molecular weight estimation was also performed by the gel-filtration method with Sephadex G-100 according to Andrews (14) using the following proteins as a marker: bovine serum

albumin, ovalbumin, α-chymotrypsinogen-A, myoglobin, and cytochrome c. The hydrodynamic parameters, i.e., $s^\circ_{20,w}$, $D^\circ_{20,w}$ and $[\eta]$ gave a molecular weight by the following equations (15):

$$M = \frac{s^\circ RT}{D^\circ(1 - \bar{v}\rho_\circ)} \quad , \qquad \text{(Eq 1)}$$

$$M = \frac{6.58 \times 10^{-16}}{(D^\circ_{20,w})^3[\eta]} \quad , \qquad \text{(Eq 2)}$$

and

$$M = \frac{4690(s^\circ_{20,w})^{3/2}[\eta]^{1/2}}{(1 - \bar{v}\rho_\circ)^{3/2}} \quad . \qquad \text{(Eq 3)}$$

Frictional coefficient ratio of the enzyme, f/f_{min}, was calculated from the sedimentation coefficient using the equation,

$$f/f_{min} = \frac{(4/3)^{1/3}}{6\eta(\pi N)^{2/3}} \frac{(1 - \bar{v}\rho_\circ)M^{2/3}}{\bar{v}^{1/3}s^\circ_{20,w}} \quad , \qquad \text{(Eq 4)}$$

or from the diffusion coefficient using the equation,

$$f/f_{min} = \frac{(4/3)^{1/3}kT}{6\eta\pi^{2/3}(\bar{v}M/N)^{1/3}D^\circ_{20,w}} \quad , \qquad \text{(Eq 5)}$$

or from the sedimentation coefficient and diffusion coefficient using the equation,

$$f/f_{min} = \frac{1}{6\eta\pi}\left(\frac{RT}{D^\circ N}\right)^{2/3}\left[\frac{4\pi(1 - \bar{v}\rho_\circ)}{3\bar{v}s^\circ_{20,w}}\right]^{1/3} \quad , \qquad \text{(Eq 6)}$$

where η is the viscosity of solvent, N is Avogadro's number and k is Boltzmann's constant. f/f_{min} is the frictional coefficient for an anhydrous sphere and the frictional coefficient for an hydrated sphere is denoted by symbol f_o. The frictional coefficient ratio was also obtained by Polson's emprical relationship (16),

$$f/f_{min} = \frac{\log[\eta]/\bar{v}}{0.61} \quad . \qquad \text{(Eq 7)}$$

Reconstituted ^{57}Fe-SOD was prepared as follows: Azotobacter Fe-SOD (202 mg) was dissolved in 40 ml of 7.5 M guanidine-HCl containing 75 mM Tris-HCl, pH 7.1, 2 mM EDTA and 10 mM dithiothreitol (DTT), then was incubated at 25°C for 17 hours under anaerobic conditions. The enzyme was dialyzed against 50 mM Tris-HCl, pH 7.1, containing 2 mM EDTA and 1 mM DTT then against 50 mM Tris-HCl. The dialyzed apoenzyme was concentrated in a collodion bag under reduced pressure then was incubated in 15 ml of the mixture of 10 mM $^{57}FeCl_3$, 5 mM ascorbate, 10 mM ammonium sulfate, 9 mM DTT, 100 mM Tris-HCl, pH 7.1 and 7.5 M guanidine-HCl at 25°C for 18 hours under anaerobic conditions. The ^{57}Fe-enzyme was dialyzed successively against each 50 ml of 5, 4, 3, 2, 1, 0.8, 0.6 and 0.3 M guanidine-HCl containing 100 mM Tris-HCl, pH 7.1, and 5 mM DTT under anaerobic conditions. Finally, the enzyme was dialyzed against 10 mM potassium phosphate, pH 7.8, and the dialyzed solution was centrifuged to remove denatured protein. The supernatant was applied to a small column of DEAE-Sephadex (1 x 10 cm) which had been equilibrated with 10 mM potassium phosphate, pH 7.8. The column was washed with 10 mM phosphate then with 30 mM phosphate and the enzyme was eluted with 100 mM phosphate, pH 7.8.

The apoenzyme had no enzymatic activity and its iron content was 0.07 atom per molecule. Absorption spectrum of the apoenzyme showed no peak at 350 nm due to the iron chromophore. The absorption spectrum of the reconstituted ^{57}Fe-SOD, its ratio, A_{280}/A_{350} (30.8) and the EPR spectrum, were indistinguishable from those of the native enzyme. The specific activity of the ^{57}Fe-enzyme was 90% of that of the native enzyme. The reconstituted enzyme contained 2.2 atom iron per molecule. The yield of the ^{57}Fe-enzyme was 54% of the native enzyme.

Mössbauer data were obtained at 4.2K (liquid helium), 77K (liquid nitrogen) and 195K (dry ice-acetone) with a Elron 5011 Mössbauer resonance analyzer consisted of an electromagnetic velocity transducer (linear velocity type), a gamma-ray counting system and a liquid helium Dewar, as described previously (17). The velocity transducer was used in conjunction with a 400 channel analyzer operating in the multi-scaler mode. The source was ^{57}Co (30 mCi) diffused into metallic Cu and the temperature of the source was 293K. All isomer shifts are quoted

relative to iron metal.

The concentrated ^{57}Fe-SOD (1.46 mM) in 10 mM potassium phosphate, pH 7.8, was located into a Lucite absorber cell (5 mm thick and 15 mm in diameter), frozen rapidly in liquid nitrogen, and then placed on a cell-holder in a low temperature Dewar for the measurements.

Electron paramagnetic resonance (EPR) measurements were performed at 4.2K and 77K with a Varian E-9 spectrometer with 100 kHz field modulation.

3. RESULTS

Large-Scale Purification of Fe-SOD from Azotobacter

Unless noted, all steps were performed at 0-4°C and potassium phosphate buffer was used at pH 7.8. The frozen cells (2.5 kg) were suspended in 3 liters of 50 mM phosphate containing 0.5 mM EDTA, and disrupted continuously by agitating at 2,000 rpm with glass beads (0.1-0.2 mm in diameter) using a Dyno-mill equipped with 0.6 liter grinding container. Deoxyribonuclease (10 mg) was added to the extract and the solution was stirred for 30 min at room temperature. Then, the solution was centrifuged at 12,000 g for 30 min and the cell debris was discarded.

To the supernatant solution solid ammonium sulfate was added to 40% saturation, and the precipitate formed was removed by centrifugation at 12,000 g for 20 min. Again, solid ammonium sulfate was added to the supernatant to make 80% saturation. After stirring, the precipitate was collected by centrifugation, dissolved in and dialyzed against 30 mM phosphate containing 0.1 mM EDTA.

After removal of insoluble materials by centrifugation, the dialyzed solution was applied onto a column of DEAE-Sephadex (14 x 60 cm) previously equilibrated with 30 mM phosphate containing 0.1 mM EDTA. The column was washed with 40 liters of the equilibrating buffer, and then SOD was eluted wih 20 liters of 200 mM phosphate containing 0.1 mM EDTA. No SOD activity was detected in unadsorbed fraction or eluate of

1.0 M KCl containing 200 mM phosphate and 0.1 mM EDTA after the elution of the enzyme with 200 mM phosphate. SOD was collected by centrifugation after the addition of ammonium sulfate to 90% saturation, dissolved in and dialyzed against 30 mM phosphate, containing 0.1 mM EDTA.

Dialyzed enzyme was applied onto a column of DEAE-Sephadex (5 x 50 cm) previously equilibrated with 30 mM phosphate containing 0.1 mM EDTA. The column was eluted with a linear gradient elution of 5 liters of phosphate which was composed of 2.5 liters of 30 mM phosphate and 2.5 liters of 200 mM phosphate, each containing 0.1 mM EDTA. SOD was eluted at 74 mM phosphate with a single peak. The active fractions were pooled, concentrated by the addition of ammonium sulfate at 80% saturation and dialyzed against 10 mM phosphate containing 0.1 M KCl.

After clarified by centrifugation, the concentrated enzyme solution (125 ml) was divided into two portions and each was separately applied on a column of Sephadex G-100 (5 x 80 cm) equilibrated with 10 mM phosphate containing 0.1 M KCl. The brown SOD was eluted with the same buffer and the fractions having a constant ratio of A_{280}/A_{350} were pooled. The enzyme obtained from the separate runs was combined, concentrated with ammonium sulfate and dialyzed against 10 mM phosphate. At this step, the homogeneity of the enzyme was attained on the basis of

Table I

Summary of the Purification of Fe-SOD from Azotobacter vinelandii

Purification step	Total[a] protein (mg)	Total[b] activity (units x 10^{-3})	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Homogenate	284,706	12,185	43	100	1
40 to 80% $(NH_4)_2SO_4$	96,154	9,257	96	76	2.3
Stepwise DEAE-Sephadex	29,512	7,977	270	65	6.4
Linear gradient DEAE-Sephadex	5,040	7,583	1,505	62	36
Sephadex G-100	1,657	7,472	4,509	61	107

a) Protein was determined by the Lowry method from homogenate to linear gradient DEAE-Sephadex steps and in the final step spectrophotometrically using an $A^{1\%}_{1\ cm}$ at 280 nm of 26.81.

b) McCord-Fridovich unit.

the result in electrophoresis. Table I summarizes the purification of the enzyme.

General Properties of Azotobacter Fe-SOD

Purity

The purified enzyme gave a single protein band on polyacrylamide gel disc electrophoresis (Fig. 1). The location of the purified enzyme in the gel was identical with the active zone of the crude extract, indicating no modification of the enzyme during the purification procedure. Isoelectric focusing on polyacrylamide gel using Ampholine showed a single band (data not shown). The homogeneity of the purified enzyme was also confirmed by equilibrium centrifugation and sedimentation velocity. The slope obtained from log(fringe displacement) plotted as a function of the square of the distance from the center of rotation showed a straight line (Fig. 2). Sedimentation pattern indicated a single symmetrical peak (Fig. 3).

Stability

The enzyme was stable at room temperature at least for several days: no loss of the activity was observed during the present measurements. The stored enzyme under ammonium sulfate-precipitated state at 4°C or frozen state at -20°C held the full activity at least for a year. The ratio of absorbance at 280 mm and 350 nm was constant after

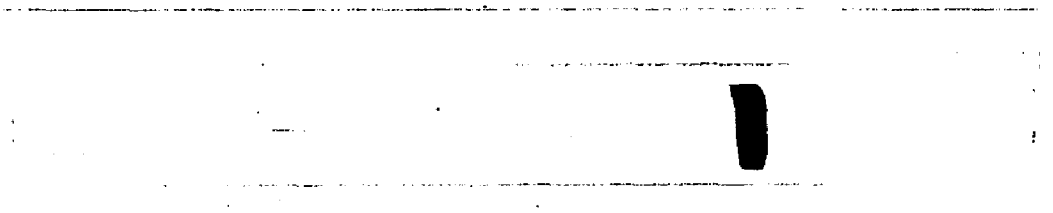

Fig. 1. Polyacrylamide gel disc electrophoresis of Azotobacter Fe-SOD. The purified enzyme (50 μg) was subjected to electrophoresis. Protein was stained with Coomassie Brilliant Blue R-250. Cathode, left; anode, right.

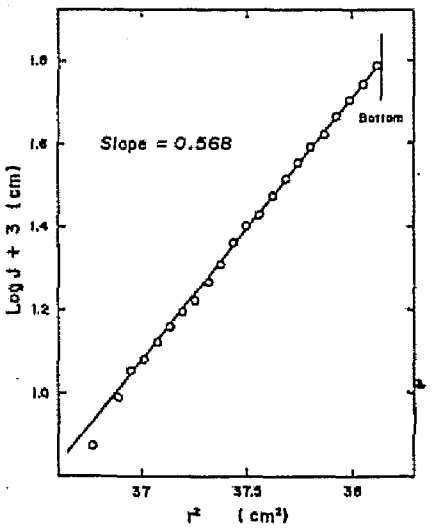

Fig. 2. Sedimentation equilibrium of Azotobacter Fe-SOD. The purified enzyme (0.362 mg/ml) in 0.1 M KCl and 5 mM potassium phosphate, pH 7.8, was equilibrated at a rotor speed of 23,371 rpm at 20°C. Log (fringe displacement, J) is plotted as a function of the square of the distance from the center of rotation (r^2).

one-year preservation in ammonium sulfate-precipitated state, indicating no loss of metal from the active site.

Molecular weight and subunit structure

The molecular weight of the enzyme was determined by the sedimentation equilibrium. A molecular weight of 40,200 was estimated from the slope of the log(fringe displacement) plotted against the square of the distance from the center of rotation (Fig. 2) and a partial specific volume (0.73) measured experimentally (Fig. 7). The molecular weight of the purified enzyme was estimated to be 38,000 by gel filtration with a Sephadex G-100 column (data not shown). The molecular weight of the enzyme was also calculated from hydrodynamic parameters and the values are shown in Table III.

The subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis. The enzyme gave one band both in the presence and in the absence of 2-mercaptoethanol, corresponding to a position of molecular weight of 21,000 (data not shown). Thus, Azotobacter SOD is composed of two subunits of equal size without a disulfide bridge.

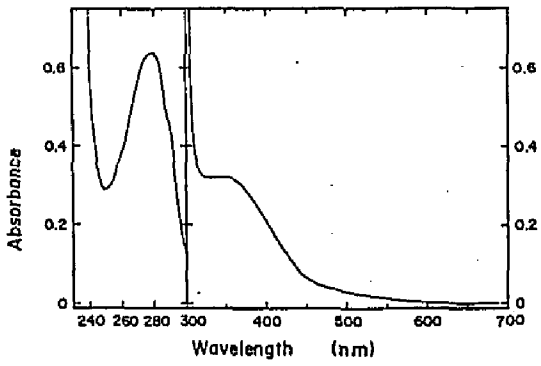

Fig. 3. Absorption spectrum of Azotobacter Fe-SOD. The ultraviolet and visible spectra were recorded at 0.241 and 3.622 mg/ml, respectively, in 10 mM potassium phosphate, pH 7.8, at 25°C. The light path was 10 mm.

Dry weight measurement

The enzyme (1.8 mg) in duplicate were dialyzed against distilled water, and dryed at 105°C until its weight reached minimum value after the measurement of absorbance at 280 nm in 10 mM potassium phosphate, pH 7.8. $A^{1\%}_{1\ cm}$ at 280 nm is calculated to be 26.81.

Absorption spectrum

The ultraviolet spectrum of the enzyme in 10 mM potassium phosphate, pH 7.8, showed an absorption maximum at 280 nm with shoulders at 260 nm and 290 nm (Fig. 3). The absorbance coefficient at 280 nm was calculated to be 107,000 $M^{-1}cm^{-1}$ based on a molecular weight of 40,200 and dry weight. The visible spectrum showed a weak and broad absorption from 320 nm to 600 nm with a shoulder around 350 nm which is characteristic of Fe-SOD. The absorbance coefficient at 350 nm was 3,620 $M^{-1}cm^{-1}$. The ratio of absorbance at 280 nm to 350 nm was 29.60.

Metal analysis

The purified enzyme was dialyzed against 10 mM Tris-HCl, pH 7.8, for 4 days with several changes of the buffer and was digested in 60% perchloric acid, in two runs. The results were corrected for the blank which contained the same volumes of dialysis buffer and perchloric acid. The atoms of iron to mole of enzyme ratio was estimated to be 1.9 ± 0.1 on the basis of a molecular weight of 40,200. Manganese, copper, zinc

and magnesium were below detection or were insignificant. Assay for sulfide after acidification of the enzyme indicated the absence of acid-labile sulfide.

Amino acid analysis

The enzyme was dialyzed against water for 4 days and three samples of the enzyme in acid-washed tubes were hydrolyzed by 1 ml of 6 M HCl,

Table II

Amino Acid Composition of Fe-SOD from Azotobacter vinelandii

Amino acid	g residue per 100 g protein	Calculated number of residues per 40,000 g of protein	Nearest integer	Residue weight
Lysine	6.788	21.18	21	2692
Histidine	3.127	9.12	9	1234
Arginine	1.651	4.23	4	625
Aspartic acid	11.770	40.91	41	4719
Threonine[a)]	5.688	22.50	23	2325
Serine[a)]	3.492	16.04	16	1393
Glutamic acid	9.344	28.95	29	3744
Proline	4.910	20.22	20	1942
Glycine	4.848	33.99	34	1940
Alanine	5.700	32.08	32	2275
Valine[b)]	5.070	20.46	20	1983
Methionine[c)]	0.258	0.79	1	131
Isoleucine[b)]	4.359	15.41	15	1697
Leucine	9.194	32.50	33	3734
Tyrosine	7.500	18.38	18	2937
Phenylalanine	7.365	20.02	20	2944
Half-cystine[c)]	0.411	1.61	2	204
Tryptophan[d)]	7.849	16.86	17	3166
Iron	0.279		2	112
Total	99.603			39,797

a) The values extrapolated to zero hydrolysis time.

b) The values at 72 hours hydrolysis.

c) Determined as methionine sulfone and cysteic acid after performic acid oxidation.

d) Spectrophotometric determination according to Edelhoch (19).

in vacuo, at 110°C for 24, 48, and 72 hours. One sample was treated with performic acid according to Moore (18) before 18 hours-hydrolysis with HCl. After removal of HCl, in vacuo, the hydrolysate was subjected to a Hitachi KLA-5 amino acid analyzer in duplicate. The time-dependent losses and the increse during hydrolysis were corrected by extrapolation to zero hydrolysis time and by employing the values at 72 hours of hydrolysis, respectively. Half-cystine and methionine were estimated as cysteic acid and methionine sulfone, respectively. Tryptophan was determined from ultraviolet absorption in 6 M guanidine-HCl (19).

The amino acid composition of Azotobacter Fe-SOD (Table II) well resembled that of Pseudomonas ovalis (20).

Sulfhydryl groups

The contents of sulfhydryl group was estimated by 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) in the presence or absence of denaturating reagent. The addition of DTNB to 6.58 μM Azotobacter Fe-SOD in 0.1 M sodium phosphate, pH 8.0, did not cause any increase of absorbance at 412 nm. Thus, the native enzyme sulfhydryl group is not exposed and not reactive with the thiol reagent. However, when the enzyme was incubated in 0.9% SDS or 6.5 M guanidine hydrochloride for 20 min, it showed an increase of absorbance corresponding to 1.53 and 1.56 moles of sulfhydryl group per mole of enzyme, respectively (data not shown). These observations indicate tht 2 half-cystine residues of the enzyme (Table II) exist as two sulfhydryl groups which are reactive with thiol reagents only under denaturation conditions.

Isoelectric point

Isoelectric point of the enzyme was estimated by isoelectric focusing with Ampholine (pH 3.5-10.0). The isoelectric focusing was performed in 2% Ampholine on 5% polyacrylamide gel 9 cm-rode at 200 V for 4.5 hours and the location of the enzyme was visualized by protein staining or activity staining. The pH gradient in the gel was monitered by cutting the gel into each 5 mm-segment and sorking them into 1 ml of CO_2-free water for 2 days. The isoelectric point was estimated to be 4.08. The staining both for protein and activity gave a single band having a identical pI (data not shown).

Molecular size and shape of Azotobacter Fe-SOD were estimated from hydrodynamic parameters. For these calculations, sedimentation coefficient, diffusion coefficient, partial specific volume and intrinsic viscosity were measured independently.

Sedimentation coefficient

The sedimentation coefficient, $s^{\circ}_{20,w}$, in water at 20 °C, extrapolated to zero protein concentration was found to be 3.24 S from three

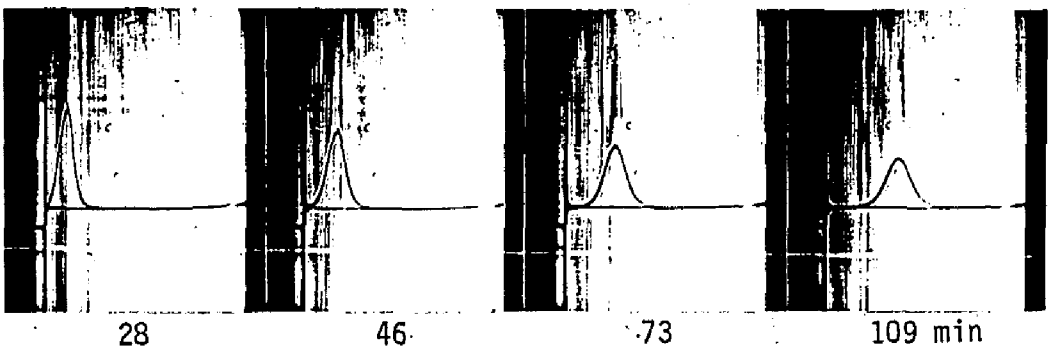

Fig. 4. Sedimentation pattern of Azotobacter Fe-SOD. The purified enzyme was used at a concentration of 9.87 mg/ml in 0.1 M KCl and 5 mM potassium phosphate, pH 7.8. Photographs were taken at 28, 46, 73, 109 min after reaching 60,000 rpm at 20°C with a phase plate angle of 75°. Direction of sedimentation is from left to right.

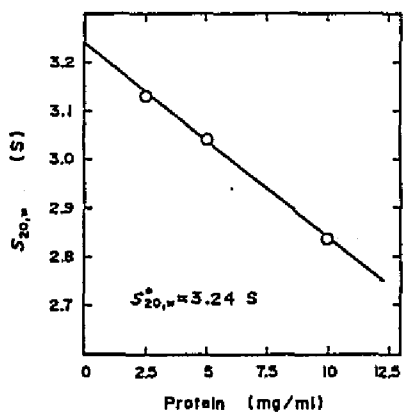

Fig. 5. Concentration dependence of sedimentation coefficient of Azotobacter Fe-SOD. The purified enzyme at a concentration of 2.53, 5.06, and 9.87 mg/ml in 0.1 M KCl and 5 mM potassium phosphate, pH 7.8, was sedimented at a rotor speed of 60,000 rpm at 20°C.

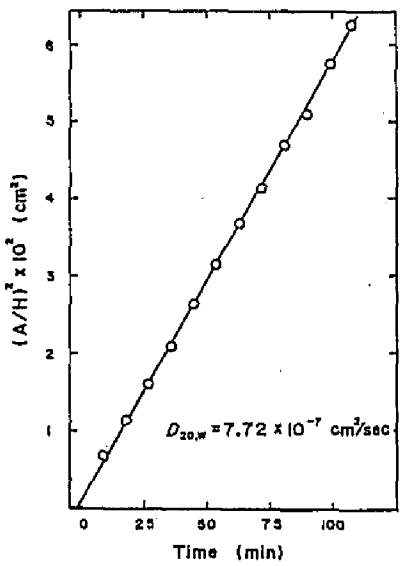

Fig. 6. Determination of diffusion coefficient of Azotobacter Fe-SOD by analytical centrifugation. The purified enzyme was used at a concentration of 9.87 mg/ml in 0.1 M KCl and 5 mM potassium phosphate, pH 7.8. Centrifugation was performed at a rotor speed of 6,520 rpm using a double-sector synthetic boundary cell at 20°C. $(A_{sch})^2/(dc/dr)^2_{max}$ is plotted against time in sec. Where A_{sch} is area under a schlieren peak in cm^2, and $(dc/dr)_{max}$ is the maximum height of the schlieren peak.

ultracentrifugation runs at protein concentrations of 2.53, 5.06, and 9.87 mg/ml in 0.1 M KCl and 5 mM potassium phosphate, pH 7.8 (Fig. 4 and 5).

Diffusion coefficient

The diffusion profile of the enzyme was observed as peak shapes detected with schlieren optics. Diffusion coefficient, $D_{20,w}$ calculated by the peak height-area method was 7.72×10^{-7} cm^2/sec (Fig. 6).

Partial specific volume

The partial specific volume was measured with the protein in a concentration range from 0.5% to 1.8%. The partial specific volume, $\bar{v}$, obtained was 0.733 cm^3/g (Fig. 7). The partial specific volume was also calculated from amino acid composition of the enzyme and the value was 0.731.

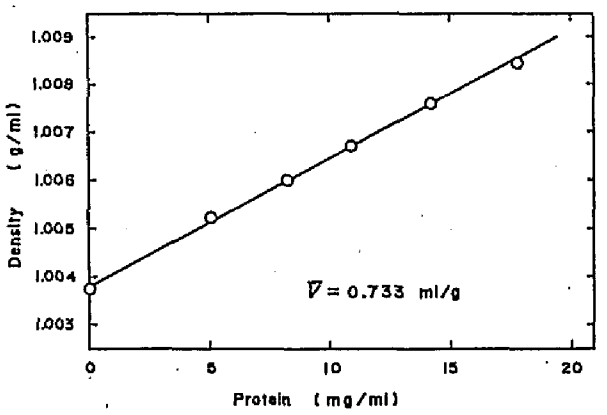

Fig. 7. Determination of partial specific volume of Azotobacter Fe-SOD with a pycnometer. The purified enzyme was used at a concentration of 5.1, 8.2, 10.9, 14.2, and 17.8 mg/ml in 0.1 M KCl and 5 mM potassium phosphate, pH 7.8. Partial specific volume was calculated from the slope and the intercept according to the equation: $\rho = \rho_o + (1 - \bar{v}\rho_o)c$, where, ρ is density in grams per cubic centimeter (solution), ρ_o is density of solvent in g/cm^3 and c is concentration in g/ml of solution.

Intrinsic viscosity

Viscosity was measured over the concentration range from 0.5% to 1.1%. Fig. 8 illustrates the reduced viscosity, η_{sp}/c, of the enzyme plotted as a function of protein concentration. A linear line was obtained, and intrinsic viscosity, $[\eta]$, was estimated to be 3.68×10^{-2} dl/g by extrapolation to zero concentration.

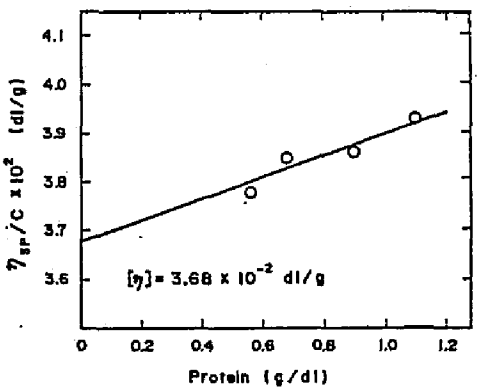

Fig. 8. Concentration dependence of reduced viscosity of Azotobacter Fe-SOD. The viscosity of the purified enzyme in 0.1 M KCl and 5 mM potassium phosphate, pH 7.8, was determined using an Ubbelohde semi-microdilution viscometer. The reduced viscosity, η_{sp}/c, was plotted against various concentrations of the enzyme.

Molecular size and shape

In order to certify the accuracy of s°, D and [η], molecular weight, M, of the enzyme was calculated from these parameters using eq 1, eq 2 and eq 3, and compared with that obtained by sedimentation equilibrium (M = 40,200). The values of 38,100, 38,900 and 37,800 were obtained from s° and D, D and [η], and s° and [η], respectively (Table III). Specific volume of 0.733 was used in these calculations. The values were almost the same as that obtained by sedimentation equilibrium, but still less about 1,000-2,000. To avoid systematical error, a molecular weight of 38,200, averaged among calculated values, was employed in the following calculation.

Frictional coefficient ratio, f/f_{min}, of the enzyme was calculated to be 1.28 from s°, $\bar{v}$ and M by eq 4; 1.22 from D, $\bar{v}$ and M by eq 5; and 1.24 from s° and D by eq 6 (Table III). Averaged f/f_{min} of 1.25 was obtaiend. A value of 1.12 was indicated by Polson's emprical relationship (eq 7).

Table III

Hydrodynamic properties of Azotobacter Fe-SOD

$s^{\circ}_{20,w}$	3.24 S
$D_{20,w}$	7.72×10^{-7} cm^2/sec
[η]	3.68×10^{-2} dl/g
$\bar{v}$ (measured)	0.733 ml/g
$\bar{v}$ (amino acid composition)	0.731 ml/g
f/f_{min} (s,$\bar{v}$ and M)	1.28
f/f_{min} (D,$\bar{v}$ and M)	1.22
f/f_{min} (s and D)	1.24
M (s and D)	38,100
M (D and [η])	38,900
M (s and [η])	37,800
M (gel filtration)	38,000
M (amino acid analysis)	39,800
M (sedimentation equilibrium)	40,200
M (SDS-electrophoresis)	42,000

The molecular shape of Azotobacter Fe-SOD was estimated by the frictional ratio, and by the intrinsic viscosity, respectively, according to the Perrin's and Simha's equations with "an rigid ellipsoid of revolution" model (15). In this model, experimentally determined f/f_{min} of a protein represents not only its deviation from spherical and compact state, but also the degree of solvation; it is a function of both parameters. Thus, without data of hydration of a protein, the contribution of deviation in shape to frictional ratio cannot be estimated accurately. Therefore, two extreme cases for the enzyme are considered here; i.e. zero and maximum solvation. To separate the contribution of solvation and asymmetry of a protein to frictional ratio, the following equation was introduced by Oncley (21):

$$f/f_{min} = \frac{f}{f_o}\left(\frac{\bar{v} + \delta v_o}{\bar{v}}\right)^{1/3} ,$$

where f_{min} is the frictional coefficient of unsolvated spherical hydrodynamic particle, and f_o is that of solvated sphere. δ is the degree of solvation expressed in g of water per gram of protein. v_o is the specific volume of solvent. These symbols are consistent with the definition of Tanford (13) and different from the original ones (21). Thus, f/f_o of more than unity represents a deviation in shape of protein from solvated sphere, and is independent of the degree of solvation. When the shift of f/f_{min} from unity is assumed to be entirely asymmetry, the protein particle can be considered as an ellipsoid of revolution having an observed f/f_{min}. Thus, axial ratio, a/b, of the enzyme (b is the equatorial semiaxis of the ellipsoid, and a is the semiaxis of revolution) was calculated to be 4.9 for prolate ellipsoid and 0.19 for oblate ellipsoid by the diagram of Oncley (21). When a deviation in f/f_{min} is assumed to be ascribed to solvation, the protein particle can be considered as a solvated sphere having an observed diffusion coefficient. In this case, the degree of solvation, δ, was maximum and calculated to be 0.70 g per gram of protein. The radius of sphere, r, was 26 Å from the Stokes' equation ($f = 6\pi\eta r$) and $f = kT/D^\circ$.

Intrinsic viscosity, $[\eta]$, is also a measure of shape of protein, and also depends on two factors; factor ν which is representative of the

shape of hydrodynamic particle, and solvation. $[\eta]$ is related with ν and δ by the following equation:

$$[\eta] = \nu(\bar{v} + \delta v_o) \quad .$$

ν is the Simha factor which is a function of axial ratio and its value for unsolvated spherical particle is 2.5. If the enzyme is assumed to be not solvated ($\delta = 0$), Simha factor was calculated to be 5.0, corresponding to the axial ratio of 4.3 for prolate ellipsoid and 0.18 for oblate ellipsoid from the diagram of Oncley (21). In the case of maximum solvation ($\nu = 2.5$), the protein molecule may be considered to be a sphere having observed $[\eta]$. The degree of solvation was determined to be 0.74 and the radius of the sphere was 29 Å from the equaiton, $[\eta] = \nu(N/M)(4/3)\pi r^3$.

The shape of the enzyme was also analyzed by using β-function of Scheraga and Mandelkern with "an effective hydrodynamic ellipsoid" model (22). β-function allows the determination of axial ratio without knowledge of solvation, and is calculated by the following equations:

$$\beta \equiv \frac{Ns[\eta]^{1/3}\eta_o}{M^{2/3}(1 - \bar{v}\rho)} = \frac{D[\eta]^{1/3}M^{1/3}\eta_o}{kT} \qquad \nu = (\beta/\gamma F)^3 \quad ,$$

where $F = f_{min}/f$, $\gamma = N^{1/3}/(16200\pi^2)^{1/3}$, and η_o is solvent viscosity. The β-values of the enzyme were calculated to be 2.15 from D and $[\eta]$ and to be 2.16 from s° and $[\eta]$, respectively. These values correspond to the axial ratio of 3 for prolate of ellipsoid, and may exclude the possibility for oblate of ellipsoid, since β of 2.14 is the upper-limit for oblate of ellipsoid with any axial ratio. Simha factor was calculated to be 5.2 from β-value and the observed f/f_{min}, indicating axial ratio of 4.5 for prolate of ellipsoid on the assumption of zero solvation ($\delta = 0$). The difference of the axial ratio between that obtained from β-value only and that calculated from Simha factor may correspond to the contribution of solvaiton to ν, and 35% solvation of the enzyme was estimated from the diagram of Oncley (21).

The recalculation of axial ratio from f/f_{min} and $[\eta]$ on the assumption of 35% solvation of the enzyme gave the axial ratio of 3 from both parameters. The agreement of axial ratio with that calculated from

β-function indicates the proper estimation for solvation of the enzyme.

The effective hydrodynamic volume, V_e, was calculated from the Simha factor using the equation, $V_e = 100[\eta]M/(\nu N)$. The calculation gave the values of 4.72×10^{-20} ml. Thus, the molecualr dimension and shape of the enzyme were estimated from the axial ratio and the effective hydrodynamic volume. The calculated values for the dimension of the molecule are 30 Å x 30 Å X 90 Å as a prolate ellipsoid of revolution indicating that the enzyme is a compact globular protein.

Electronic State of Iron in Active Site of Azotobacter Fe-SOD

Electron paramagnetic resonance spectroscopy

The EPR spectrum of the native Fe-SOD indicates that the iron in the enzyme is a ferric high-spin state, $(t_{2g})^3(e_g)^2$, (S = 5/2) in a nearly rhombic symmetry characterized by E/D = 0.25 (Fig. 9). The ground state ($^6A_1$) splits into three Kramers doublets in the ligand field of low symmetry described by the spin Hamiltonian with S=5/2:

$$H = D[S_z^2 - S(S+1)/3] + E(S_x^2 - S_y^2) \quad ,$$

where E and D are the zero-field splitting parameters. Eigenfunctions are written in the following notation:

$$\Psi_{\pm,i} = a_i|S_z=\pm 5/2\rangle + b_i|S_z=\pm 1/2\rangle + c_i|S_z=\mp 3/2\rangle \quad ,$$

here the subscripts i=1, 2, 3 label the three doublets. The parameter $\lambda = E/D$ of the spin Hamiltonian was calculated using the eigenfunction coefficients (a_i, b_i, and c_i) reported by Wickman et at. (23). The spectra were well fitted with $\lambda = 0.25$. The observed and calculated g-values with E/D = 0.25 for the transitions from the three doublets were listed in Table IV. In the spectrum at 77 K, the signals at 4.03, 3.72, 4.77 were ascribed to g_{x2}, g_{y2} and g_{z2} of the middle level of the Kramers doublet. These values were fairly agreed with the calculated values, 4.08, 3.76 and 4.76 for g_{x2}, g_{y2} and g_{z2}, respectively. Almost

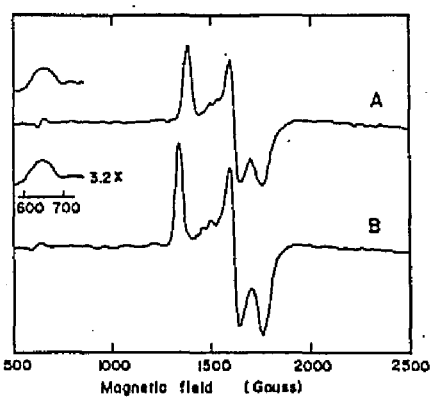

Fig. 9. Electron paramagnetic resonance spectra of Azotobacter Fe-SOD. A, 0.80 mM Native enzyme in 0.1 M Tris-HCl, pH 7.8; B, 0.87 mM reconstituted ^{57}Fe-enzyme in the same buffer. The spectra were recorded at 4.2K with the following instrumental setting: microwave power, 0.5 mW; microwave frequency, 9.274 GHz; modulation amplitude, 1 G; receiver gain, 10^4; field modulation, 100 kHz; time constant, 0.3 sec; and sweep rate, 500 G/min. The signals at g = 9.8 were similarly obtained except that the modulation amplitude and time constant were 3.2 G, and 1 sec, respectively.

same spectrum was obtained at 4.2K, showing the resonance at 4.09, 3.78, 4.80. These values were assigned to be g_{x2}, g_{y2} and g_{z2}, respectively, for the middle Kramers doublet with E/D = 0.25. In addition to these signals, the signal at 9.82 which corresponds to g_{y1} of the lowest level of the doublet was observed. None of the transitions in the lowest and

Table IV

Apparent g-Values of Azotobacter Fe-SOD

SOD[a]	Temp.(K)	Three levels of Kramers doublet								
		ground			1st excited			2nd excited		
		g_x	g_y	g_z	g_x	g_y	g_z	g_x	g_y	g_z
Native Fe-SOD	77	-[b]	-	-	4.03	3.72	4.77	-	-	-
	4.2	-	9.82	-	4.09	3.78	4.80	-	-	-
^{57}Fe-SOD	77	-	-	-	3.97	3.63	4.80	-	-	-
	4.2	-	9.85	-	3.76	4.10	4.94	-	-	-
Calc.[c]		0.47	9.84	0.04	4.08	3.76	4.76	1.45	0.92	9.34

a) Spectra were obtained in 0.1 M Tris-HCl, pH 7.8.

b) Not observed under the present conditions.

c) Calculated using λ (= E/D) of 0.25 with spin Hamiltonian: $H = S_z^2 - S(S+1)/3 + \lambda(S_x^2 - S_y^2)$.

in the highest Kramers doublet except g_{y1} was observed under the present conditions where the temperature were 77 or 4.2K and the microwave power was 0.5 mW.

The substituted ^{57}Fe-SOD revealed the identical EPR spectrum with that of native Fe-SOD indicating no alteration by substituting the metals.

Mössbauer spectroscopy

The Mössbauer spectra of the oxdized and reduced Fe-SOD were measured at different low temperatures, and the Mössbauer parameters are summarized in Table V. The spectrum of the reduced enzyme exhibited a pair of absorption lines, with a large isomer shift of 1.10 mm/sec and a large quadrupole splitting of 2.98 mm/sec at 77K (Fig. 10). The spectrum was similar to those of hemoglobin and reduced Japanese-radish peroxidase (24), indicating that the electronic state of the iron ion in

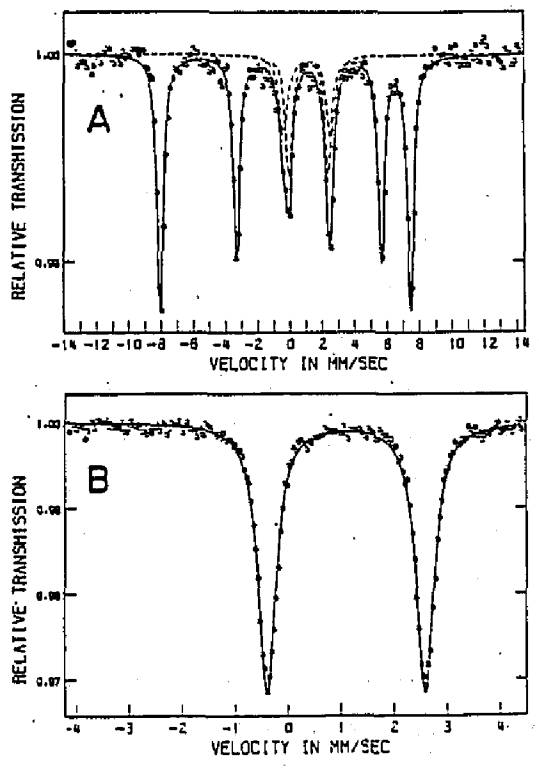

Fig. 10. Mössbauer spectra of Azotobacter Fe-SOD. A, Oxidized ^{57}Fe-enzyme (2.62 mM) in Tris-HCl, pH 7.8, at 4.2K; B, dithionite-reduced enzyme (2.62 mM) in the same buffer at 77K. Notice the difference in scale of velocity.

Table V

Mössbauer Parameters of Azotobacter Fe-SOD

Redox state	Temp.	Isomer shift	Quadrupole splitting	Internal magnetic field	Intensity
	(K)	(mm/s)	(mm/s)	(kOe)	
Native Fe-SOD	4.2	0.45	1.46	481	88%
		1.09	2.96	-	12%
	77	Broadening spectrum			
		1.13	2.83	-	
	195	Broadening spectrum			
		1.05	2.63	-	
Reduced Fe-SOD	77	1.10	2.98	-	
	195	1.06	2.70	-	

the reduced enzyme is a ferrous high-spin state, $(t_{2g})^4(e_g)^2$, (S = 2). Temperature dependence of the quadrupole splitting and the independence of isomer shift (Table V) were consistent with its ferrous high-spin state.

The Mössbauer spectrum of the oxidized enzyme was measured at three different temperatures. The spectrum at 4.2K exhibited six well separated spitting lines due to magnetic interaction (Fig. 10). As the temperature was raised, the absorption lines collapsed into two or three overlapped broad lines, indicating the overlapping of a different spectrum to that of the oxidized enzyme. The Mössbauer parameters, isozmer shift and quadrupole splitting, of the superimposed spectrum were almost identical to that of the fully reduced enzyme by dithionite (Table V). Thus, it appears that 12% of the reduced enzyme is present in the oxidized enzyme. The parameters of the oxidized enzyme at 4.2K indicate that the enzyme is a ferric high-spin state, $(t_{2g})^3(e_g)^2$, (S = 5/2) which is consistent with the EPR measurement. The broadening of the spectra at 77 and 175K is attributable to strong magnetic hyperfine interaction with the long spin-lattice relaxation time. It is well established that the ferric high-spin state has a net magnetic moment and zero orbital angular momentum, and therefore, the electronic spin system is well insulated from the lattice, and the spin-lattice relaxa-

tion through the spin-orbit interaction is slow. The internal magnetic field of the oxidized enzyme was calculated to be 481 KOe. The externally apllied magnetic field of 1 T did not affect the spectra of the oxdized enzyme at 4.2, 77, and 175K.

4. DISCUSSION

The present results clearly show that Azotobacter vinelandii contains Fe-SOD but lacks Mn-SOD. The occurrence of Mn-SOD has been reported in Azotobacter chroococcum (25). This conclusion was deduced from the pinkish color of the final preparation, although the absorption spectrum presented in ref 25 did not show the absorption maximum around 480 nm which is characteristic of the Mn-enzyme. The electrophoresis of crude extracts from A. chroococcum and A. indica followed by the activity staining showed a single band which was inactivated by hydrogen peroxide treatment (data not shown). Hydrogen peroxide inactivates the Fe-enzyme but not Mn-enzyme (26). The above results indicate that superoxide dismutase in genus Azotobacter including A. chroococcum is the Fe-enzyme.

The physicochemical properties of Azotobacter vinelandii Fe-SOD well resembled those of the other Fe-SOD so far purified (1). The metal analysis revealed 1.9 g-atoms iron per mole of enzyme, indicating one Fe atom in a subunit. The recent voltametric analysis of the present preparation by Sawyer et al. (27) confirmed this stoichiometry of metal to subunit. The statistical analysis (SΔQ) based on the amino acid composition of Azotobacter Fe-SOD suggested that Azotobacter Fe-enzyme is most similar to Pseudomonase Fe-enzyme (7) among Fe-SOD in amino acid sequence. This might be rationalized in terms of the evolutional relationship, since both organisms are classified into closer groups (28).

The experimentally obtained sedimentation coefficient, s°, diffusion coefficient, D , intrinsic viscosity, $[\eta]$, and specific volume, $\bar{v}$, of Azotobacter Fe-SOD are summarized in Table III. The measured specific volume well coincided with that calculated from the amino acid

composition. Using these hydrodynamic parameters, molecular weight of Azotobacter Fe-SOD was calculated according to the equations described in MATERIALS AND METHODS. The calculation gave molecular weights of 38,000-39,000, which were fairly agreeable to those obtained experimentally by gel-filtration, SDS-electrophoresis, sedimentation equilibrium, and amino acid analysis. This indicates the validity of those hydrodynamic parameters for the estimation of molecular dimension and shape of Azotobacter Fe-SOD. The dimension of the enzyme was estimated to be 30 Å x 30 Å x 90 Å as a prolate ellipsoid of revolution. Thus, Azotobacter Fe-SOD is a compact, globular protein.

The implication that the Fe-SOD is a compact protein should be commented here. The disproportionation of suproxide catalyzed by SOD is a nearly diffusion-limited reaction (29-33). Thus, the catalytic efficiency of the enzyme is directly correlated with the accesibility of the substrate, i.e. the superoxide anion radical. The more accesible the superoxide, the higher the enzymatic efficiency. Therefore, the compactness of the protein might be one of the strategies to deal with the very short-lived radical substrate. In conjunction with this, Koppenol suggested the significance of the small dipole moment of Cu,Zn-SOD (34). The small or zero dipole moment facilitates the enzyme to catalyze the reaction with superoxide which comes from every directions because the enzyme is not allied to a possible electric field. Thus, this suggests the importance of the rotational diffusion of the enzyme during catalysis.

In addition to this, the translational diffusion of the enzyme also involves in the enzymatic efficiency. In this sense, one might have a doubt about the enzymatic function of SOD as a catalyst for superoxide disproportionation, because even aquo Cu^{2+} possesses the catalytic activity which is comparable to Cu,Zn-SOD with an apparent second-order rate constant of 8.1×10^9 $M^{-1}s^{-1}$ (35), and because smaller molecule has advantage over macromolecule in respect of diffusion-limited reaction. However, while the apparent rate constant of aquo Cu^{2+} for superoxide disproportionation is dependent on proton availability, SOD is independent of pH, at least up to 8 (31-33). This property is characteristic of SOD and may bring about the physiological significance as the

catalyst inside cells. Therefore, it might be concluded that during the evolution SOD had acquired the efficient proton donating mechanism, and amino acid residues were folded so that higher structure would be more suitable to the superoxide disproportionation. The compactness of SOD may render the merit in the enzymatic reaction.

It has been believed that there was no gross difference in the molecular weight of both dimeric Fe- and Mn-SOD. The equilibrium centrifugation for molecular weight determination has so far indicated the molecular weight of about 40,000 irrespective of the type of enzyme. However, the complete sequencing for Escherichia coli (3) and Bacillus stearothermophillus (4) Mn-SOD have recently revealed higher molecular weight of 45,980 and 45,487, respectively. This discrepancy can be attributed to, in part, improper assumption of the partial specific volume in the previous determinations (37). The equilibrium centrifugation method requires an accurate partial specific volume of the protein. Using the experimentally determined partial specific volume for the first time, the molecular weight of Azotobacter Fe-SOD was estimated to be 40,200, verifying the molecular weight estimation for this type of enzyme. Thus, the difference in molecular weight of subunits of the Azotobacter Fe- and E. coli and B. stearothermophillus Mn-SOD is 2,000-5,000, which corresponds 10-20 amino acid residues. Although the assignment of the positions where extra residues are distributed awaits the elucidation of complete amino acid sequence, it can be assumed that most insertions took place in the region of position 30-40 to COOH-terminal, since partial sequences of the NH_2-terminal region (up to 30-40) of Fe- and Mn-SOD have a high homology (1).

The structure of the metal binding sites of Cu,Zn-SOD has been elucidated to be approximately square planer for copper and approximately tetrahedral arrangement for zinc. However, less information about Fe-SOD is available; even iron ligands have not been completly assigned. The EPR spectrum of oxidized Azotobacter Fe-SOD showed that the iron occurs as ferric high-spin in distorted ligand field characterized by the the zero field splitting parameter of E/D = 0.25. Similar E/D have been obtained from Spirulina platensis (38), Plectonema boryanum (26) and Photobacterium leiognathi (39) Fe-SOD, indicating that

the rhombically distorted ligand configuration is conserved in all Fe-SOD.

The Mössbauer spctrum of the oxidized Azotobacter Fe-SOD was well understood in terms of theoretical calculation. When the zero-field splitting of the spin Hamiltonian is large enough ($D \tilde{=} 1.5$ cm^{-1} in Fe-SOD), the Mössbauer spectrum of $^6A_1$ state can be calculated by the following Hamiltonian with effective spin of 1/2 operating upon each Kramers doublets:

$$H = K_i + \beta_e H \cdot g_i \cdot S + I \cdot A_i \cdot S + I \cdot P \cdot I - g_n \beta_n H \cdot I \quad ,$$

where

$$I \cdot P \cdot I = (QV_{zz}/4)[I_z^2 - (5/4) + \eta(I_x - I_y)/3] \quad ,$$

$$\eta = (V_{xx} - V_{yy})/V_{zz} \quad ,$$

β_e and β_n are the Bohr and nuclear magnetons, g_i and g_n are the electronic and nuclear g-factors, and the remaining symbols have their conventional meaning; the g-tensor is known from the EPR data. Here each term, from left in right-hand of the equation, indicates the ligand-field splitting, the electron Zeeman interaction, the electron nuclear hyperfine interaction, the nuclear quadrupole splitting, and the nuclear Zeeman interaction, respectively. In $^6A_1$ state, A_i is proportional to g_i, considering only Fermi contact term, and assymmetrical parameter, η, of quardrupole splitting is given by 3λ. Thus, the Mössbaure spectrum of the oxidized enzyme is compatible with that of EPR of the oxidized enzyme.

The disproportionation reaction of superoxide catalyzed by Fe-SOD has been considered to proceed via outer sphere mechanism, while the reaction of Cu,Zn-SOD can be explanined in terms of inner-sphere mechanism (40). During redox shuttle of the Cu,Zn-enzyme, coordination bond of the histidine ligand is broken (the common ligand with Zn), which results in more axial configuration of the ligands around copper (1 and also ref therein). It is not likely, however, that the Fe-enzyme involves large rearrangement of the iron ligands upon oxidation and reduction by superoxide. Mössbaur spectroscopy of Fe (II) gives

complimental information to EPR spectroscopy, since Fe (II) is EPR-silent. The Mössbaur parameters, isomer shift and quadrupole splitting, of the reduced Azotobacter Fe-SOD indicates that iron occurs in a ferrous high-spin state, and furthermore, a large quadrupole splitting suggsts an anisotropy of the iron-ligand structure. This indicates that the ligand configuration of the reduced enzyme is not so different from that of the oxidized one.

Azotobacter is an aerobic diazotroph and several protection mechanisms of dinitrogen fixing system against oxidizing stress have been proporsed (41). Vigorous respiratory activity which produces ATP for dinitrogen fixation plays a role in scavenging molecular oxygen inside the cell. It seems that the sharp concentration gradient of O_2 is formed from periplasmic region toward interia of the cell, and thereby autooxidation of compounds for the dinitrogen fixing system is suppressed. The efficient scavenging of O_2 is ascribed to the low k_m of cytochrome c oxidase. However, the respiration may inevitably produce active oxygen, i.e. superoxide. Thus, SOD may also participate in the defense system against oxygen toxicity by converting superoxide to hydrogen peroxide and molecular oxygen. One of the reaction products, oxygen, will be again removed by cytochrome c oxidase. Although the actual concentration of SOD in Azotobacter cell is not known, a rough calculation indicates a concentration of about 0.1 mM. SOD at this concentration does not permit superoxide to diffuse exceeding 0.08 μm toward interia cell (see APPENDIX), since the production site of superoxide should be restricted to peripheral region of the cell. This indicates that the concentration of superoxide in the central region of the cell is very low or zero taking into account the cell size of 1 x 4 μm. In addition, hydrogen peroxide-scavenging enzymes such as catalase and peroxidase may also be involved in the defense system, because toxic hydrogen peroxide is one of the products of superoxide disproportionation. The scavenging of hydrogen peroxide by catalase again generates O_2, which may be removed by cytochrome c oxidase. Thus, cytochrome c oxidase, SOD, catalase, and peroxidase are cooperatively scavenging molecular oxygen and active oxygen. This situation is illustrated in Fig. 11. It should be emphasized that the concentration gradients of

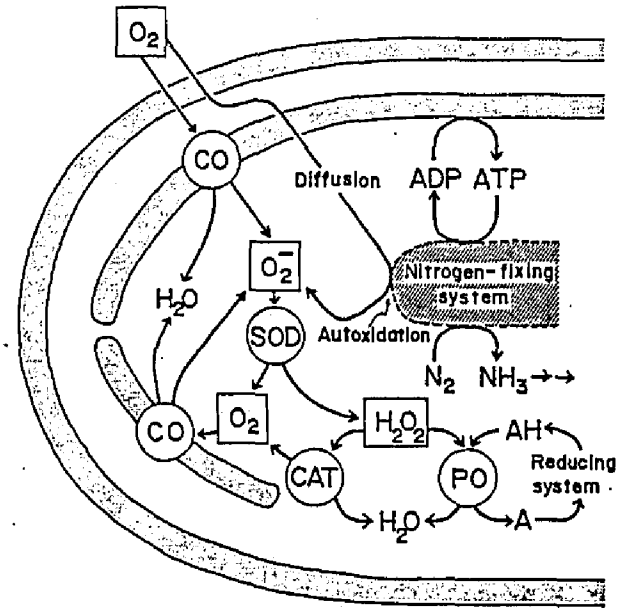

Fig. 11. Cooperative scavenging of molecular and active oxygen by SOD, catalase, peroxidase and cytocrome c oxidase in Azotobacter cell. SOD, Fe-superoxide dismutase; CAT, catalase; PO, peroxidase; CO, cytochrome c oxidase; AH, electron donor.

molecular oxygen and the active oxygen such as superoxide and hydrogen peroxide are formed from peripheral region to interia of the cell due to the cooperative scavenging by above enzymes, and the central region of the cell is anaerobic. This may enable dinitrogen fixation, which is potentially O_2-labile, with concomitant supply of ATP by vigorous respiration.

Note: After being done this experiment, X-ray diffraction studies on Fe-SOD from E. coli (42) and P. ovalis (43) have been reported. The dimension and shape of Azotobacter Fe-SOD estimated by hydrodynamic method were compatible with those of E. coli and Pseudomonas Fe-SOD.

5. SUMMARY

Azotobacter contained a large amount of Fe-superoxide dismutase comprising about 2% of total solbule proteins. Gram-scale purification of this enzyme was carried out, and examined its physicochemical properties.

The enzyme is a dimer having a molecular weight of 40,200 made of two identical subunits, and contained 1.9 atoms of iron per molecule. The enzyme is stable and no loss of the metal was observed. Its isoelectric point was 4.1, and amino acid composition was described. Sedimentation coefficient, $s^{\circ}_{20,w}$, diffusion coefficient, $D_{20,w}$, partial specific volume, $\bar{v}$, and intrinsic viscosity, $[\eta]$, were determined to be 3.24 S, 7.72×10^{-7} cm^2/sec, 0.733 cm^3/g and 3.68×10^{-2} dl/g, respectively. The molecular dimension of the enzyme was estimated by the hydrodynamic parameters to be 30 Å x 30 Å x 90 Å as a prolate ellipsoid of revolution with an axial ratio, a/b, of 3.

EPR spectrum of the oxidized enzyme revealed that the iron occurs in ferric high-spin state (S = 5/2) with an E/D of 0.25. Mössbaure spectra were obtained with reconstituted ^{57}Fe-enzyme. The spectrum of the reduced enzyme at 77K showed isomer shift, δ, of 1.10 mm/sec and quadrupole splitting, ΔE_Q, of 2.98 mm/sec, indicating that the iron is a ferrous high-spin state (S = 2). The spectrum of the oxidized enzyme at 4.2K showed magnetically splitted six absorption lines. Isomer shift, quadrupole splitting, and internal magnetic field were 0.45 mm/sec, 1.46 mm/sec and 481 kOe, respctively. These parameters, in accordance with the EPR analysis, indicates Fe being ferric high-spin state in near rhombic symmetry of ligand field.

6. REFERENCES

(1) Steinman, H.M. (1982) in Superoxide Dismutase (Oberley, L.W., ed.), pp. 11-68, CRC Press, Florida.

(2) Gregory, E.M. (1985) Arch. Biochem. Biophys. 238, 83-89.

(3) Steinman, H.M. (1976) J. Biol. Chem. 253, 8708-8720.

(4) Brock, C.J., and Walker, J.E. (1980) Biochemistry 19, 2873-2882.

(5) Kusunose, E., Ichihara, K., Noda, Y., and Kusunose, M. (1976) J.Biochem. 80, 1343-1352.

(6) Bridgen, J., Harris, J.I., and Kolb, E. (1976) J. Mol. Biol. 105, 333-335.

(7) Yamakura, F., Suzuki, K., and Mitsui, Y. (1976) J. Biol. Chem. 251, 4792-4793.

(8) Kimura, M. (1968) Nature (London) 217, 624-626.

(9) Burns, R.C., and Hardy, R.W.F. (1972) Methods Enzymol. 24, 480-496.

(10) Yphantis, D.A. (1964) Biochemistry 3, 297-317.

(11) Chervenka, C.H. (1969) A Manual of Methods for the Analytical Ultracentrifuge, pp. 83-86, Beckman Instruments, California.

(12) Cohn, E.J., and Edsall, J.T. (1941) Proteins, Amino Acids and Peptides, p. 357, Reinhold, New York.

(13) Tanford, C. (1961) Physical Chemistry of Macromolecules, pp. 371-456, John Wiley and Sons, New York.

(14) Andrews, P. (1964) Biochem. J. 91, 222-233.

(15) Edsall, J.T. (1953) in The Proteins (Neurath, H., and Bailey, K., eds.), pp. 549-726, Academic Press, New York.

(16) Polson, A. (1967) Experientia 23, 815-816.

(17) Maeda, Y. (1968) J. Phys. Soc. Japan 24, 151-159.

(18) Moore, S. (1963) J. Biol. Chem. 238, 235-237.

(19) Edelhoch, H. (1967) Biochemistry 6, 1948-1954.

(20) Yamakura, F. (1976) Biochim. Biophys. Acta 422, 280-294.

(21) Oncley, J.L. (1941) Ann. N.Y. Acad. Sci. 41, 121-150.

(22) Scheraga, H.A., and Manderkern, L. (1953) J. Amer. Chem. Sci. 75, 179-184.

(23) Wickman, H.H., Klein, M.P., and Shirley, D.A. (1965) J. Chem. Phys. 42, 2113-2117.

(24) Maeda, Y., Higahimura, T., and Morita, Y. (1969) Annu. Repts. Res. Reactor Inst. Kyoto Univ. 2, 67-72.

(25) Buchanan, A.G., and Lees, H. (1980) Can. J. Microbiol. 26, 441-447.

(26) Asada, K., Yoshikawa, K., Takahashi, M., Maeda, T., and Enmanji, K. (1975) J.Biol.Chem. 250, 2801-2807.

(27) Barrette, W.C., Sawyer, D.T., Fee, J.A., and Asada, K (1983) Biochemistry 22, 624-627.

(28) Becking, J.H. (1974) in Bergey's Mamual of Determinative Bacteriology, 8th ed. (Buchanan, R.E., and Gibbons, N.E., eds.), pp. 253-256, Williams and Wilkins, Baltimore.

(29) Klug, D., Rabani, J., and Fridovich, I. (1972) J. Biol. Chem. 247, 4839-4842.

(30) Rotilio, G., Bray, R.C., and Fielden, E.M. (1972) Biochim. Biophys. Acta 268, 605-609.

(31) Klug, D., Fridovich, I., and Rabani, J. (1973) J. Amer. Chem. Soc. 95, 2786-2790.

(32) Pick, M., Rabani, J., Yost, F., and Fridovich, I. (1974) J. Amer. Chem. Soc. 96, 7329-7333.

(33) Lavelle, F., McAdam, M.E., Fielden, E.M., Roberts, P.B., Puget, K., and Michelson, A.M. (1977) Biochem. J. 161, 3-12.

(34) Koppenol, W.H. (1981) in Oxygen and Oxy-Radicals in Chemistry and Biology (Rogers, M.A.J., and Powers, E.L., eds.), pp. 671-674, Academic Press, New York.

(35) Rabani, J., Klug-Roth, D., and Lilie, J. (1973) J. Phys. Chem. 77, 1169-1175.

(36) Keele, B.B., Jr., McCord, J.M., and Fridovich, I. (1970) J. Biol. Chem. 245, 6176-6181.

(37) Brock, C.J., Harris, J.I., and Sato, S. (1976) J. Mol. Biol. 107, 175-178.

(38) Lumsden, J., and Hall, D.O. (1974) Biochem. Biophys. Res. Commun. 58, 35-41.

(39) Puget, K., and Michelson, A.M. (1974) Biochimie 56, 1255-1267.

(40) Fee, J.A. (1977) in Superoxide and Superoxide dismutases (Michelson, A.M., McCord, J.M., and Fridovich, I., eds.), pp. 173-192,

Academic Press, London.
(41) Burns, R.C., and Hardy, R.W.F. (1975) Nitrogen Fixation in Bacteria and Higher Plants, pp. 102-104, Springer-Verlag, Berlin.
(42) Stallings, W.C., Powers, T.B., Pattridge, K.A., Fee, J.A., and Ludwig, M.L. (1983) Proc. Natl. Acad. Sci. USA 80, 3884-3888.
(43) Ringe, D., Petsko, G.A., Yamakura, F., Suzuki, K., and Ohmori, D. (1983) Proc. Natl. Acad. Sci. USA 80, 3879-3883.

APPENDIX

Calculation for the movable distance of superoxide anion radical

In order to estimate the superoxide concentration inside cells, a model calculation was conducted. As a zero-order approximation, the following assumptions were made: (a) By respiration, some fraction of oxygen is converted to superoxide (flux of O_2^-) and the site of this production is restricted to membrane of cell surface (a model for single cell prokaryotes). Thus, simple one-dimensional diffusion-reaction equation can be employed. (b) The diminution of the superoxide radical in a cell is considered only by spontaneous and SOD-catalyzed disproportionation. Thus,

$$\frac{\partial C(x,t)}{\partial t} = D\frac{\partial^2 C(x,t)}{\partial x^2} - 2k_{sp}C^2(x,t) - k_{en}C(x,t)[SOD] \qquad (A1)$$

was obtained. Where, C(x,t) is a concentration of the superoxide radical which is a function of position (x) and time (t). The position is taken so as to make 0 at membrane and positive toward interia of a cell. D is diffusion coefficient of O_2^- (1×10^{-10} m^2/sec, at 25°C, when $\eta'/\eta = 25$). The k_{sp} and k_{en} are second-order rate constants for spontaneous and SOD-catalyzed dismutation, respectively. The values of $k_{sp} = 6.3 \times 10^4$ and $k_{en} = 2.0 \times 10^9$ $M^{-1}sec^{-1}$ (pH 7.8) were used (31). In equilibrium, $\partial C(x,t)/\partial t = 0$. Thus, A1 is rewritten as

$$D\frac{d^2C(x)}{dx^2} = 2k_{sp}C^2(x) + k_{en}C(x)[SOD] \quad , \qquad (A2)$$

where, C(x) is a function of only x. Since $k_{sp} << k_{en}$, the first term in right hand of A2 can be neglected. Thus,

$$\frac{d^2C(x)}{dx^2} - \frac{k_{en}[SOD]}{D} C(x) = 0 \quad . \tag{A3}$$

A3 is a second-order linear homogeneous differential equation and can be solved analytically under the following boundary conditions: x = 0; C(0) = C_i and x = L; dC(x)/dx = 0, where C_i is the concentration of superoxide at x = 0 and L is the distance at dC(x)/dx = 0. Thus, the solution of A3 is

$$C(x) = \frac{C_i \cosh[m(1 - x/L)]}{\cosh m} \quad , \quad \text{where } m = \sqrt{k_{en}[SOD]L^2/D} \quad . \tag{A4}$$

On the assumption that the flux of O_2^- is constant, the movable distance of O_2^- is defined as position (x) at $C_i/2$. Thus, the diffusion distance of O_2^- was calculated to be 0.08 μm at [SOD] = 0.1 mM, and 0.25 μm at [SOD] = 10 μM.

CHAPTER XVI

REACTIONS OF Mn- AND Fe-SUPEROXIDE DISMUTASES WITH HYDROGEN PEROXIDE

1. INTRODUCTION

Superoxide dismutase (SOD) catalyzes the disproportionation of the superoxide anion radicals according to the following equation (1);

$$O_2^{\cdot-} + O_2^{\cdot-} + 2\,H^+ \xrightarrow{SOD} O_2 + H_2O_2 \qquad [1]$$

The reaction mechanism of Cu,Zn-, Mn- and Fe-SOD has been interpreted in terms of the oxidation-reduction cycle (2) as

$$E\text{-}M^{n+} + O_2^{\cdot-} \longrightarrow E\text{-}M^{(n-1)+} + O_2 \qquad [2]$$

$$E\text{-}M^{(n-1)+} + O_2^{\cdot-} + 2\,H^+ \longrightarrow E\text{-}M^{n+} + H_2O_2 \qquad [3]$$

where $E\text{-}M^{n+}$ is oxidized Cu,Zn-, Mn- or Fe-enzyme and $E\text{-}M^{(n-1)+}$ is the respective reduced counterpart. The Mn-enzyme has been shown to undergo the following reactions in addition to the above cyclic reaction when $O_2^{\cdot-}$ concentration is high (3);

$$E\text{-}Mn^{2+} + O_2^{\cdot-} \xrightarrow{first} E\text{-}Mn\text{-}O_2$$

$$E\text{-}Mn\text{-}O_2 \xrightarrow{slow} E\text{-}Mn^{3+} + H_2O_2$$

where $E\text{-}Mn\text{-}O_2$ is an intermediate complex, but its structure is not determined.

The second-order rate constants of the reactions 2 and 3 are almost the same, being $2 \times 10^9\ M^{-1}sec^{-1}$ at pH 7.8 (2) and indicates that the reactions 2 and 3 are diffusion-controlled ones. Since the apparent rate constant of eq 1 is $2 \times 10^9\ M^{-1}sec^{-1}$ (4), the slower reaction of

either eq 2 or 3 determines the overall reaction. Although the reverse reaction of eq 1 is unfavorable in such a diffusion-controlled reaction, the reverse reaction of eq 3 has been demonstrated by the reduction of tetranitromethane by O_2^- in the presence of H_2O_2 (5).

Hydrogen peroxide which is one of the reaction products of eq 1 inactivates Cu,Zn- (6) and Fe-SOD (7), but not Mn-SOD (7). Several inactivation mechanisms for the Cu,Zn-enzyme by hydrogen peroxide have been proposed: involvement of bound-oxidant or free oxidant which is formed by the interaction of the enzyme with hydrogen peroxide (7,8). For the Fe-enzyme, however, no attempt has been done to elucidate the inactivation process. The reason why the Mn-enzyme is stable to hydrogen peroxide is obscure.

Under these circumstances, I studied the reaction of Fe- and Mn-SOD with hydrogen peroxide to clarify the inactivation mechanism of the Fe-enzyme and the reason for the stability of the Mn-enzyme against hydrogen peroxide.

2. MATERIALS AND METHODS

Cytochrome c (type III) and catalase, and xanthine oxidase were obtained from Sigma and Boehringer, respectively. Hydrogen peroxide was a product of Mitsubishi Gas Chemical Co., LTD. A molar absorbance coefficient of 43.6 $M^{-1}cm^{-1}$ at 240 nm was used for hydrogen peroxide.

Porphyra yezoensis Mn-SOD and Azotobacter venelandii Fe-SOD were obtained as previously described (Chapter XV and XIV). Porphyra and Azotobacter enzymes contained 1.4 atoms Mn and 1.9 atoms Fe per molecule, respectively. The concentration of the enzyme was expressed throughout in terms of metal concentration.

The assay of SOD and the definition of the enzymatic activity were the same as the previous chapters. Absorption spectra were recorded using a Shimadzu UV-200 or Union Giken SM401 spectrophotometers. Oxygen was determined with a Clark-type oxygen electrode at 25°C.

3. RESULTS

Effects of hydrogen peroxide on Mn- and Fe-SOD activities

Fig. 1 demonstrates the stability of Porphyra Mn-SOD in H_2O_2 up to 10 mM for 1 hour at 25°C. At higher concentrations more than 10 mM, the enzyme was inactivated to some extent, and showed 40% activity in 50 mM H_2O_2. To the contrary, Azotobacter Fe-SOD was inactivated by H_2O_2. Fig. 2 shows the pseudo-first order plot obtained with more than 350-fold excess of H_2O_2 over the enzyme concentration. The inactivation was first order with respect to both H_2O_2 and enzyme concentrations. The apparent second order rate constant for the inactivation (k_{inact}) was calculated using the equation, $k_{obs} = k_{inact}[H_2O_2]$, from the slope of the observed pseudo-first order rate constants (k_{obs}) plotted as a function of H_2O_2 concentration (Fig. 2, inserted). Thus, k_{inact} was 2.4 $M^{-1}sec^{-1}$ at pH 7.8 and 25°C.

Spectral changes of Mn- and Fe-SOD upon the titration with hydrogen peroxide

Hydrogen peroxide bleaches the visible chromophore of both Mn- and Fe-SOD (3,9). H_2O_2 is favorable to two-electron reduction since the

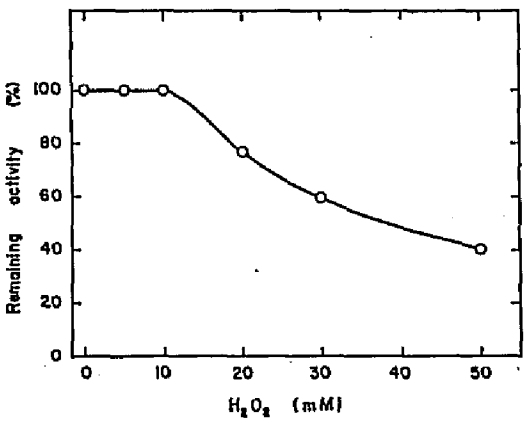

Fig. 1. Effect of hydrogen peroxide on the enzymatic activity of Porphyra Mn-SOD. The enzyme (1.04 μM) was incubated for 1 hour at 25°C with H_2O_2 at indicated concentrations in 10 mM potassium phosphate, pH 7.8. The aliquot of the sample was withdrawn after the incubation, and the remaining activity was measured in the xanthine-xanthine oxidase-cytochrome c assay system containing 500 units catalase.

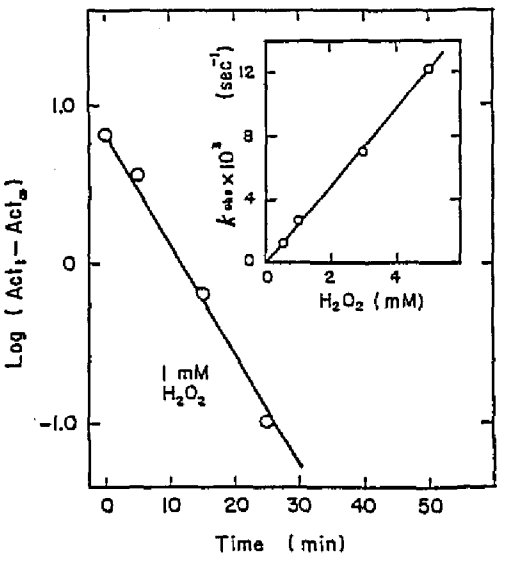

Fig. 2. Inactivation of Azotobacter Fe-SOD by hydrogen peroxide. The enzyme (2.84 μM) was incubated with H_2O_2 at 0.5, 1, 3, and 5 mM in 10 mM potassium phosphate, pH 7.8, at 25°C and the remaining activity was measured using 10 μl sample at the indicated intervals in the assay system containing 500 units catalase. The pseudo-first order rate constants for the inactivation were obtained from the plots of Log($Act_t - Act_\infty$) against time. Since the inactivation of the enzyme was incomplete leaving 3-9% of the initial activity, the final remaining activity was taken as the activity at infinite time (Act_∞). A typical result for 1 mM H_2O_2 is indicated. (Inserted), the observed pseudo-first order rate constants were plotted as a function of H_2O_2 concentration. The second order rate constant for the inactivation was obtained from the slope.

oxidation-reduction potential (E°') for O_2/H_2O_2 couple is 0.31 V as compared to 0.89 V for O_2^-/H_2O_2 couple at pH 7.0 (10). These bleachings, however, have been considered to be associated with one-electron reduction of E-Mn^{3+} or E-Fe^{3+}, because the enzymes undergo one-electron oxidation-reduction shuttle of E-M^{3+} and E-M^{2+} during catalysis (2,3,9), and have E°' of about 0.27 V at pH 7.0 (11).

The absorption maximum at 480 nm of the Mn-enzyme was decreased by addition of H_2O_2 (Fig. 3A). One mol of the enzyme was reduced by 0.5 mol of H_2O_2 (Fig. 3A, inserted). The bleached enzyme did not undergo further spectral change upon standing, at least, for 15 min. Further addition of dithionite to the H_2O_2-bleached enzyme slightly decreased the absorption at 480 nm (Fig. 3A). Thus, the enzyme was bleached to

20% the absorbance at 480 nm with H_2O_2, and further bleached to 10% with dithionite.

The absorption shoulder of the Fe-enzyme at 350 nm was decreased progressively upon each addition of H_2O_2, and finaly decreased to 40% the initial absorbance. Addition of ascorbate did not affect the final

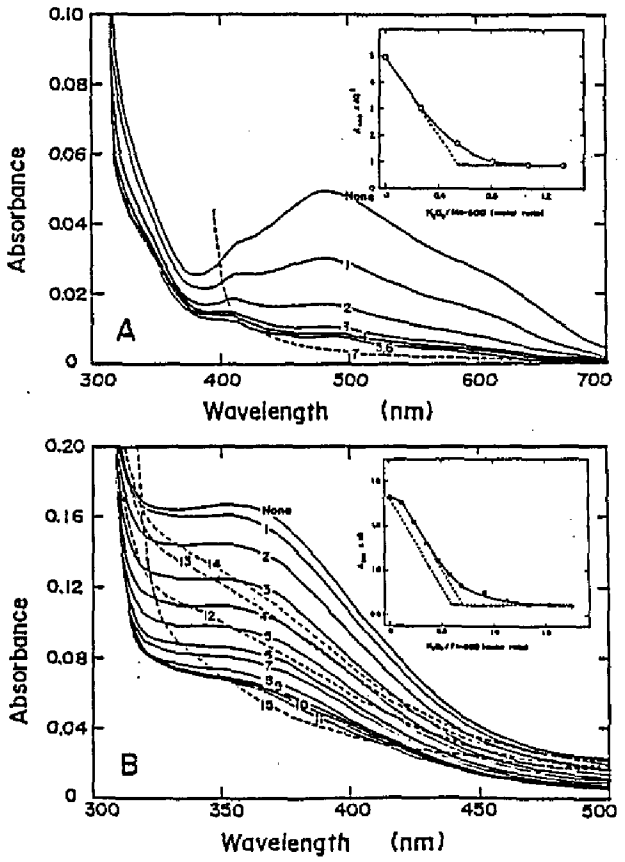

Fig. 3. Spectroscopic titration of Mn- and Fe-SOD by hydrogen peroxide. Porphyra Mn-SOD (50.6 μM) (A) in 0.7 ml of 10 mM potassium phosphate, pH 7.8, and Azotobacter Fe-SOD (94.6 μM) (B) in 0.9 ml of the same buffer were successively titrated with 5 or 10 μl of 2 mM H_2O_2, respectively. (A) 1 to 5, each 5 μl H_2O_2; 6, 5 was standed for 15 min; 7, solid dithionite was added. (B) 1 to 6, each 5 μl H_2O_2; 7-11, each 10 μl; 12, same as 11 except it was standed for 15 min; 13, 30 min; 14, 45 min; 15, solid sodium ascorbate was added. (Inserted), the bleachings of the absorption at 480 nm for Mn-SOD (A) and at 350 nm for Fe-SOD (B) were plotted as a function of molar ratio of H_2O_2 against the enzyme, respectively. The correction was made for the dilution due to the addition of H_2O_2. Each spectrum was taken after 30 sec for Mn-SOD or 1 min for Fe-SOD after the addition of H_2O_2.

TABLE I

Stoichiometry of Oxygen Evolution from Hydrogen Peroxide by the Reaction with Porphyra Mn-SOD[a)]

Mn-SOD	50 nmol H_2O_2		O_2 evolved	Ratio	
	unreacted[b)]	used[c)]		O_2/H_2O_2 used	H_2O_2 used/Mn
	(nmol)				
25 nmol	36	14	12	0.9	0.6
40 nmol	30	20	22	1.1	0.5

a) Ten microliters of 5 mM H_2O_2 (50 nmol) was added into 25 or 40 nmol of the enzyme in 1 ml of 10 mM potassium phosphate, pH 7.8, and oxygen evolved was measured by an oxygen electrode at 25°C.

b) Unreacted H_2O_2 was calculated from the O_2 evolved by adding 1000 units catalase.

c) Used H_2O_2 was calculated by substracting the unreacted H_2O_2 from 50 nmol of H_2O_2 added.

absorption, indicating that the enzyme was fully reduced (Fig. 3B). At an early stage of the titration, the Fe-enzyme consumed H_2O_2 without absorption change. This may be attributed to non-functional iron bound to the enzyme, which was observed in the reconstituted Fe-enzyme (Chapter XV). Therefore, the correction corresponding about 10% of H_2O_2 consumed was made. Thus, one mol of the Fe-enzyme was reduced with 0.6 mol of H_2O_2 (Fig. 3B, inserted). These results indicate that one mol of the metals in the Mn- or Fe-enzyme is reduced by 0.5 mol of H_2O_2.

Stoichiometry of oxygen evolution from hydrogen peroxide by the reaction with Mn- and Fe-SOD

When 50 nmol of H_2O_2 was added to 25 or 40 nmol of the Mn-enzyme, O_2 corresponding to half mol of the enzyme was evolved. Consumption of H_2O_2 corresponding to half mol of the enzyme was confirmed by addition of catalase at the end of O_2 evolution (Table I). Thus, the ratio of O_2 evolved/H_2O_2 consumed was approximatry 1, and the ratio of H_2O_2 reduced/Mn is about 0.5; one mol of E-Mn reacts with 0.5 mol of H_2O_2 to produce 0.5 mol of O_2.

A slight excess H_2O_2 resulted in the further reactions with the Fe-enzyme. Therefore, O_2 evolution was studied under the conditions of $[\text{Fe-SOD}] >> [H_2O_2]$. When Fe-SOD at 40-160 μM was used, 5 μM oxygen was

evolved from 5 μM H_2O_2 (Fig. 7B), and the O_2 evolved was proportional to H_2O_2 added (Fig. 7E). Thus, one mol of H_2O_2 produces one mol of O_2 as the Mn-enzyme.

Rate constants for bleaching at 480 nm of Mn-SOD and for oxygen production

The second order rate constant for the bleaching of the Mn-enzyme at 480 nm was determined under the second order conditions, since the reaction was too fast to follow with a conventional spectrophotometer under pseudo-first order conditions. The reaction was conducted at the same concentration of H_2O_2 as the enzyme (Fig. 4). The plot of $1/(A_t - A_\infty)$ vs. time gave a straight line over 80% of total change, indicating that the reaction was second order (Fig. 4, inserted). From the slope the apparent second order rate constant was estimated to be 3.8×10^2 $M^{-1}sec^{-1}$.

The second order rate constant for the O_2 production was also determined under the second order reaction conditions (data not shown).

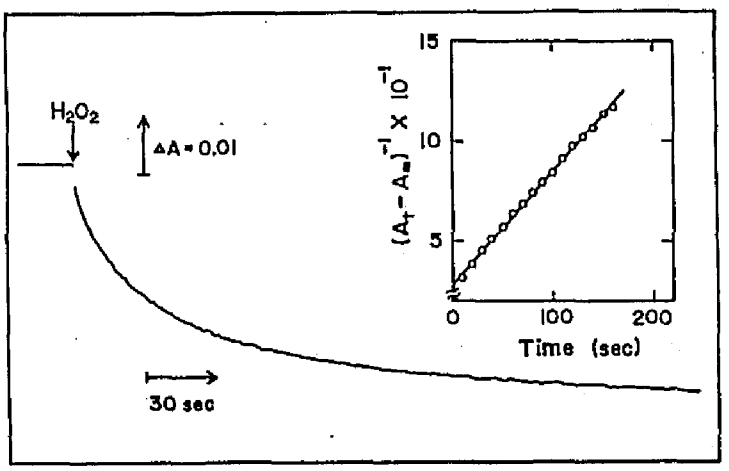

Fig. 4. The bleaching at 480 nm of Mn-SOD by hydrogen peroxide. The reaction was started by adding 54.7 μM H_2O_2 into 0.9 ml of 10 mM potassium phosphate, pH 7.8, containing 53.8 μM enzyme at 25°C. The absorbance change was recorded several second after mixing. (Inserted), the second order reaction plot. The apparent second order rate constant was calculated from a slope according to the equation, $(A_t - A_\infty)^{-1} = kt + (A_0 - A_\infty)^{-1}$, where A_t is absorbance at 480 nm at time t, A_0 is the initial absorbance and A_∞ is the final absorbance. A_∞ was taken as the value of 0.0165 at 72 min.

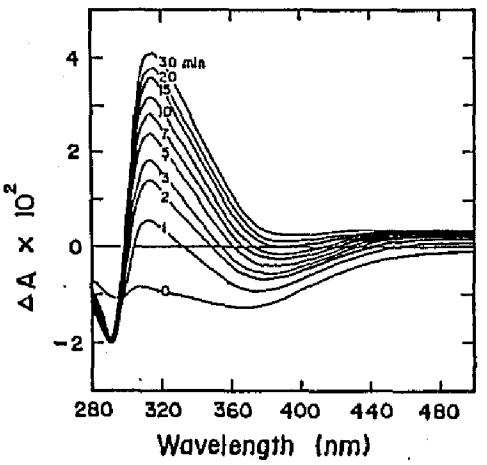

Fig. 5. Difference absorption spectra of Fe-SOD in the presence and absence of hydrogen peroxide. The scan speed was 10 nm/sec. The reaction was started by adding 5 μl of 100 μM H_2O_2 into 25.4 μM Fe-SOD in 0.8 ml of 10 mM potassium phosphate, pH 7.8. Spectra were obtained at 0 (ca. 10 sec), 1, 2, 3, 5, 7, 10, 15, 20, 30 min.

The slope of the plot for $1/([O_2]_t - [O_2]_\infty)$ vs. time was straight and the apparent second order rate constant of 3.2×10^2 $M^{-1}sec^{-1}$ was obtained. Thus, the both rate constants were equal within exprimental error and the bleaching and O_2 evolution are concomitant processes.

Time course of absorption change of Fe-SOD by hydrogen peroxide

The addition of H_2O_2 to the Fe-enzyme resulted in a rapid bleaching followed by a slower increase in absorption at 350 nm (Fig. 3B). At this stage, the difference spectra revealed a new absorption peak at 310-315 nm (Fig. 5). The bleaching at 350 nm and appearance of new absorption at 315 nm were well separated in terms of the reaction time, and the rate constants for both processes could be separately determined. The bleaching process was followed under the second order conditions using 48 μM enzyme and 48 μM H_2O_2. The second order rate constant for the bleaching at 350 nm was determined to be 4.5×10 $M^{-1}sec^{-1}$ (data not shown).

The kinetics for the slow change at 315 nm was studied in the presence of large excess H_2O_2. Under these conditions the fast phase (bleaching at 350 nm) finished in a few seconds and the slow phase procceded for 30-60 min. The semilogarithmic plot of absorption change

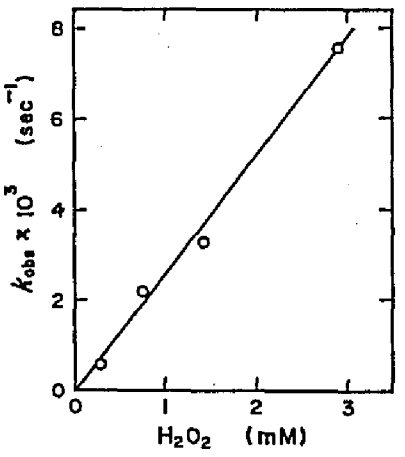

Fig. 6. Apparent second order rate constant for absorbance change at 315 nm of Fe-SOD caused by hydrogen peroxide. The reaction was started by adding indicated amount of H_2O_2 into 72 μM Fe-SOD in 10 mM potassium phosphate, pH 7.8, at 25°C, under the conditions of pseudo-first order reaction. The observed pseudo-first order rate constant (k_{obs}) was replotted against $[H_2O_2]$.

at 315 nm vs. time showed a straight line, confirming a pseudo-first order reaction (data not shown). The pseudo-first order rate constants were plotted as a function of H_2O_2 concentration (Fig. 6). A line having zero intercept showed that the slow process was first order with respect to $[H_2O_2]$. The k_{obs} was independent of the enzyme concentration in a range of 10-38 μM in the presence of 2.9 mM H_2O_2, confirming the attainment of pseudo-first order reaction conditions (data not shown). The apparent second order rate constant was 2.6 $M^{-1}sec^{-1}$.

Oxygen evolution from hydrogen peroxide by Fe-SOD

When the reaction was performed in excess enzyme, oxygen evolution from H_2O_2 was first order with respect to the enzyme concentration. The dependence of k_{obs} upon the enzyme concentration give a straight line with [SOD] (Fig. 7A). The apparent second order rate constant for oxygen evolution was estimated to be 4.7 x 10 $M^{-1}sec^{-1}$, which was the same as that for the 350 nm-bleaching within experimental error. Thus, the bleaching at 350 nm and O_2 evolution are concomitant processes. The stoichiometry for O_2 evolution from H_2O_2 in the presence of excess

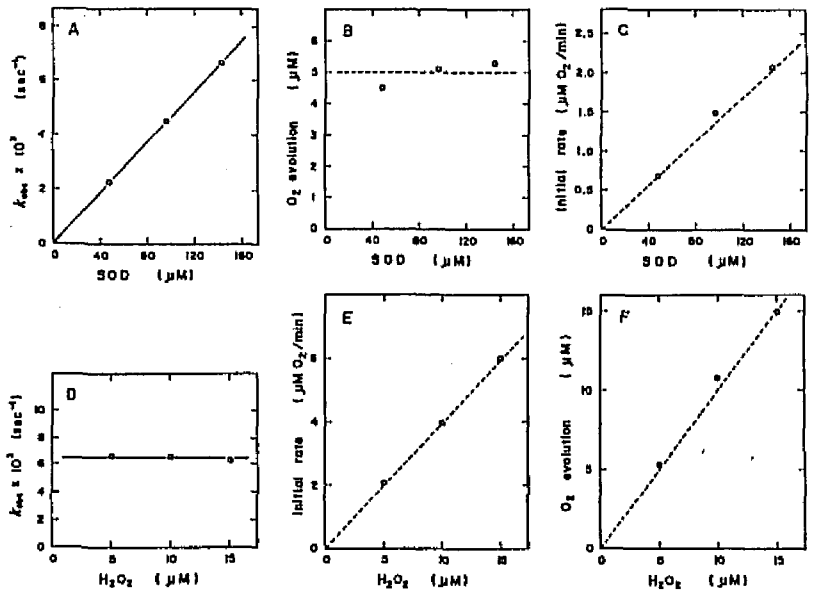

Fig. 7. Oxygen evolution from H_2O_2 by Fe-SOD under the conditions of pseudo-first order reaction, $[SOD] >> [H_2O_2]$. The reaction was started by adding 5 μM H_2O_2 into various amount of the enzyme (A,B,C) or various concentrations of H_2O_2 into 144 μM Fe-SOD (D,E,F) in 1 ml of 10 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA and 1 mM KCN at 25°C. Cyanide was added to suppress a possible decomposition of H_2O_2 due to catalase bound to a membrane of oxygen electrode. Catalase added to the reaction mixture to determine remaining H_2O_2 adhered to the membrane and it was difficult to remove it completely by washing, even with a detergent. Control experiment with or without 1 mM cyanide using a new electrode membrane indicated that cyanide did not affect the reaction rate for oxygen evolution. The observed pseudo-first order rate constant (k_{obs}) for oxygen evolution was obtained from a plot of $Log([O_2]_\infty - [O_2]_t)$ against time. The semilogarithmic plot was linear until 95% of oxygen evolution. (A), dependence of observed pseudo-first order rate constants (k_{obs}) upon enzyme concentration. (B,E), amount of oxygen evolved. Dotted line indicates the amount of H_2O_2 added. (C,F), initial velocity for oxygen evolution. Initial velocity was obtained from a tangent of the trace for oxygen evolution at 30 sec after the start of the reaction. Dotted line was calculated from a equation, $d[O_2]/dt = k_{app}[SOD][H_2O_2]$ with inital concentration of both SOD and H_2O_2, and second order rate constant (k_{app}) obtained from the slope in (A). (D), independence of k_{obs} upon $[H_2O_2]$.

enzyme was 1:1 as mentioned before (Fig. 7B, E). The k_{obs} was independent of H_2O_2 concentrations between 5-15 μM with 144 μM enzyme (Fig. 7D). The initial velocity was well agreed with that calculated using an

equation, $d[O_2]/dt = k_{app}[SOD][H_2O_2]$. (Fig. 7C, F). All of these data suggest that the reaction with excess enzyme was first order with respect to both enzyme and H_2O_2 concentrations.

On the contrary, when the reaction was conducted in excess H_2O_2, the kinetics for O_2 evolution was complicated. The semilogarithmic plots of O_2 produced vs. time obtained with H_2O_2 at a concentration range of 0.2 - 4 mM and 20 μM enzyme gave a straight line (data not shown). The k_{obs} obtained from these slopes were dependent on $[H_2O_2]$ with a finite intercept (Fig. 8A), suggesting the involvment of a reversible second order reaction. From the slope and intercept apparent rate constants for the forward and reverse reactions were calculated to be 2.6 $M^{-1}sec^{-1}$ and 1 x $10^{-3}sec^{-1}$, respectively (Fig. 8A). Under these

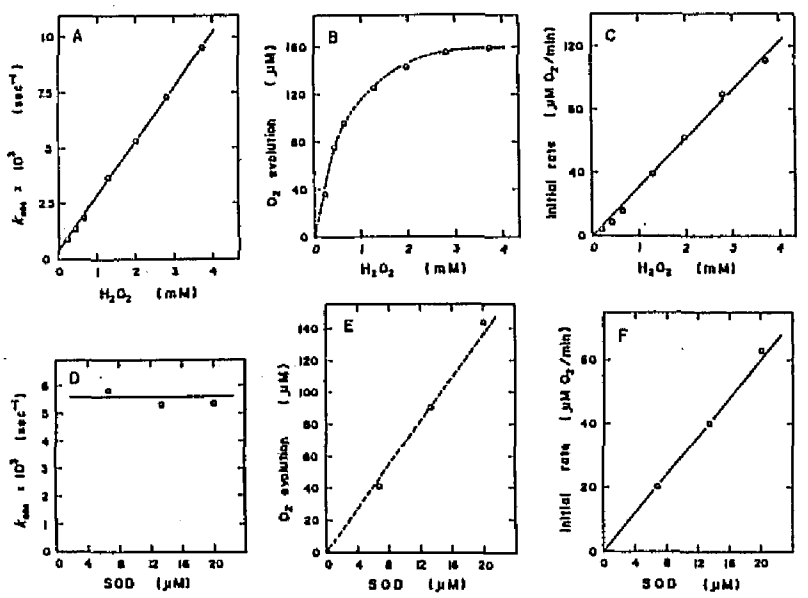

Fig. 8. Oxygen evolution from H_2O_2 by Fe-SOD under the conditions of pseudo-first order reaction, $[H_2O_2]>>[SOD]$. The reaction was initiated by introducing various amount of H_2O_2 into 20 μM Fe-SOD (A,B,C) or 2 mM H_2O_2 into various concentrations of Fe-SOD (D,E,F) in 1 ml of 10 mM potassium phosphate, pH 7.8, containing 0.5 mM EDTA and 1 mM KCN at 25°C. The observed pesudo-first order rate constant (k_{obs}) for oxygen evolution was obtained as in Fig 7. (A), dependence of observed pseudo-first order rate constants (k_{obs}) upon H_2O_2 concentration. (B,E), amount of oxygen evolved. Dotted line indicates the calculated values using eq 18 with k_3 and k_{-3} (Details are given in the text). (C,F), initial velocity for oxygen evolution. Initial velocity was obtained as in Fig 7. (D), independence of k_{obs} upon [SOD].

conditions, the k_{obs} was independent of [SOD] (Fig. 8D). However, the initial velocity was ten times higher than that expected from an equation, $d[O_2]/dt = k_{app}[SOD][H_2O_2]$ on the assumption of a simple second order reaction (Fig. 8C, F). Thus, it is evident that a catalytic process for O_2 evolution was involved in overall reaction and this should not be a rate-limiting step. The apparent rate constant (k_{cat}) was 2.5 x 10 $M^{-1}sec^{-1}$ (Fig. 8C, F). Fig. 8B demonstrates catalytic O_2 evolution from H_2O_2 with 20 μM enzyme. Under these conditions, O_2 evolution was saturated at about 4 mM H_2O_2, indicating the presence of decay process for the catalyst responsible for H_2O_2-decomposing cycle. O_2 evolved was proportional to the enzyme concentration (Fig. 8E).

Fe-SOD is not inactivated by hydrogen peroxide when [hydrogen peroxide]>> [SOD]

It has been revealed that the reaction of Fe-SOD with H_2O_2 consisted of two steps: the first step is the reduction of the enzyme without inactivation and the second step involves alteration of the active site associated with the inactivation. Thus, it could be anticipated that half-equimolar or less H_2O_2 reduces the enzyme but not inactivates it. The results in Fig. 9 shows that this was the case. The enzyme was not inactivated by H_2O_2 below 10 μM for 24 hours when

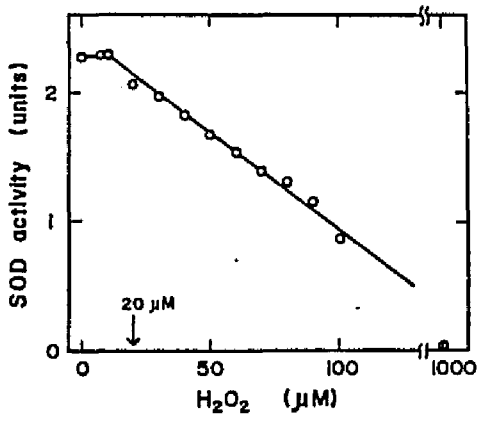

Fig. 9. Titration of Fe-SOD by hydrogen peroxide for its activity. Fe-SOD (20 μM) was incubated with indicated concentrations of H_2O_2 in 100 μl of 10 mM potassium phosphate, pH 7.8, for 24 hours at 0°C. After incubation, the aliquot was diluted with above buffer, and 10 μl of sample was measured for the remaining activity in the xanthine-xanthine oxidase-cytochrome c assay system.

Fe-SOD was 20 μM. The enzyme was inactivated linearly as a function of H_2O_2 concentration from 10 μM to 150 μM. The requirement of more than 10-fold [H_2O_2] over [SOD] to inactivate the enzyme is consistent with the occurrence of catalytic H_2O_2-decomposing activity during inactivation (see DISCUSSION).

Structure of hydrogen peroxide-inactivated Fe-SOD

The structural change of Fe-SOD was investigated using native and SDS-polyacrylamide gel electrophoresis after incubating the enzyme (20 μM) with H_2O_2 at various concentrations (5 μM-1 mM) for 24 hours. The native electrophoresis showed that the protein-stained band of the enzyme was weakened as a function of H_2O_2 concentration from 10 μM, and disappeared completely at 200 μM H_2O_2 (data not shown). The reverse linear relationship was observed between density of the band and the remaining enzymatic activity, indicating that the inactivation was associated with drastic change of protein structure.

To the contrary, SDS-polyacrylamide gel electrophoresis gave a single protein band corresponding to a subunit molecular weight of 20,000, irrespective of H_2O_2 concentration in a range of 5 μM to 1 mM (data not shown). Thus, the cleavage of peptide bond did not occur during the inactivation process. Taking together with the results of native polyacrylamide gel electrophoresis, it can be concluded that the inactivation of Fe-SOD by H_2O_2 results in the destruction of protein structure but not in the fragmentation of the enzyme.

4. DISCUSSION

Present results clearly indicate that hydrogen peroxide reduces both Fe- and Mn-SOD at first. The Fe-enzyme undergoes further reaction but the Mn-enzyme does not. Therefore the additional reaction of the Fe-enzyme should be associated with its inactivation.

The reaction of the Mn-enzyme with H_2O_2 can be interpreted using the following model:

$$E\text{-}M^{n+} + H_2O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-}M^{(n-1)+} + O_2^{\cdot-} + 2\,H^+ \qquad [4]$$

$$E\text{-}M^{n+} + O_2^{\cdot-} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}M^{(n-1)+} + O_2 \qquad [5]$$

Overall reaction is:

$$2\,E\text{-}M^{n+} + H_2O_2 \longrightarrow 2\,E\text{-}M^{(n-1)+} + O_2 + 2\,H^+ \qquad [6]$$

where, $E\text{-}M^{n+}$ is the oxidized form of Mn-SOD. Here, the forward reaction (eq 4) is the rate-limiting step in the overall reaction (eq 6). Thus, one mol of H_2O_2 reduces one mol of the Mn-enzyme producing one mol of $O_2^{\cdot-}$ (eq 4). In turn, one mol of $O_2^{\cdot-}$ reduces one mol of the Mn-enzyme at diffusion-controlled rate ($k_2 = 2 \times 10^9\ M^{-1}sec^{-1}$) (eq 5). Because k_1 and the spontaneous disproportionation rate constant of $O_2^{\cdot-}$ are too small as compared with k_2, all the $O_2^{\cdot-}$ produced in the reaction 4 is converted to O_2 in the reaction 5. Under the above model, the reduction of the Mn-enzyme by H_2O_2 and the O_2 evolution from H_2O_2 are second order reaction, and the rates for both processes are first order with respect to both enzyme and H_2O_2 concentrations. Furthermore, the second order rate constants for both processes should be the same (see APPENDIX):

$$-\frac{1}{2}\frac{d[E\text{-}M^{n+}]}{dt} = k_1[E\text{-}M^{n+}][H_2O_2] \qquad [7]$$

$$\frac{d[O_2^{\cdot-}]}{dt} = k_1[E\text{-}M^{n+}][H_2O_2] \qquad [8]$$

Titration of the Mn-enzyme by H_2O_2 showed that one mol of H_2O_2 reduced two mol of the enzyme (Fig. 3A). The stoichiometry of O_2 evolution from H_2O_2 indicated that one mol of H_2O_2 reacted with two mol of the enzyme producing one mol of O_2 (Table I). The reduction of the enzyme and oxygen evolution were second order reaction and the rate constants for both processes were same. Thus, the second order plot for reduction (Fig. 4) and oxygen production (data not shown) of the Mn-enzyme gave a straight line, and the rate constants for the both processes were 3.8×10^2 $M^{-1}sec^{-1}$ and 3.2×10^2 $M^{-1}sec^{-1}$, respectively. These results indicate the validity of the above model for the reaction of Mn-SOD with H_2O_2.

Alternatively, if $O_2^{\cdot-}$ is not liberated from the active site of the enzyme, eqs 4 and 5 could be modified to:

$$E\text{-}M^{n+} + H_2O_2 \rightleftharpoons E\text{-}M^{(n-1)+}\text{-}O_2 + 2H^+ \quad [4']$$

$$E\text{-}M^{n+} + E\text{-}M^{(n-1)+}\text{-}O_2 \rightleftharpoons 2\ E\text{-}M^{(n-1)+} + O_2 \quad [5']$$

where $E\text{-}M^{(n-1)+}\text{-}O_2$ is a enzyme-H_2O_2 or enzyme-O_2 complex. If the reaction 4' is assumed to be the rate limiting step, the rate equation for eqs 4' and 5' gives the same expression as eqs 7 and 8. However, this possibility can be excluded since $O_2^{\cdot-}$ was detected in the reverse reaction of eq 3 of Cu,Zn-SOD by tetranitromethane as a trapper (5).

When $[SOD] >> [H_2O_2]$, the reaction of Fe-SOD with H_2O_2 can be explained by the same scheme (eqs 4, 5 and 6, here, $E\text{-}M^{n+}$ is oxidized form of Fe-SOD) as that for Mn-SOD. Thus, the reaction followed second order kinetics, and was first order with respect to both enzyme and H_2O_2 concentrations (Fig. 7). The stoichiometry of O_2 evolution from H_2O_2 and the reduction of the Fe-chromophore (Figs. 3B and 7) were consistent with the reaction scheme. The second order rate constants for the reduction (Fig. 6) and oxygen evolution (data not shown) were same: 4.5 x 10 and 4.7 x 10 $M^{-1}sec^{-1}$, respectively.

Under the conditions of large excess H_2O_2 over Fe-SOD, after the rapid reduction of the enzyme by H_2O_2, the reduced enzyme underwent further reaction with H_2O_2. The increase of absorption at 315 nm was

first order with respect to $[H_2O_2]$ and its second order rate constant was 2.6 $M^{-1}sec^{-1}$ (Fig 6), which was coincided with the overall inactivation rate constant (k_{inact}) of 2.4 $M^{-1}sec^{-1}$ (Fig 2). Thus, this process should be associated with the inactivation. The 315 nm absorption form of the enzyme may be the inactivated one which Fe-ligands were altered. During the inactivation of the enzyme, O_2 evolution was occurred. O_2 evolved form H_2O_2 exceeded the stoichiometric amount of the enzyme (Fig. 8B,E), indicating the catalytic H_2O_2-decomposition. This catalytic O_2 evolution showed an exponential decay, suggesting the first order decay of the catalyst. The plot of k_{obs} for O_2 evolution, which reflects the decay of the catalyst, vs. $[H_2O_2]$ gave the second order rate constant of 2.5 $M^{-1}sec^{-1}$ (Fig. 8A). This was similar to those values for the increase of absorption at 315 nm and for the overall inactivation. Thus, these processes are concomitant processes and the catalyst may be the reduced Fe-SOD. Furthermore, the plot in Fig 8A showed a clear intercept which gave a rate constant of 1×10^{-3} sec^{-1}. This is consistent with a second order reversible reaction with H_2O_2 to form a complex followed by formation of the inactivated enzyme. The lack of a directly observable intermediate would imply that association rate is slower than dissociation rate leading to the enzyme inactivation. Thus, the following scheme can explain the inactivation process of Fe-SOD.

$$E\text{-}Fe^{2+} + H_2O_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\text{-}Fe^{2+}\text{-}H_2O_2 \xrightarrow{k_4} E\text{-}Fe^{*}(\text{inactive}) \qquad [9]$$

$$E\text{-}Fe^{2+} + H_2O_2 + 2\,H^{+} \xrightarrow{k_5} E\text{-}Fe^{4+} + 2\,H_2O \qquad [10]$$

$$E\text{-}Fe^{4+} + H_2O_2 \xrightarrow{k_6} E\text{-}Fe^{2+} + O_2 + 2\,H^{+} \qquad [11]$$

Here, $E\text{-}Fe^{*}$ is the inactivated 315 nm absorption form. The reaction 9 is the rate limiting step for the O_2 evolution: $k_3 << k_4$ and k_{cat} under large excess $[H_2O_2]$. Eqs 10 and 11 comprize the catalytic cycle for O_2 evolution and its rate equation is (see APPENDIX):

$$\frac{d[O_2]}{dt} = k_{cat}[H_2O_2][E\text{-}Fe^{2+}] \quad , \qquad [12]$$

where $k_{cat} = k_5 k_6/(k_5 + k_6)$. In eq 9, when $k_3 << k_4$, the rate equation for the decay of $E\text{-}Fe^{2+}$ becomes to be first order with respect to both $[E\text{-}Fe^{2+}]$ and $[H_2O_2]$, and can be written as:

$$-\frac{d[E\text{-}Fe^{2+}]}{dt} = k_d[H_2O_2][E\text{-}Fe^{2+}] \quad , \qquad [13]$$

where k_d is apparent second order decay rate constant. Under large excess $[H_2O_2]$, eq 13 is reduced to be:

$$-\frac{d[E\text{-}Fe^{2+}]}{dt} = k_d'[E\text{-}Fe^{2+}] \quad ,$$

where $k_d' = k_3[H_2O_2] + k_{-3}$. By integration, we get:

$$[E\text{-}Fe^{2+}] = [E\text{-}Fe^{2+}]_i \exp(-k_d' t) \quad , \qquad [14]$$

where $[E\text{-}Fe^{2+}]_i$ is the initial concentration of $E\text{-}Fe^{2+}$ which corresponds to the $[E\text{-}Fe^{3+}]$ added. Thus, substituting eq 14 into eq 12 gives:

$$\frac{d[O_2]}{dt} = k_{cat}[H_2O_2][E\text{-}Fe^{2+}]_i \exp(-k_d' t) \quad . \qquad [15]$$

By integrating with the initial conditions that $[O_2]$ at $t = 0$ is zero and with the assumption that $[H_2O_2]$ is constant, one get:

$$[O_2] = k_{cat}[E\text{-}Fe^{2+}]_i \frac{[H_2O_2]}{k_{-3} + k_3[H_2O_2]} \{1 - \exp(-k_d' t)\} \quad . [16]$$

Eqs 12 and 16 indicate that the kinetics for O_2 evolution follows the first order with respect to $[E\text{-}Fe^{2+}]$ with the rate constant k_d under large excess $[H_2O_2]$. Thus, the slope and intercept of the plot for k_{obs} ($= k_d$) vs. $[H_2O_2]$ shown in Fig. 8A give k_3 and k_{-3}, respectively.

When $t \longrightarrow 0$, eq 15 gives:

$$\frac{d[O_2]}{dt} = k_{cat}[H_2O_2][E\text{-}Fe^{2+}]_i \quad . \qquad [17]$$

Thus, eq 17 indicates that initial rate of O_2 evolution is proportional to both $[H_2O_2]$ and $[E\text{-}Fe^{2+}]_i$. When $t \longrightarrow \infty$, eq 16 is reduced to be:

$$[O_2] = k_{cat}[E\text{-}Fe^{2+}]_i \frac{[H_2O_2]}{k_{-3} + k_3[H_2O_2]} \quad . \qquad [18]$$

Eq 18 implies that O_2 evolved has a saturation profile with respect to $[H_2O_2]$. Thus, at higher $[H_2O_2]$, $[O_2]$ evolved becomes constant $(=k_{cat}[E\text{-}Fe^{2+}]_i/k_3)$.

The second order rate constant for catalytic H_2O_2-decomposition (k_{cat}) was determined from a slope of initial velocity plotted as a function of [SOD] or $[H_2O_2]$ according to eq 17 (Fig. 8C,F). Both plots gave k_{cat} of 2.5 x 10 $M^{-1}sec^{-1}$. Using eq 18 and k_{cat} and k_3, the O_2 evolved from H_2O_2 was simulated (Fig. 8B). Curve-fitting of the calculated and observed O_2 evolution was well agreeable when k_{-3} of 2 x 10^{-3} sec^{-1} was used, which was the same order of magnitude as that obtained from a plot of k_{obs} (for O_2 evolution) vs. $[H_2O_2]$ (Fig. 8A). Thus, all of the experimental results satisfy eqs 9, 10 and 11.

The inactivation of Cu,Zn-SOD by H_2O_2 has been studied by several investigators. Hodgson and Fridovich (6,8) have proposed the inactivation mechanism as:

$$E\text{-}Cu^{2+} + H_2O_2 \longrightarrow E\text{-}Cu^{+} + O_2^{\cdot-} + 2\,H^{+} \qquad [19]$$

$$E\text{-}Cu^{+} + H_2O_2 \longrightarrow E\text{-}Cu^{2+}\text{-}OH\cdot + OH^{-} \qquad [20]$$

$$E\text{-}Cu^{2+}\text{-}OH\cdot + ImH \longrightarrow \text{inactivated } E\text{-}Cu^{2+} + Im + H_2O \qquad [21]$$

Reduced Cu,Zn-SOD reacts with H_2O_2 to form bound OH· and, in turn, this bound OH· oxidizes adjacent imidazole (ImH) group of the histidine residue which is one of the ligands to Cu. The pH dramatically affects the inactivation rate and the inactivation undergoes rapidly as pH is increased.

The present results clearly showed that during the reaction of Fe-SOD with H_2O_2, the catalytic O_2 evolution occurred. From the initial velocity of O_2 evolution it is suggested the catalytic process turned over about 10 times during the decay of the catalyst. The catalyst responsible to the catalatic activity seems to be reduced Fe-SOD

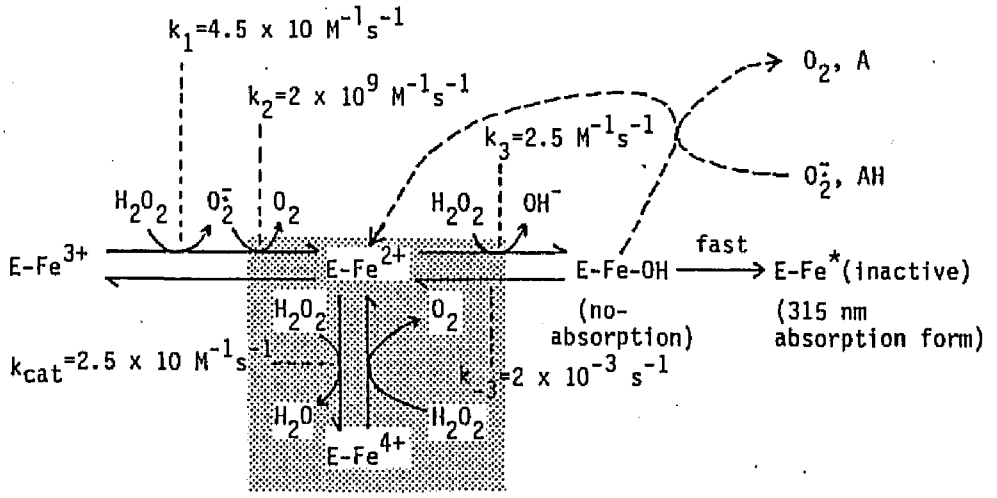

Fig. 10. Proposed reaction scheme of Fe-SOD with H_2O_2. ▒▒, Catalatic H_2O_2-decomposing activity; ---→, possible reaction; AH, reductant (such as ascorbate, NADPH, glutathione, etc.).

although catalatic activity of Fe^{2+}-complex has not been known so far. To the contrary, it is well established that Fe^{3+}-centered catalysts decompose H_2O_2 catalatically; for example, catalase (10^7), metmyoglobin (10^2) and aquo-ferric ion (10^{-1} $M^{-1}sec^{-1}$) (12). In the present scheme (Fig. 10), I tentatively propose that H_2O_2 is decomposed catalatically by two-electron redox reaction between Fe^{2+} and Fe^{4+} of the enzyme. However, this does not exclude the possibility of E-Fe-H_2O_2 complex as a catalytic intermediate. The elucidation for the nature of catalytic intermediate is subjected to further investigation. On the other hand, it seems that catalytic O_2 evolution process is absent in the reaction of Cu,Zn-SOD with H_2O_2 (13).

Detailed mechanism of the inactivation of Fe-SOD by H_2O_2 in eq 9 is not clear, but with the analogy for Cu,Zn-SOD (eqs 20 and 21) it is likely that bound oxidant formed from H_2O_2 is responsible to the inactivation. Although ligands of Fe-SOD are not determined yet, some of them have been suggested to be histidine residues (14). Thus, the destruction of ligand-histidine may be involved in the inactivation process. Then, conformational change occurred around active site may trigger drastic unfolding of the protein.

Among three types of SOD, Cu,Zn- and Fe-SOD are H_2O_2-sensitive,

and have similar inactivation rate constants. Thus, these SOD should be protected from the inactivation stress of H_2O_2 by H_2O_2-decomposing system including catalase and peroxidase. Although content of SOD is almost constant comprising 0.1-1% of total soluble proteins irrespective of organisms, content of catalase or peroxidase varies from species to species (15). Therefore, it seems that SOD cannot escape from the H_2O_2-inactivation stress. In fact, a finite concentration of H_2O_2 was detected in animal cells (16). Under these circumstances, catalatic activity of Fe-SOD may render the merit to H_2O_2-sensitive Fe-SOD to cope with a little H_2O_2 escaped from its scavenging system.

The highly reactive bound-OH· which is formed during interaction of SOD with H_2O_2 competes for the reactions between the metal-ligand residues and exogenous reductant (6,8). When the reductant is added, the bound oxidant oxidizes the reductant instead of the ligand, and the oxidant complex goes back to native state without a loss of SOD activity. Therefore, inactivation rate of Fe-SOD may be slower <u>in situ</u> than that expected from the mass action rule according to the simple second order reaction (eq 13). This possibility is included in the present scheme for inactivation of Fe-SOD with H_2O_2 (Fig. 10). Furthermore, if the concentration of H_2O_2 is low enough and comparable to the concentration of SOD (ca. 10-100 μM), SOD is not inactivated (Fig. 9). Even at a little higher concentration of H_2O_2, the catalatic rate of Fe-SOD is higher 10 times than the inactivation rate, and moreover the flux of H_2O_2 entering H_2O_2-decomposing cycle of Fe-SOD becomes higher due to the reverse reaction (k_{-3}) (Fig. 8).

Catalatic activity of Fe-SOD will be more important in anaerobic organisms where Fe-SOD is present but catalase is absent (17, 8). In anaerobic cells, the production of superoxide seems to be low, but once it is generated, H_2O_2 which is derived from the disproportionation of superoxide catalyzed by SOD, will accumulate unless H_2O_2 is scavenged. Thus, it is tempting to assume that the catalatic activity of Fe-SOD is responsible for the scavenging of H_2O_2 in anaerobes. In addition, under reductive environment inside the cell, many reductants may prevent the H_2O_2-inactivation of Fe-SOD by restoring the enzyme-bound oxidant complex to native enzyme.

5. SUMMARY

The reactions of Mn- and Fe-superoxide dismutases (SOD) with H_2O_2 were kinetically investigated. At low concentrations of H_2O_2, both enzymes were reduced following the second order kinetics (Mn-SOD, 3.8 x 10^2; Fe-SOD, 4.5 x 10 $M^{-1}sec^{-1}$). When excess H_2O_2 was added, Mn-SOD showed no other reaction but Fe-SOD underwent further reactions which involved the enzyme inactivation. After rapid bleaching at 350 nm, a new peak at 315 nm appeared at a second order rate constant of 2.6 $M^{-1}sec^{-1}$, which was the same as that for overall inactivation rate constant (2.4 $M^{-1}sec^{-1}$). In addition, catalatic O_2 evolution from H_2O_2 occurred during inactivation (k_{cat} = 2.5 x 10 $M^{-1}sec^{-1}$) and this activity was decayed with the same rate constant as that for appearance of peak at 315 nm. Thus, an intermediate formed during the inactivation process is responsible for H_2O_2-decomposing catalyst and this was assigned to reduced Fe-SOD. The reaction scheme which satisfies the experimental results is proposed.

6. REFERENCES

(1) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.

(2) Klug, D., Fridovich, I., and Rabani, J. (1973) J. Amer. Chem. Soc. 95, 2786-2790.

(3) Pick, M., Rabaini, J., Yost, F., and Fridovich, I. (1974) J. Amer. Chem. Soc. 96, 7329-7333.

(4) Klug, D., Rabani, J., and Fridovich, I. (1972) J. Biol. Chem. 247, 4839-4842.

(5) Hodgson, E.K., and Fridovich, I (1973) Biochem. Biophys. Res. Commun. 54, 270-274.

(6) Hodgson, E.K., and Fridovich, I. (1975) Biochemistry 14, 5294-5299.

(7) Adada, K., Yoshikawa, K., Takahashi, M., Maeda, T., and Enmanji, K. (1975) J. Biol. Chem. 250, 2801-2807.

(8) Hodgson, E.K., and Fridovich, I. (1975) Biochemistry 14, 5299-5303.

(9) Lavelle, F., McAdam, M.E., Fielden, E.M., Roberts, P.B., Puget, K., and Michelson, A.M. (1977) Biochem. J. 161, 3-12.

(10) Sawyer, D.T., Richens, D.T., Nanni, E.J., Jr., and stallings, M.D. (1980) in Chemical and Biochemical Aspects of Superoxide dismutase (Bannister, J.V., and Hill, H.A., eds.), pp. 1-26, Elsevier North Holland, New York.

(11) Barrette, W.C., Sawyer, D.T., Fee, J.A., and Adada, K. (1983) Biochemistry, 22, 624-627.

(12) Jones, P., and Wilson, I. (1978) in Iron in Model and Natural Compounds (Sigel, H., ed.), pp. 185-240, Marcel Dekker, New York.

(13) Fee, J.A, and DiCorleto, P.E. (1973) Biochemistry 24, 4893-4899.

(14) Harris, J.I., Auffret, A.D., Northrup, F.D., and Walker, J.E. (1980) Eur. J. Biochem., 106, 297-303.

(15) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(16) Oshino, N., and Chance, B. (1977) in Biochemical and Medical Aspects of Active Oxygen (Hayaishi, O., and Asada, K., eds), pp. 191-207, Japan Scientific Societies Press, Tokyo.

(17) McCord, J.M., and Fridovich, I. (1971) Proc. Natl. Acad. Sci. USA 68, 1024-1027.

(18) Gregory, E.M., Moore, W.E.C., and Holdeman, L.V. (1978) Appl. Environ. Microbiol. 35, 988-991.

APPENDIX

SCHEME I

The rate equations for individual reactions (eqs 4 and 5) can be written by:

$$-\frac{d[E\text{-}M^{n+}]}{dt} = k_1[E\text{-}M^{n+}][H_2O_2] - k_{-1}[E\text{-}M^{(n-1)+}][O_2^-] + k_2[E\text{-}M^{n+}][O_2^-] - k_{-2}[E\text{-}M^{(n-1)+}][O_2] \quad [A1]$$

$$\frac{d[O_2^-]}{dt} = k_1[E\text{-}M^{n+}][H_2O_2] - k_{-1}[E\text{-}M^{(n-1)+}][O_2^-] - k_2[E\text{-}M^{n+}][O_2^-] + k_{-2}[E\text{-}M^{(n-1)+}][O_2] \; . \quad [A2]$$

If k_1, $k_{-2} \ll k_2$, then $d[O_2]/dt = 0$. Substituting eq A2 into eq A1 gives:

$$-\frac{d[E\text{-}M^{n+}]}{dt} = 2k_2[E\text{-}M^{n+}][O_2^-] \; . \quad [A3]$$

From eq A2, we obtain:

$$[O_2^-] = \frac{k_1[E\text{-}M^{n+}][H_2O_2] + k_{-2}[E\text{-}M^{(n-1)+}][O_2]}{k_{-1}[E\text{-}M^{(n-1)+}] + k_2[E\text{-}M^{n+}]} \; . \quad [A4]$$

By substituting eq A4 into eq A3, we get:

$$-\frac{d[E\text{-}M^{n+}]}{dt} = \frac{2k_1[E\text{-}M^{n+}][H_2O_2] + 2k_{-2}[E\text{-}M^{(n-1)+}][O_2]}{\frac{k_{-1}}{k_2}\frac{[E\text{-}M^{(n-1)+}]}{[E\text{-}M^{n+}]} + 1} \; . \quad [A5]$$

Since $k_{-1}/k_2 = 1$ (2), $0 < E\text{-}M^{(n-1)+}/E\text{-}M^{n+} < 1$, and $k_{-2} < k_1$ (2), eq A5 is reduced to be:

$$-\frac{1}{2}\frac{d[E\text{-}M^{n+}]}{dt} = k_1[E\text{-}M^{n+}][H_2O_2] \quad . \qquad [A6]$$

Eq A6 is the rate equation for reduction of $E\text{-}M^{n+}$ in eq 6 and indicates that eq 6 follows second order reaction kinetics.

The rate equation for O_2 evolution is written by

$$\frac{d[O_2]}{dt} = k_2[E\text{-}M^{n+}][O_2^-] - k_{-2}[E\text{-}M^{(n-1)+}][O_2] \quad , \qquad [A7]$$

and transformed into using eq A4:

$$\frac{d[O_2]}{dt} = \frac{k_1[E\text{-}M^{n+}][H_2O_2] - \frac{k_{-1}}{k_2}\frac{[E\text{-}M^{(n-1)+}]}{[E\text{-}M^{n+}]} \cdot k_{-2}[E\text{-}M^{(n-1)+}][O_2]}{\frac{k_{-1}}{k_2}\frac{[E\text{-}M^{(n-1)+}]}{[E\text{-}M^{n+}]} + 1} \quad . \qquad [A8]$$

Applying the assumption which is used in eq A6, eq A8 becomes to be:

$$\frac{d[O_2]}{dt} = k_1[E\text{-}M^{n+}][H_2O_2] \quad . \qquad [A9]$$

From eqs A6 and A9,

$$\frac{d[O_2]}{dt} = -\frac{1}{2}\frac{d[E\text{-}M^{n+}]}{dt} \quad . \qquad [A10]$$

Thus, the oxygen evolution, as well as the reduction of $E\text{-}M^{n+}$, is a second order reaction between the oxidized enzyme and hydrogen peroxide, and the second order rate constants for the oxygen evolution and the reduction process, respectively, should be the same if the model is correct.

SCHEME II

Under turnover conditions, the reciprocal overall reaction velocity for the cycle consisted of eqs 10 and 11 can be explained in terms of the sum of the reciprocal reaction velocity for each reaction (eqs 10 and 11). Thus, the rate equation is

$$\frac{[E]_o}{v} = \frac{1}{k_3[H_2O_2]} + \frac{1}{k_4[H_2O_2]}$$

$$= \frac{k_3 + k_4}{k_3 k_4} \frac{1}{[H_2O_2]} \quad , \qquad [A11]$$

where $[E]_o$ is total concentration of $E\text{-}Fe^{2+}$ which is equal to the initial concentration of $E\text{-}Fe^{3+}$, and v is the initial rate velocity for the overall reaction. Eq A11 is transformed to

$$v = \frac{d[O_2]}{dt} = \frac{k_3 k_4}{k_3 + k_4}[H_2O_2][E]_o \quad . \qquad [A12]$$

CHAPTER XVII

Phylogenic Distribution of Three Types of Superoxide Dismutase in Organisms and in Cell Organelles: Concluding Remarks

Three distinct types of superoxide dismutase (SOD) have been isolated from various kinds of organisms. Each type contains different prosthetic metals; Cu,Zn-, Fe- and Mn-enzymes. In addition to the prosthetic metals, these three types are distinguished by their amino acid sequences; the sequence of the Cu,Zn-enzyme has no homology with sequences of the Fe- and Mn-enzymes (1-3). Although a high degree of homology between the Fe- and Mn-enzymes has been shown, the micro-environments of the ligands for metal binding should be specific for each type, because the reconstitution of the active Fe- and Mn-enzymes from their respective apoenzymes is successful only with the original metal ions (4,5 but see 6). Further, the Fe- and Mn-enzymes from the same organisms have been immunologically distinguished (7).

In respect to the catalysis of the disproportionation of O_2^-, however, little difference has been found among the three types. Their reaction rate constant between O_2^- and the enzyme is about 10^9 $M^{-1}s^{-1}$ at a neutral pH. The sole difference is that the catalytic rate of the Cu,Zn-enzyme is constant between pH 5.5 and 10 and that of the Fe- and Mn-enzymes becomes progressively lower as the pH is raised. Nevertheless, distribution of the three types of SOD in the organisms is characteristic of the stage of evolution and of the cell organelles. This characteristic distribution must reflect the evolutionary history of the acquisition of the defenses against oxygen toxicity due to the increase in atmospheric oxygen by photosynthetic organisms, and should provide insight into the physiological functions of SOD and its molecular evolution.

The distribution of the three types of enzyme in organisms has been determined by its isolation and characterization. Further, the insensitivity of the Mn-enzyme to both cyanide and H_2O_2, and the sensitivi-

ties of the Cu,Zn-enzyme to cyanide and the Cu,Zn- and Fe-enzymes to H_2O_2 (8) allow me to distinguish the three types of enzyme in crude extracts. Different ratios of inhibition by azide with the three types of enzyme also can be used to distinguish them (9). The present chapter summarizes the distribution of the three types of SOD in organisms that range from anaerobic prokaryotes to higher organisms deduced from my results and the literatures. In addition, the distribution of the enzyme in chloroplasts and mitochondria is described. Based on the distribution of the three types of enzyme, their physiological functions and evolutionary aspects are discussed.

SOD in anaerobic bacteria; the Fe-enzyme

The presence of SOD in photosynthetic anaerobes, sulfate reducing bacteria, and the fermentative, anaerobic Clostridium was first reported by Hewitt and Morris (10). Since then the enzyme has been found in obligate anaerobes including bacteroids and in other fermentative bacteria (11-16). The enzyme also occurs in anaerobically grown protozoa (17). I isolated the enzyme from photosynthetic anaerobes; the purple sulfur bacterium Chromatium vinosum (Chapter VIII), and the green sulfur bacterium Chlorobium thiosulfatophilum (Chapter IX). Both bacteria contain only the Fe-enzyme. Further, Hatchikian and Henry (18) have characterized the SOD from the sulfate reducing bacterium Desulfovibrio desulfuricans, as the Fe-enzyme.

The molecular properties of the Fe-SOD from these anaerobes are similar to those from aerobic prokaryotes (18-20), although there is a slight difference between the Fe-enzymes from prokaryotes and from the eukaryotic alga Euglena (Chapter XI). The amino acid sequence of the Fe-enzyme from Euglena differs from sequences of the prokaryotic enzymes according to the statistical analysis of the amino acid composition (21). The amino acid sequence of the NH_2-terminal region of the Fe-SOD from the sulfate reducing bacterium has a high degree of homology with that of the Fe- and Mn-enzymes from the other organisms, including vertebrates (22). The sequence analysis in the NH_2-terminal region of my preparations from both anaerobic sulfur bacteria by the late Dr. J.I. Harris has indicated a similar high degree of homology (Fig. 1, Ref 23).

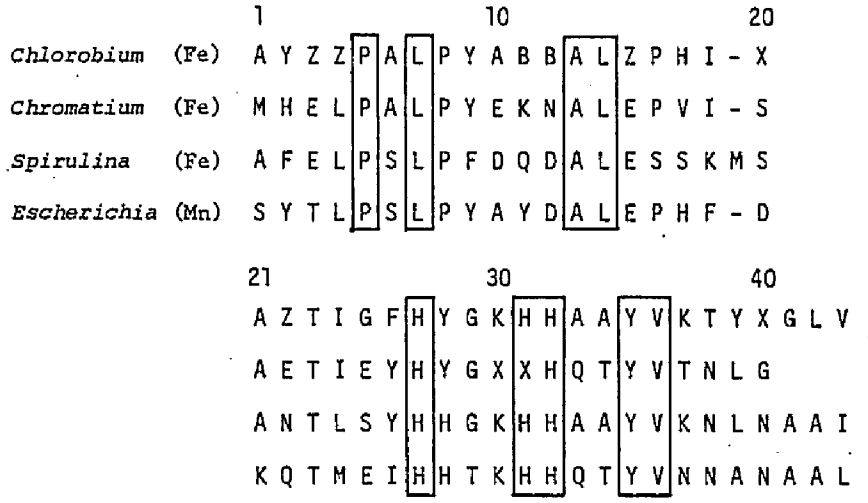

Fig. 1. NH_2-terminal sequence of Fe- and Mn-superoxide dismutases from anaerobic and aerobic bacteria, and cyanobacterium. Cited from Ref 23.

Thus, anaerobes contain only the Fe-enzyme that has properties similar to those of the Fe-enzyme from other organisms.

It admits no doubt that anaerobic prokaryotes are the most primitive organisms on the evolutionary tree; therefore, the Fe-enzyme of anaerobes may be the ancestor of the Fe- and Mn-enzymes of aerobes. On the other hand, to what extent the environmental concentration of oxygen was raised by abiotic reactions such as the photodissociation of water vapor when anaerobes appeared on the earth before the blue-green algae, is an open question (24,25). The presence of SOD in anaerobes suggests that there was an accumulation of oxygen in the early Precambrian period even if at an extremely low concentration, if the SOD in anaerobes functions to protect the cells from the active oxygen produced by temporary exposure to oxidized environments (25). Recently some correlation between the SOD content and the air-tolerance of anaerobic bacteria has been shown, which supports the above role for the enzyme (14). However, circumstantial evidence that the Fe-enzyme is biosynthesized under the strict anaerobic conditions such as in a medium of sulfur bacteria containing sulfide and thiosulfate has cast a doubt on the above role for the enzyme. The contents of SOD in anaerobic Chromatium and Chlorobium are 7 and 13 units per mg protein (19,20), and the content in aerobically-grown Escherichia coli is about 20 units.

Catalysis of oxidation-reduction other than the disproportionation of O_2^- by the enzyme cannot be excluded, at least, in anaerobes. I found that the Fe-enzyme has a weak catalase activity (Chapter XVI).

SOD in facultative and aerobic bacteria; the Fe- and Mn-enzymes

In contrast to anaerobic bacteria, aerobic and facultative anaerobic bacteria contain the Fe- or Mn-enzyme or both. The only exceptions to this are Photobacterium leiognathi (26) and Caulobacter crescentus (27) that contain the Cu,Zn-enzyme in addition to the Fe-enzyme. However, Photobacterium is a symbiont with fish and gene-transfer of the Cu,Zn-enzyme from the host fish has been suggested (28).

The Mn-enzyme has been isolated from E. coli (29), Mycobacterium phlei (30), M. sp. strain Takeo (31), M. lepraemurium (32), Streptococcus mutans (33), S. faecalis (34), Bacillus stearothermophilus (35), Thermus aquaticus (36), T. thermophilus (37) and Rhodopseudomonas spheroides (38). In contrast, the Fe-enzyme has been purified from Mycobacterium tuberculosis (39), Photobacterium sepiae (40), P. leiognathi (40), E. coli (41,42), Pseudomonas ovalis (43), Bacillus megaterium (44) and cyanobacteria (8).

Some comments on the above list are in order: a) Britton et al. (34) carried out the H_2O_2-test on twenty four species of aerobic bacteria, and showed that most Mn-SOD-containing bacteria are gram-positive, and that the Fe-SOD- or both-enzyme-containing bacteria are generally gram-negative. The above list shows the same trends, but there are several exceptions as in the results of Britton et al. b) In contrast to anaerobic photosynthetic bacteria, aerobic photosynthetic bacteria contain the Mn-enzyme. c) Three thermophilic bacteria have the Mn-enzyme and the luminescent bacteria the Fe-enzyme. d) The enzyme content in M. lepraemurium is the highest in any of the bacteria surveyed so far; the Mn-enzyme constitutes 6% of its soluble protein (32).

Because of the broad divergence of prokaryotes and the difficulty in correlating their evolutionary relationships, more extensive surveys of the distribution of Fe- and Mn-SOD in prokaryotes are needed and the type of enzyme would provide one taxonomic criterion for prokaryotes. I

have determined the type of enzyme in aerobic diazotrophs in terms of its relationship to the oxygen concentration in the cells.

SOD in aerobic diazotrophs; the Fe-enzyme

Most diazotrophs (Clostridium, Chromatium, Chlorobium and Desulfovibrio) are anaerobes, but, bacteria such as Azotobacter, Rhizobium and the blue-green algae are aerobes. Nitrogenase is a highly oxygen labile enzyme (45); therefore, for the fixation of nitrogen, the concentration of oxygen inside the cells must be extremely low or near zero, even in the aerobic diazotrophs. As described above the SOD in anaerobes is the Fe-enzyme. Facultative anaerobic bacteria such as E. coli contain only the Fe-enzyme when cells have been cultured under anaerobic states. Biosynthesis of the Mn-enzyme and of "hybrid" Fe-SOD (46) is induced by O_2 (47) or O_2^- (48). Thus, the Fe-enzyme appears to be biosynthesized when the oxygen concentration inside the cells is extremely low. Aerobic diazotrophs should contain only the Fe-enzyme because of the very low oxygen concentration inside the cells; I found that this is the case (Chapter X). The isolation and properties of the Azotobacter enzyme and the Mössbauer spectrum of the ^{57}Fe-reconstituted enzyme was described in Chapter XV. The bacteroids from soybean root-nodules, Rhizobium japonicum, also contain only the Fe-enzyme (Chapter X).

Thus, anaerobes, anaerobically grown-facultative anaerobes, and aerobic diazotrophs contain only the Fe-SOD and lack the Mn-enzyme. The property common to the three bacterial groups is an extremely low or nearly zero concentration of O_2 or O_2^- inside the cells. I propose that the biosynthesis of the Fe-enzyme occurs only when the concentration of O_2 or O_2^- is nearly zero inside the cells, and that the Mn-enzyme is formed only in cells that contain O_2 or O_2^- above a definite concentration. If further evidence supports this proposal, the type of SOD should be useful in predicting the concentration of O_2 or O_2^- inside the cells.

SOD in algae; the Fe- and Mn-enzymes

Prokaryotic blue-green algae contain only the Fe- and Mn-enzymes as do other aerobic prokaryotes (13,49). The Fe-enzymes from Plectonema

boryanum (8,50), Spirulina platensis (38) and Anacystis nidulans (51) have been characterized.

Eukaryotic algae, except the phragmoplast algae (Chapter III and 52), lack the cyanide-sensitive enzyme and contain the Fe- and/or Mn-enzymes (Chapter II and 11-13,49,52). The Fe-enzyme from Euglena (Chapter XI) and the Mn-enzyme from the red algae Porphyridium cruentum (53) and Porphyra yezoensis (Chapter XIV) have been isolated. In Euglena, the Fe-enzyme localizes in the chloroplast stroma.

SOD in protozoa; the Fe- and Mn-enzymes

No Cu,Zn-SOD is detectable in Euglenophyta (13), Flagellate; Tritrichomonas foetus, Monocercomonas sp. (17) and Crithidia fasciculate (13), and Ciliate; Tetrahymena pyriformis (17). These organisms contain only the cyanide-insensitive enzyme; the Fe-enzyme has been isolated from Euglena (Chapter XI). Thus, like most eukaryotic algae, protozoa are a group of eukaryotes that lack the Cu,Zn-enzyme and contain only the Fe- and Mn-enzymes. It should be noted here that anaerobically grown T. foetus contains SOD (17), but, which type has not been determined. This indicates that the presence of the enzyme in anaerobically grown cells is not limited to prokaryotes. On the other hand, the content of the enzyme in Euglena cultured under photoautotrophic conditions is higher than that in cells cultured under heterotrophic conditions (Chapter II), which reflects the photoproduction of O_2^- in chloroplasts (54-55).

SOD in animals; the Cu,Zn- and Mn-enzymes

In contrast to protozoa, the animals, including invertebrates and vertebrates, tested so far contain the Cu,Zn-enzyme in addition to the Mn-enzyme. Although the mammalian enzymes have been extensively studied, only a few surveys have been done on invertebrates. The enzyme has been detected only in the hemerythrocytes of sipunculids that contain hemerythrin in place of hemoglobin (56), and in the sea anemone (57). However, no determination of the type of enzyme has been made. I surveyed the type of SOD in twenty four species of primitive marine invertebrates with the cyanide-test (Chapter IV). Even the most

primitive animals such as Porifera and Coelenterata have the Cu,Zn-SOD and, as expected, more evolved invertebrates, Mollusca, Echinodermata and Arthropoda, also contain the Cu,Zn-enzyme. Thus, animals acquired the Cu,Zn-enzyme at the early step of evolution from unicellular protozoa to multicellular primitive invertebrates. The fossil record of Porifera and Coelenterata shows that they appeared on the earth in the Cambrian period.

SOD has been purified from many mammalian species and tissues. A complete list is beyond the scope of this chapter; I cite only the classic work on the Cu,Zn-enzyme from bovine erythrocytes (58) and the Mn-enzyme from human liver (59). A survey of the enzyme in vertebrates, including fish has been conducted (60,61), and the Cu,Zn-enzyme has been isolated from swordfish (62). In liver mitochondria the Mn-enzyme localizes in the matrix, whereas, the Cu,Zn-enzyme is in the intermembrane space (59,63,64). The Mn-enzyme also has been found in the cytosol of the liver (59). To my knowledge, however, no Fe-SOD has been detected in animal tissues.

SOD in fungi and slime molds; the Cu,Zn- and Mn-enzyme

The Cu,Zn-enzyme has been isolated from Saccharomyces (65,66), Fusarium (67) and Neurospora (68) and the Mn-enzyme has been isolated from the luminescent fungus, Pleurotus olearius (40) and from the mitochondria of yeast (69). In addition, I have tested two species of slime molds (Myxomycota) and fifty five species of fungi (Eumycota) covering Mastigomycotina, Zygomycotina, Ascomycotina, Basidiomycotina, and Deuteromycotina for their sensitivity to cyanide in the extracts (Chapter IV). Without exception, enzyme activity was inhibited by cyanide which indicates the presence of the Cu,Zn-enzyme in Myxomycota and Eumycota. Thus, among the microorganisms, only those of this group contain the Cu,Zn-enzyme. No one has reported the Fe-enzyme in fungi.

A classification of organisms based on recent biological data has been proposed by Whittaker, who divides the organisms into the five kingdoms; Monera, Protista, Plantae, Fungi and Animalia (70). In this classification the fungi are regarded as an independent kingdom characterized by its evolutionary development in the absorption of nutrients,

and one of the three kingdoms of higher organisms. Yamanaka has proposed that eukaryotic algae and protozoa appeared earlier than fungi from his analysis of the reactivity of the cytochrome c of algae and yeast with cytochrome oxidase (71). Thus, several lines of evidence indicate that the fungi are developed organisms; therefore, the absence of the Cu,Zn-enzyme in algae and protozoa and its presence in fungi is not surprising.

SOD in land plants; the Cu,Zn-, Mn- and Fe-enzymes

All the land plants tested so far including the mosses, ferns and seed plants (gymnosperms and angiosperms) contain the Cu,Zn-enzyme in addition to the cyanide-insensitive enzyme (Chapter II). The enzyme has been demonstrated both in leaves and in other tissues such as seeds, fruits, shoots, roots (Chapter V and see 72-77). Isozymes of the Cu,Zn-enzyme have been found in many higher plants, and purified and characterized from wheat (74), maize (78,79), spinach (Chapter V and 80) and rice (Chapter VI). These isozymes were shown to be organelle-specific. Cytosolic and stromal Cu,Zn-SOD isozymes were significantly differ in their NH_2-terminal animo acid sequences and distinguished immunologically, but their properties are very similar to those of the enzyme from animals and fungi (Chapter V and VI). Both types of the isozyme were also detected in the phragmoplast green alga Spirogyra (Chapter III and V). Thus, it is indicated that both groups of Cu,Zn-SOD isozymes had been separated at an early phase of the evolution, i.e. before the emergence of invertebrates, fungi and land plants. Stromal Cu,Zn-SOD was also purified from the fern Equisetum arvense (Chapter VII). Cyanide-insensitive SOD has been detected in wheat (74), Jerusalem artichoke (81), corn (73), and peas (82). The H_2O_2-test shows the presence of the Mn-enzyme and the absence of the Fe-enzyme in most land plants including kidney bean (83) and tea (84). The Mn-enzyme has been purified from pea (85) and spinach (86). However, the Fe-enzyme was detected in several vascular plnats and purified from mustard (87), water lily (88), and tomato (89) and spinach (Chapter XIII). Furthermore, the occurrence of Fe-SOD in the moss Marchantia polymorpha has been shown (Chapter XII). Thus, the Fe-enzyme is present phylogene-

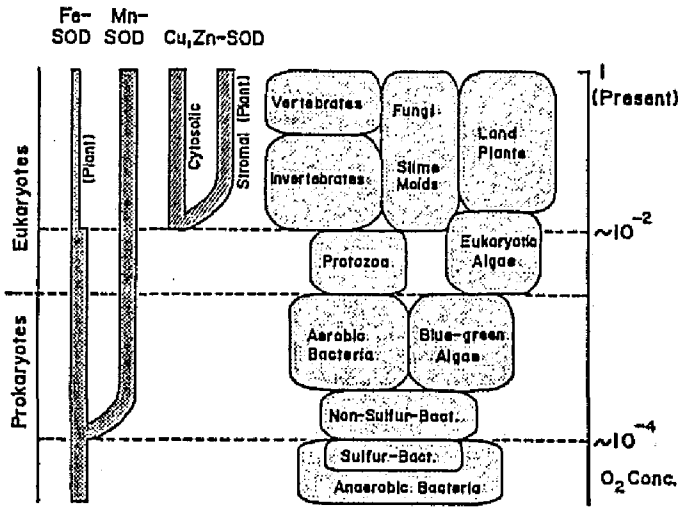

Fig. 2. Phylogenic distributions of three types of superoxide dismutase in organisms at various stages of evolution. O_2 Conc. shows the concentrations of O_2 in the atomosphere, relative to the present concentration, when the indicated organisms appeared on the earth.

tically in several plants. The localization of the Fe-enzyme in the chloroplast strom of plants is also the same as in eukaryotic algae (Chapter XI and XIII, and 90); the Mn-enzyme is in the mitochondrial matrix and the Cu,Zn-enzyme is in the intermembrane space (81,91). And, the Cu,Zn-enzyme is also localized in chloroplast stroma (80) and cytosol (Chapter V). Fig. 2 summarizes the distribution of three types of SOD in organisms at different levels of evolution.

Organelle distribution of SOD

As described above, prokaryotic and eukaryotic algae contain the Fe- and Mn-enzymes, whereas land plants have the Cu,Zn- and Mn-enzymes. In addition, several land plants also possess the Fe-enzyme. Since superoxide radical is produced inside the chloroplasts by autooxidation of the primary electron acceptor in photosystem I (55), it is interesting to determine the type of SOD in chloroplasts. In blue-green algae, both the Fe- and Mn-enzymes were found in soluble fraction, and any association of the enzymes to membrane was not observed (Chapter XIII). The aerobic bacteria Eschericia coli also contains both the enzymes in cytosol (92). In eukaryotic algae, the chloroplast stromal SOD of

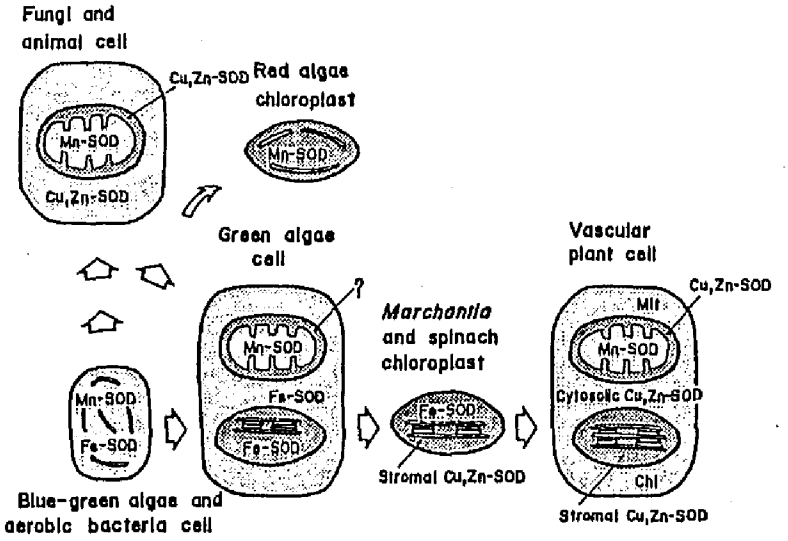

Fig. 3. Sub-chloroplast and sub-mitochondrial distribution of three types of superoxide dismutase in organisms at various stages of evolution.

Euglena was the Fe-enzyme (Chapter XI), whereas that of the red alga Porphyra yezoensis was suggested to be the Mn-enzyme (Chapter XIV). Localization of Cu,Zn-SOD in chloroplasts stroma from higher plants has been shown (80,91,93-96). In addition, the presence of the Fe-SOD in stroma of a moss (Chapter XII) and several angiosperms (Chapter XIII and 90) has been revealed.

Thus, the cytosol of blue-green algae and the stroma of green algae, and of some land plant chloroplasts contain the Fe-enzyme, providing evidence for the symbiotic orgin of the chloroplasts of green algae from prokaryotic cyanobacteria. The stroma enzyme in eukaryotic algae, however, is replaced by the Cu,Zn-enzyme in most land plants. My preliminary results also show that Euglena mitochondria contain the Fe-enzyme, which suggests the intermembrane space Fe-SOD of algal and protozoal mitochondria is replaced by the Cu,Zn-enzyme in plants, fungi, and animals. In mitochondria from animals, fungi, and plants the Mn-enzyme is localized in the matrix (59,63,64,69,81,91). Sub-chloroplast and -mitochondrial distributions of the three types of enzyme in organisms at different stages of evolution are schematically summarized in Fig. 3.

Evolutional stress of hydrogen peroxide on SOD

The distribution of three types of SOD in organisms and in cell organelles and their molecular evolution raises basic questions; why did organisms evolve three types of enzyme that have similar catalytic properties in respect of the disproportionation of O_2^-? And why did the Fe-enzyme disappear and a Cu,Zn-enzyme was acquired in higher organisms? The most basic and simplest answer to these questions is that each type of enzyme has its own biological function that includes not only the scavenging of O_2^- but some unknown reaction. In this respect, I will propose the following hypotheses.

Hydrogen peroxide inactivates the Fe- and Cu,Zn-enzymes but not the Mn-enzyme (8,97). Thus, H_2O_2, which is one of the products of disproportionation reaction of superoxide should have affected the evolution of SOD in conjunction with the acquisition of the H_2O_2-scavenging system and with its scavenging efficiency. Table I shows that the stromal Cu,Zn-SOD isozymes are more resistant to H_2O_2 than the cytosolic Cu,Zn-enzymes (Chapter V and VI). The production of superoxide in chloroplasts is inevitable (55) and its concentration is likely higher in chloroplasts than in cytosol. Thus, H_2O_2 has determined the evolution of SOD as a evolutional stress so as to make the enzyme more resistant against it. Furthermore, I found that the Cu,Zn-SOD has a ascorbate peroxidase activity, by which ascorbate prevent the enzyme from the inactivation by H_2O_2.

Table I

Comparison of the Inactivation Rate Constants by H_2O_2 among Cytosolic and Stromal Cu,Zn-Superoxide Dismutase Isozymes from Angiosperms

Plant	Cu,Zn-SOD ($M^{-1}s^{-1}$)		Temp. (°C)
	Cytosol	Stroma	
Wheat[a)]	5.3	2.1	38
Maize[b)]	0.6	0.3	25
Spinach	0.64	0.28	25
Rice	0.76	0.43	25

a) Ref 74.

b) Ref 79.

When the Fe-enzyme appeared firstly in anaerobic bacteria, these organisms seemed to be devoid of H_2O_2-decomposing system such as catalase and peroxidase. Then, how H_2O_2 was scavenged? I found that the Fe-enzyme has a weak catalase activity but the Mn-enzyme does not (Chapter XVI). Thus, in these organisms, the catalase activity of the SOD might have a advantage under the H_2O_2-stress. The reason of the replacement of the Fe-enzyme to the Cu,Zn-enzyme in chloroplast stroma is obscure, but one of the reason seems to be that the Cu,Zn-enzyme is more resistant against H_2O_2 than the Fe-enzyme. The determination of the biological role of the Fe-enzyme in anaerobes provide clues toward the answer of above questions. Further, the reductase activity of the SOD using H_2O_2 as a reductant (unpublished observation) also deserve further investigation.

Concluding remarks

The distribution of three types of SOD in organisms from anaerobic prokaryotes to higher organisms is illustrated in Fig. 2. In addition to anaerobes, the aerobes such as aerobic diazotrophs whose concentration of oxygen inside the cells is supposed to be near zero contain only the Fe-SOD. Aerobic and facultative bacteria, prokaryotic algae, protozoa and most eukaryotic algae contain the Fe- or Mn-enzyme or both; the Cu,Zn-SOD is absent in these organisms except two bacteria. The bacterial Cu,Zn-enzyme has been suggested to be due to the gene-transfer from eukaryotes. Anaerobically grown facultative prokaryotes contain only the Fe-enzyme, and biosynthesis of the Mn-enzyme is inducecd by oxygen. The Cu,Zn-enzyme has been found in animals, fungi and plants, even in such primitive organisms, as Porifera, Coelenterata, slime molds, ferns, and mosses. Phragmoplast green algae and land plants contain the cytosolic and stromal Cu,Zn-SOD isozymes. Animal, fungi, and most land plants lack the Fe-enzyme, and the Mn-enzyme occurs mainly in the mitochondrial matrix, but some moss and vascular plants possess the Fe-enzyme. SOD in the intermembrane space of the mitochondria and in the stroma of chloroplasts (cytosol in the blue-green algae) is the Fe-enzyme in algae and protozoa and the Cu,Zn-enzyme in animals, fungi and plants (Fig. 3).

The Fe- and Mn-enzymes in aerobes may have evolved from a common ancestor, the Fe-enzyme of anaerobes, because of the high degree of homology of the amino acid sequences of the two types of enzyme. Divergence of the Mn-enzyme from the Fe-enzyme should reflect an increse in the oxygen concentration of the biosphere. When aerobic prokaryotes appeared on the earth and to what extent the free oxygen was produced by abiotic reactions are points of controversy (24,25), but, I may assume that divergence of the Mn-enzyme from the Fe-enzyme came in the early Precambrian period, 30 to 20 x 10^8 years ago when the oxygen concentration was about 10^{-4} that of the present atmospheric level (98). The acquisition of the Cu,Zn-enzyme is believed to have occurred in the Cambran period, 6 to 4.5 x 10^8 years ago, because of the appearance of primitive animals such as Porfera and Coelenterata in fossils. According to Berkner and Marshall, the oxygen concentation in the atmosphere at that time was 10^{-2} of the present concentration (98).

REFERENCES

(1) Steinman, H.M., and Hill, R.L. (1973) Proc. Natl. Acad. USA 70, 3725-3729.

(2) Bridgen, J., Harris, J.I., and Northrop, F. (1975) FEBS Lett. 49, 392-395.

(3) Steinman, H.M. (1978) J. Biol. Chem. 253, 8708-8720.

(4) Ose, D.E., and Fridovich, I. (1976) J. Biol. Chem. 251, 1217-1218.

(5) Yamakura, F., Suzuki, K., and Omori, O. (1978) J. Japan Biochem. Soc. 50, 971.

(6) Gregory, E.M. (1985) Arch. Biochem. Biophys. 238, 83-89.

(7) Okada, S., Kanematsu, S., and Asada, K. (1979) FEBS Lett. 103, 106-110.

(8) Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y. and Enmanji, K. (1975) J. Biol. Chem. 250, 2801-2807.

(9) Misra, H.P., and Fridovich, I. (1978) Arch. Biochem. Biophys. 189, 317-322.

(10) Hewitt, J., and Morris, J.G. (1975) FEBS Lett. 50, 315-317.

(11) Lumsden, J. and Hall, D.O. (1975) Nature 257, 670-672.

(12) Asada, K., Kanematsu, S., Takahashi, M. and Kono, Y. (1976) in Iron and Copper Proteins (Yasunobu, K.T., Mower, H.F., and Hayaishi, O., eds.), pp. 551-564, Plenum Press, New York.

(13) Asada, K., Kanematsu, S., and Uchida, K. (1977) Arch. Biochem. Biophys. 179, 243-256.

(14) Tally, F.P., Golden, B.R., Jacobus, N.V., and Gorbach, S.L. (1977) Infec. Immunity 16, 20-25.

(15) Carlsson, J., Werthau, C., and Beckman, G. (1977) J. Clin. Microbiol. 6, 280-284.

(16) Gregory, E.M., Moore, W.E.C., and Holdeman, L.V. (1978) Appl. Environ. Microbiol. 35, 988-991.

(17) Lindmark, D.G., and Muller, M. (1974) J. Biol. Chem. 249, 4634-4637.

(18) Hatchikian, E.C., and Henry, Y.A. (1977) Biochimie 59, 153-161.

(19) Kanematsu, S., and Asada, K. (1978) Arch. Biochem. Biophys. 185, 473-482.

(20) Kanematsu, S., and Asada, K. (1978) FEBS Lett. 91, 94-98.
(21) Kanematsu, S., and Asada, K. (1979) Arch. Biochem. Biophys. 195, 535-545.
(22) Bruschi, M., Hatchikian, E.C., Bonicel, J., Bovier-Lapierre, G., and Couchoud, P. (1977) FEBS Lett. 76, 121-124.
(23) Harris, J.I., Auffret, A.D., Northrup, F.D., and Walker, J.E. (1980) Eur. J. Biochem. 106, 297-303.
(24) Schwarz, R.M., and Dayhoff, M.O. (1978) Science 199, 395-403.
(25) Towe, K.M. (1978) Nature 274, 657-661.
(26) Puget, K., and Michelson, A.M (1974) Biochem. Biophys. Res. Commun. 58, 830-838.
(27) Steinman, H.M. (1982) J. Biol. Chem. 257, 10283-10292.
(28) Bannister, J.V., and Parker, M.W. (1985) Proc. Natl. Acad. Sci. USA 82, 149-152.
(29) Keele, B.B., McCord, J.M., and Fridovich, I. (1970) J. Biol. Chem. 245, 6176-6181.
(30) Chikata, Y., Kusunose, E., Icihara, K., and Kusunose, M. (1975) Osaka City Med. J. 21, 127-136.
(31) Kusunose, M., Noda, Y., Ichihara, K., and Kusunose, E. (1976) Arch. Microbiol. 108, 65-73.
(32) Ichihara, K., Kusunose, E., Kusonose, M., and Mori, T. (1977) J. Biochem. Tokyo 81, 1427-1433.
(33) Vance, P.G., Keele, B.B., and Rajagopalan, K.V. (1972) J. Biol. Chem. 247, 4782-4786.
(34) Britton, L., Malinowshi, D.P., and Fridovich, I. (1978) J. Bacteriol. 134, 229-236.
(35) Harris, J.I. (1977) in Superoxide and Superoxide Dismutases (Michelson, A.M., McCord, J.M., and Fridovich, I., eds.), pp. 151-157, Academic Press, New York.
(36) Sato, S., and Harris, J.I. (1977) Eur. J. Biochem. 73, 373-381.
(37) Sato, S., and Nakazawa, K. (1978) J. Biochem. Tokyo 83, 1165-1171.
(38) Lumsden, J., Cammack, R., and Hall, D.O. (1976) Biochim. Biophys. Acta 438, 380-392.
(39) Kusunose, E., Ichihara, K., Noda, Y., and Kusunose, M. (1976)

J. Biochem. Tokyo 80, 1343-1352.

(40) Puget, K., Lavelle, F., and Michelson, A.M. (1977) in Superoxide and Superoxide Dismutases (Michelson, A.M., McCord, J.M., and Fridovich, I., eds.), pp. 139-150, Academic Press, New York.

(41) Yost, F.J., and Fridovich, I. (1973) J. Biol. Chem. 248, 4905-4908.

(42) Slykhouse, T.O., and Fee, J.A. (1976) J. Biol. Chem. 251, 5472-5477.

(43) Yamakura, F. (1976) Biochim. Biophys. Acta 422, 280-294.

(44) Anastasi, A., Bannister, J.V., and Bannister, W.H. (1976) Int. J. Biochem. 7, 541-546.

(45) Israel, D.W., Howard, R.L., Evans, H.J., and Russel, S.A. (1974) J. Biol. Chem. 249, 500-508.

(46) Dougherty, H.W., Sadowski, S.J., and Baker, E.E. (1978) J. Biol. Chem. 253, 5220-5223.

(47) Hassan, H.M., and Fridovich, I. (1977) J. Bacteriol. 129, 1574-1583.

(48) Hassan, H.M., and Fridovich, I. (1977) J. Biol. Chem. 252, 7667-7672.

(49) Asada, K, and Kanematsu, S. (1978) in Evolution of Protein Molecules (Matsubara, H., and Yamanaka, T., eds.), pp. 361-372, Japan Scientific Societies Press, Tokyo.

(50) Misra, H.P., and Keele, B.B. (1975) Biochim. Biophys. Acta 379, 418-425.

(51) Cseke, C., Horvath, L.I., Simon, P., Borbely, G., Keszthelyi, L., and Farkas, G. (1979) J. Biochem. Tokyo 85, 1397-1404.

(52) Henry, L.E.A., and Hall, D.O. (1977) in Photosynthetic Organelles (Miyachi, S., Fujita, Y., and Shibata, K., eds.), pp. 377-382, Japanese Soc. Plant Physiol., Kyoto.

(53) Misra, H.P., and Fridovich, I. (1977) J. Biol. Chem. 252, 6421-6423.

(54) Asada, K., and Kiso, K. (1973) Eur. J. Biochem. 33, 253-257.

(55) Asada, K., Kiso, K., and Yoshikawa, K. (1974) J. Biol. Chem. 249, 2175-2181.

(56) Manwell, C. (1977) Comp. Biochem. Physiol. 58B, 331-338.

(57) Lin, A.L. (1979) Comp. Biochem. Physiol. 62B, 425-431.
(58) McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.
(59) McCord, J.M., Boyle, J.A., Day, E.D., Rizzolo, L.J., and Salin, M.L. (1977) in Superoxide Dismutases (Michelson, A.M., McCord, J.M., and Fridovich, I., eds.), pp. 129-138, Academic Press, New York
(60) Maral, J., Puget, K., and Michelson, A.M. (1977) Biochem. Biophys. Res. Commun. 77, 1525-1535.
(61) Mazeaud, F., Maral, J., and Michelson, A.M. (1979) Biochem. Biophys. Res. Commun. 86, 1161-1168.
(62) Bannister, J.V., Anastasi, A., and Bannister, W.H. (1977) Comp. Biochem. Physiol. 56B, 235-238.
(63) Weisiger, R.A., and Fridovich, I. (1973) J. Biol. Chem. 248, 3582-3592.
(64) Peeters-Joris, C., Vandevoorde, A., and Baudhuin, P. (1975) Biochem. J. Tokyo 150, 31-39.
(65) Weser, U., Fretzdorff, A., and Prinz, R. (1972) FEBS Lett. 27, 267-269.
(66) Goscin, S.A., and Fridovich, I. (1972) Biochim. Biophys. Acta 289, 276-283.
(67) Rapp, U., Adams, W.C., and Miller, R.W. (1973) Can. J. Biochem. 51, 158-171.
(68) Misra, H.P., and Fridovich, I. (1972) J. Biol. Chem. 247, 3410-3414.
(69) Ravindranath, S.D., and Fridovich, I. (1975) J. Biol. Chem. 250, 6107-6112.
(70) Whittaker, R.H. (1969) Science 163, 150-163.
(71) Yamanaka, T., and Fukumori, Y. (1978) in Evolution of Protein Molecules (Matsubara, H, and Yamanaka, T., eds.), pp. 387-399, Japan Scientific Societies Press, Tokyo.
(72) Sawada, Y., Ohyama, T., and Yamazaki, I. (1972) Biochim. Biophys. Acta 268, 305-312.
(73) Giannopolitis, C.N, and Ries, S.K. (1977) Plant Physiol. 59, 315-318.
(74) Beauchamp, C.O., and Fridovich, I. (1973) Biochim. Biophys. Acta

317, 50-64.
(75) Baker, J.E. (1976) Plant Physiol. 58, 644-647.
(76) Hai, D.Q., Kovacs, K., Matkovics, I., and Matkovics, B. (1975) Biochem. Physiol. Pflanz. 167, 357-359.
(77) Giannopolitis, C.N. and Ries, S.K. (1977) Plant Physiol. 59, 315-318.
(78) Baum, J.A., and Scandalios, J.G. (1981) Arch. Biochem. Biophys. 206, 249-264.
(79) Baum, J.A., Chandlee, J.M., and Scandalios, J.G. (1983) Plant Physiol. 73, 31-35.
(80) Asada, K., Urano, M., and Takahashi, M. (1973) Eur. J. Biochem. 36, 257-266.
(81) Arron, G.P., Henry, J.M., Palmer, J.M., and Hall, D.O. (1976) Biochim. Soc. Trans. 4, 618-620.
(82) Del Rio, L.A., Sevilla, F., Gomez, M., Yanez, J., and Lopex, J. (1978) Planta 140, 221-225.
(83) Kono, Y., Takahashi, M., and Asada, K. (1979) Plant Cell Physiol. 20, 1229-1235.
(84) Hakamada, K., Kanematsu, S., and Asada, K. (1978) Study of Tea 54, 6-10.
(85) Sevilla, F., Lopez-Gorge, J., Del Rio, L.A. (1982) Plant Physiol. 70, 1321-1326.
(86) Hayakawa, T., Kanematsu, S., and Asada, K. (1985) Planta 166, 111-116.
(87) Salin, M.L., and Bridges, S.M. (1980) Arch. Biochem. Biophys. 201, 369-374.
(88) Salin, M.L., and Bridges, S.M. (1982) Plnat Physiol. 69, 161-165.
(89) Kwiatowski, J., Safianowska, A., and Kaniuga, Z. (1985) Eur. J. Biochem. 146, 459-466.
(90) Salin, M.L., and Bridges, S.M. (1980) Z. Pflanzenphysiol. 99, 37-45.
(91) Jackson, C., Dench, J., Moore, A.L., Halliwell, B., Foyer, C.H., and Hall, D.O. (1978) Eur. J. Biochem. 91, 339-344.
(92) Britton, L., and Fridovich, I. (1977) J. Bacteriol. 131, 815-820.
(93) Halliwell, B. (1975) Eur. J. Biochem. 55, 355-360.

(94) Doll, S., Lutz, C., and Ruppel, H.G. (1976) Z. Pflanzenphysiol. 80, 166-176.

(95) Elstner, E.F., and Heupel, A. (1975) Planta 123, 145-154.

(96) Lumsden, J., and Hall, D.O. (1975) Biochem. Biophys. Res. commun. 58, 35-41.

(97) Hodgson, E.K., and Fridovich, I. (1975) Biochemistry 14, 5299-5303.

(98) Berkner, L.C., and Marshall, L.C. (1965) J. Atmos. Sci. 22, 225-261.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Emeritus Prof. Zenzaburo Kasai and Prof. Kozi Asada of Kyoto University not only for their kind guidance but also for encouragement during the course of this work.

I am grateful to Associate Prof. Yutaka Maeda of Kyoto University for measurement of Mössbauer spectra.

I am also indebted to Prof. Keishi Shimokawa of Miyazaki University, and Associate Prof. Shoji Ida of Kyoto University for their continuous encouragement; and to my colleague of the Laboratory of Plant Photobiochemistry, Kyoto University, for their valuable discussion.

Finally, I should thank my parents for their financial support over a decade. Without the support, this study could not have been accomplished.