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SCAVENGING SYSTEMS OF HYDROGEN PEROXIDE IN CHLOROPLAST THYLAKOID MEMBRANES AND ALGAL CELLS

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Publications

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Abbreviations

AP	*p*-Aminophenol
APX	Ascorbate peroxidase
cAPX	Cytosolic APX
sAPX	Stromal APX
tAPX	Thylakoid-bound APX
AsA	L-Ascorbate
BSA	Bovine serum albumin
CAT	Catalase
CHAPSO	3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
Chl	Chlorophyll
pCMB	*p*-Chloromercuribenzoate
Cys	Cysteine
Cyt	Cytochrome
DBMIB	Dibromothymoquinone
DCIP	Dichlorophenolindophenol
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DEAE	Diethylaminoethyl
DHA	Dehydroascorbate
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
EPR	Electron paramagnetic resonance
Fd	Ferredoxin
FNR	Ferredoxin-NADP reductase
GSH	Reduced form of glutathione
GSSG	Oxidized form of glutathione
HA	Hydroxylamine
HEPES	*N*-2-Hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
HU	Hydroxyurea
HRP	Horseradish peroxidase
MES	2-(*N*-Morpholino)-ethanesulfonic acid

MDA	Monodehydroascorbate
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
PMSF	Phenylmethyl sulfonate
PS II/I	Photosystem II and I
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
Tris	Tris(hydroxymethyl)aminomethane
Tricine	*N*-Tris(hydroxymethyl)methylglycine

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Chapter-I

Introduction

Oxygen metabolism in higher plant chloroplasts

Higher plant chloroplast has two aspects in its oxygen metabolism. The first one is oxygen evolution resulting from the photooxidation of water in photosystem II. Oxygen such produced is used as the electron acceptor in an aerobic respiration which is reduced to water, coupling with the production of ATP, by the reductants produced in the glycolysis, the oxidation of fatty acids or citric acid cycle in mitochondria. Thus, oxygen evolved from chloroplasts takes part in the generation of energy in all aerobic organisms. The second one is an univalent photoreduction of dioxygen in chloroplasts, which has been referred to as the Mehler reaction (Mehler 1951a, 1951b), resulting in the production of superoxide anion radical. The superoxide anion radicals photoproduced in thylakoids are subsequently disproportionated to hydrogen peroxide and water (Asada et al. 1974). Thus, the second aspect in oxygen metabolism is, different from the first one, the reaction producing toxic, active oxygen, to all organisms. Besides higher plants, the Mehler reaction is observed also in eukaryotic algae chloroplasts and cyanobacteria (Radmer and Kok 1976, Radmer et al. 1978, Radmer and Ollinger 1980). The photoreduction of dioxygen has been shown to occur in photosynthetic electron carrier in photosystem I, center X and/or center A/B (Takahashi and Asada 1988). The production rate of superoxide anion radicals is about 10% the rate of photosynthetic carbon dioxide fixation, and increased under the conditions where plant is exposed to oxidative stress as described later.

In addition to the active oxygens, superoxide anion radical and hydrogen peroxide, produced via the Mehler reaction, the production of highly toxic singlet oxygen ($^1O_2$), which all photosynthetic organisms continuously face with, would be involved in the

second one in oxygen metabolism. Light absorbed by photosystems makes chlorophyll excited to singlet state ($^1Chl^*$) and the $^1Chl^*$ might be converted into the triplet state ($^3Chl^*$) by intersystem crossing. This allows energy transfer from chlorophyll to ground-state dioxygen ($^3O_2$) to occur with $^1O_2$ produced, which gives rise to a pattern of cellular damages similar to the effects of hydroxyl radical.

The toxicity of these active species of oxygens lies in the fact that it can react with numerous cellular components, thereby causing inactivation of enzymes, pigment bleaching, lipid peroxidations, and protein degradation (Asada and Takahashi 1987). According to the result of Kaiser (1976), hydrogen peroxide at 10 μM inhibits the photosynthetic fixation of carbon dioxide by half. Major target sites of hydrogen peroxide in chloroplasts are Calvin-cycle enzymes, fructose-1,6-bisphosphatase, glyceraldehyde-3-phosphate dehydrogenase and ribulose-5-phosphate kinase (Kaiser 1979). These enzymes have thiol groups in the reaction center and are active in their reduced forms. The inactivation of these enzymes by hydrogen peroxide is due to the oxidation of their thiol groups. Even though the photoreduction rate of dioxygen is only a small part of the total electron flow, the accumulation rate of hydrogen peroxide is 120 μM s^{-1} in the chloroplasts, if we assume a chloroplast volume of 35 μl mg Chl^{-1} and a production rate of the superoxide radicals of 30 μmol mg Chl^{-1} h^{-1}. Thus, if the photoproduced hydrogen peroxide is not immediately scavenged in chloroplasts, the photosynthetic fixation of carbon dioxide would stop within a second, resulting in the wilting of plants. Kyle (1987) has suggested that damage of D1 protein in the photosystem II reaction center might be due to attack of oxygen radical, especially superoxide anion radicals, on amino-acid residues of the peptide chain. In fact, Chen et al. (1992) have observed that in Photosystem II superoxide anion radical is produced and a rapid photodamage of the secondary donors of the photosystem II reaction center occurs by the superoxide.

The production of active oxygens in chloroplasts is inevitable under physiological conditions

The production of active oxygens, superoxide anion radicals in Mehler reaction and in photosystem II, and $^1O_2$ in photosystem II, is increased under the condition where photosystems in chloroplasts are in reduced state. And, if supply of $NADP^+$ which is physiological electron acceptor in photosystem I is limited, photoreduction of dioxygen in photosystem I is stimulated (Furbank and Badger 1983, Asada and Takahashi 1987) and electron flow to dioxygen at photosystem II acceptor side is also stimulated (Chen et al. 1992a). Further, charge recombination in photosystem II leading to non-photochemical quenching is stimulated, with the production of $^1Chl^*$ accelerated (Schreiber and Neubauer 1990). This reaction gives a large opportuniy for $^3O_2$ to react with $^3Chl^*$ resulting in the production of $^1O_2$.

Under physiological conditions, it has been reported that high light, dry and/or chilling stresses give injury to plant chloroplasts (Kyle 1987, Schöner and Krause 1990, Somersalo and Krause 1989, Wise and Nayler 1987). Under these conditions diffusion of carbon dioxide from outside of cell to chloroplasts is suppressed, resulting in an excess state of photoenergy to photosynthesis with especially superoxide anion radical and hydrogen peroxide stimulously produced. This would be one of the reason for plants to suffer from photodamages. Further, if plants are exposed to pollutant, ex. SO_2, productions of superoxide anion radical and hydrogen peroxide are enhanced in chloroplasts (Asada and Kiso 1973a,b) and oxidative damages occur in cellular components, enzymes of the Calvin cycle, fructose-1,6-bisphosphatase and ribulose bisphosphate carboxylase (Anderson and Duggan 1977, Tanaka and Sugahara 1980, Tanaka et al. 1982, Veljovic et al. 1993), photosynthetic electron transport (Daniel and Sarojini 1981, Shimazaki et al. 1984) and thylakoid ATPase (Cerovic et al. 1982). Thus, the production of active oxygen could be always involved and its rapid removal is necessary for photosynthesis.

Superoxide dismutase in higher plant chloroplasts

In 1973, Asada et al. published a first report that superoxide dismutase (SOD) is localized in higher plant chloroplasts. In higher plant chloroplasts, CuZn-SOD is localized in stroma (Asada et al. 1973, Jackson et al. 1978) and thylakoid lumen (Hayakawa et al. 1984) and in fact scavenges superoxide anion radicals photoproduced in photosystem I to hydrogen peroxide and water. Takahashi and Asada (1983) found that the superoxide photoproduced in the thylakoids is ejected to the lumen side and the permeation rate of superoxide to the stroma is very low. This observations suggest that superoxide anion radical should be scavenged at the site where it is produced and SOD should be localized in the vicinity of the production site of superoxide anion radical, photosystem I. In addition to the stroma and lumen, thylakoids bind Mn-SOD in spinach (Lumsden and Hall 1974, Hayakawa et al. 1985), but its content is too low as a component of the electron transport in the thylakoids. Thus, the thylakoid-bound Mn-SOD can catalyze the disproportionation of superoxide anion radical only when protons leaked from proton channel are available within the thylakoids.

When superoxide anion radical is produced at the rate of 240 $\mu M\ s^{-1}$ assuming a chloroplast volume of 35 $\mu l\ mg\ Chl^{-1}$, the steady-state concentration of superoxide anion radical (O_2^-) is 2.7 nM (from $v = k_{SOD}[O_2^-][SOD]$, where k_{SOD} is the reaction rate constant between SOD and O_2^-, [SOD] is the concentration of SOD in chloroplast stroma and lumen (Asada and Takahashi 1987)). This concentration is 10^{-4} order smaller than that (24 μM) estimated only by the spontaneous disproportionation of superoxide anion radical. Thus, the chloroplast SODs suppress the oxidation of chloroplast components by superoxide anion radical and the production of the hydroxyl radicals through the metal-catalyzed Harber-Weiss reaction (Baker and Gebicki 1984, Gutteridge and Bunnister 1986, Sutton and Winterbourn 1984).

Hydrogen peroxide is scavenged dependent on light in higher plant chloroplasts

In chloroplasts, hydrogen peroxide is always produced by the disproportionation of superoxide anion radical catalyzed with SOD. Asada and Badger (1984) found that, different from peroxisome where catalase scavenges hydrogen peroxide produced by glycolate oxidase in photorespiration, chloroplasts scavenge hydrogen peroxide dependent on light, but not dependent on catalase. Illuminated spinach chloroplasts evolved $^{16}O_2$ but little $^{18}O_2$ on addition of $H_2{}^{18}O_2$ and the evolution of $^{16}O_2$ could be inhibited by DCMU (Asada and Badger 1984). The stoichiometry is accounted for by the reduction of $H_2{}^{18}O_2$ by a peroxidase using a photoreductant ($e^- + H^+$),

$$2\ H_2{}^{16}O \longrightarrow 4\ H^+ + 4\ (e^-) + {}^{16}O_2 \quad \text{(PS II)}$$

$$2\ H_2{}^{18}O_2 + 4\ (e^-) + 4\ H^+ \longrightarrow 4\ H_2{}^{18}O \quad \text{(peroxidase)}$$

$$2\ H_2{}^{16}O + 2\ H_2{}^{18}O_2 \longrightarrow 4\ H_2{}^{18}O + {}^{16}O_2 \quad \text{(Sum).}$$

Foyer and Halliwell (1976) have proposed that peroxidase functioning in the light dependent-scavenging of hydrogen peroxide in chloroplasts uses ascorbate (AsA) as an electron donor, from the fact that there are AsA, glutathione (GSH), GSH reductase and dehydroasorbate (DHA) reductase in chloroplasts. Afterwards, following facts were reported as years passed; (i) in illuminated raptured chloroplasts DHA and oxidized form of GSH (GSSG) dependent oxygen evolution (Nakano and Asada 1980, Jablonski and Anderson 1978,1981), (ii) the oxidations of AsA and GSH by hydrogen peroxide in chloroplasts, and the photoreduction of the oxidized form of AsA (Jablonski and Anderson 1981, Anderson et al. 1983, Hossain et al. 1984), (iii) hydrogen peroxide-dependent oxidation of NAD(P)H in the presence of AsA (Hossain et al. 1984, Kow et al. 1982a,b), (iv) production of monodehydroascorbate (MDA) radicals by a peroxidase reaction localized in chloroplasts (Hossain et al. 1984) and a reduction of MDA radical to AsA by NAD(P)H in a ruptured chloroplasts (Hossain et al. 1984). These facts made Asada and Takahashi to propose the scavenging model of hydrogen peroxide in chloroplasts (Asada and Takahashi 1987). In illuminated chloroplasts hydrogen peroxide

produced by the disproportionation of superoxide anion radicals catalyzed with SOD is reduced by ascorbate peroxidase (APX) localized in chloroplast stroma to water using ascorbate as an electron donor. MDA radical produced in the reaction of APX is reduced to AsA by MDA reductase using NAD(P)H as an electron donor (Hossain et al. 1984, Hossain and Asada 1984). DHA, which is produced by the disproportionation of MDA escaped from the reduction by MDA reductase, is reduced to AsA by DHA reductase using GSH as an electron donor (Hossain and Asada 1984). And GSSG is reduced to GSH by GSH reductase using NAD(P)H as an electron donor (Halliwell and Foyer 1978, Connel and Mullet 1986). Thus, AsA used for APX to scavenge hydrogen peroxide is regenerated by photoreductant ($e^- + H^+ = NAD(P)H$) and functions as substrate for APX.

Ascorbate peroxidase in higher plant chloroplasts

In 1987 and 1989, Nakano and Asada, and Chen and Asada have isolated chloroplast stromal APXs from spinach and tea, respectively. Stromal APX shows the absorption spectrum of a hemoprotein and is inhibited by azide and cyanide, just as the guaiacol-specific classical plant peroxidase. Ascorbate is the most effective electron donor for stromal APX. Although monoiodeacetate and iodoacetamide have no effect, *p*-chloromercuribenzoate and 5,5'-dithio-bis-(2-nitrobenzoate) inhibit the peroxidase activity. Unlike guaiacol peroxidase and Cyt *c* peroxidase, stromal APX loses its activity in the absence of AsA as an electron donor with a half-time of about 10s. This is the reason why APX has not been found for a long time.

The following facts give further evidence that APX is the peroxidase functioning in the scavenging of hydrogen peroxide in chloroplast stroma. The capacity to photoreduce hydrogen peroxide is lost on the addition of hydrogen peroxide to intact chloroplasts in the dark, but addition of the same amounts of hydrogen peroxide to the illuminated chloroplasts shows no effect (Anderson et al. 1983, Asada and Badger 1984). During the dark treatment of intact chloroplasts with hydrogen peroxide, AsA is

oxidized by the peroxidase using photoreductant but the peroxidase is inactivated. However, the enzymes for the regeneration of AsA, MDA, DHA and GSH reductases are not affected. In fact, illumination of the chloroplasts after the treatment regenerates AsA (Hossain and Asada 1984). Thus, the peroxidase in chlroplast stroma is irreversibly inactivated under the conditions in which AsA is not regenerated, and this shows the same characteristics with those of APXs purified by Nakano and Asada (1987) and Chen and Asada (1989).

Induction of SOD and APX under environmental stress conditions

That enzymes which function in the scavenging system of superoxide anion radical and hydrogen peroxide are induced to remove the active oxygens under the condition where plants are exposed to environmental stress. In fact, following several facts were reported; (i) plant leaves show an increase in GSH and GSH reductase activity under hyperoxic conditions (Foster and Hess 1980,1982), (ii) the content of SOD is increased by chilling injury of spinach leaves (Schöner and Krause 1990) and the acclimated plant shows resistivity to cold stress, (iii) exposed to pollutants, sulfite and ozone, the activities of SOD and APX are increased (Tanaka and Sugahara 1980, Tanaka et al. 1985). Further, under water stress where diffusion of carbon dioxide is limited to chloroplasts the enzymes would be induced.

In this work

From above discussion, it is suggested that even under the favorite condition for plants dioxygen is univalently reduced to superoxide anion radical by photosystem I of thylakoids, and superoxide anion radical is disproportionated to hydrogen peroxide and water by SODs which are localized in thylakoid surface and lumen. Thus, hydrogen peroxide is inevitably produced, accompanied with the photosynthetic electron transport. The production rate of hydrogen peroxide is increased under the high light and/or carbon dioxide stress. Unless APX does not operate to scavenge the hydrogen peroxide, the photosynthetic activity is

lost in a few seconds because of the inactivation of Calvin cycle enzymes by hydrogen peroxide. This suggestion produces a new question. Hydrogen peroxide photoproduced in photosystem I should not diffuse to stroma not to inhibit Calvin cycle enzymes, but hydrogen peroxide-scavenging enzyme APX is localized in stroma and it has been supposed that stromal APX scavenges the hydrogen peroxide. What does this incompatibility mean? There are one possible answer for this question that on thylakoids is constituted unknown scavenging system of hydrogen peroxide more effective than that in stroma. To answer this question is the motive for me to do the present work.

In this work, I studied the followings to elucidate the scavenging system of hydrogen peroxide on thylakoids in chloroplasts,

(1) Existence of thylakoid-bound ascorbate peroxdiase and its physiological functions
——— (Chapter II),

(2) Purification and molecular properties of tAPX
——— (Chapter III),

(3) Inactivation mechanism of APX in the absence of AsA
——— (Chapter IV),

(4) Photoreduction of MDA radical to AsA in thylakoids
——— (Chapter V)

and I found the constitution of hydrogen peroxide-scavenging system on thylakoids and its operation. Further, to get an information about evolutional aspect of the scavenging system of hydrogen peroxide,

(5) Scavenging system of hydrogen peroxide in algae
——— (Chapter VI)

was studied, and I found that APX was acquired during the evolution of photosynthetic eukaryotic algae.

Chapter II

Thylakoid-bound Ascorbate Peroxidase in Spinach Chloroplasts and Photoreduction of Its Primary Oxidation Product Monodehydroascorbate Radicals in Thylakoids

The production of superoxide radicals in illuminated chloroplasts is an inevitable reaction, and occurs even under the favorable conditions for photosynthesis. Major producing site of superoxide radicals is the reducing side of PS I in the thylakoids, and the center X or A/B and the peripheral ferredoxin would be the univalent reductant of dioxygen. The superoxide radicals thus photoproduced are disproportionated to hydrogen peroxide and dioxygen catalyzed with the superoxide dismutases in the stroma and also in a thylakoid-bound form (Hayakawa et al. 1985). The hydrogen peroxide inhibits photosynthetic fixation of carbon dioxide with a half-inhibition at 10 μM (Kaiser 1976), which necessiates an effective scavenging system of hydrogen peroxide in chloroplasts to keep photosynthetic activity.

Chloroplasts can reduce the hydrogen peroxide by a peroxidase reaction using a photoreductant produced in the thylakoids as the electron donor (Asada and Badger 1984). The similar peroxidase system also operates in eukaryotic algae and some cyanobacteria (Chapter VI). The peroxidase participating in the scavenging in angiosperms has been identified to be ascorbate peroxidase. Its molecular properties are similar to those of yeast cytochrome *c* peroxidase rather than those of guaiacol peroxidase such as that from horseradish (Chen et al. 1992, Asada 1992). The primary oxidation product of the peroxidase reaction,

MDA radical, and its disproportionation product, DHA, are reduced to ascorbate catalyzed by respective reductases using NAD(P)H and GSH, respectively, as the electron donors. These donors are derived from the reduced ferredoxin in PS I, thus, dioxygen is finally reduced to water via superoxide and hydrogen peroxide, mediated by ascorbate (Asada and Takahashi 1987).

Spinach chloroplasts contain ascorbate peroxidase in the stroma in a soluble form (Nakano and Asada 1981), but, Groden and Beck (1979) have shown the peroxidase in a thylakoid-bound form. I confirmed the bound peroxidase only when the thylakoids were prepared using ascorbate-containing media. Further, I found the photoreduction of MDA radicals by thylakoids without participation of MDA reductase in the stroma, that is, MDA radical is the Hill oxidant. The present communication also deals with the scavenging system for the hydrogen peroxide produced in PS I by the bound-ascorbate peroxidase and the photoreduction of its primary reaction product in the thylakoids.

Materials and Methods

Fractionation of chloroplasts — Leaves of spinach obtained from a local market (30 g) were homogenized in a Polytron homogenizer for 3 s with 120 ml of the homogenizing medium (0.33 M sorbitol, 1 mM $MgCl_2$, 2 mM EDTA, 10 mM NaCl, 0.5 mM KH_2PO_4, 1 mM ascorbate, 50 mM MES-KOH, pH 6.2). The homogenate was filtered through cheesecloth and centrifuged at 2,000 x g for 30 s. The sediments were suspended in 30 ml of the assay medium (0.33 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4, 1 mM ascorbate and 50 mM HEPES-KOH, pH 7.6), and centrifuged at 2,000 x g for 60 s. The sedimented chloroplasts were suspended in 3 to 4 ml of the assay medium and layered on a discontinuous gradient composed of 90% (1 ml), 70% (3 ml), 40% (4 ml) and 10% (2 ml) Percoll in the assay medium. The Percoll tube was then centrifuged at 4,000 x g for 15 min, and the intact chloroplast layer between 40% and 70% Percoll was collected. The chloroplasts were washed with 30 ml of the assay medium by centrifugation at 2,000

x g for 2 min, and resuspended in 1 to 2 ml of the same medium. Thylakoid and stroma fractions were separated by centrifugation at 100,000 x g for 1 h after the intact chloroplasts were disrupted by osmotic shock in a hypotonic medium (50 mM HEPES-KOH, pH 7.6, 10 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4 and 1 mM ascorbate). Then the thylakoids were suspended in the assay medium. Stroma and grana thylakoids were separated by the treatment with a Yeda pressure cell followed by centrifugation according to Takano et al.(1982), but the ascorbate-containing medium (50 mM Tricine-KOH, pH 7.6, 150 mM NaCl, 2 mM $MgCl_2$ and 1 mM ascorbate) was used.

Intactness of the Percoll-purified chloroplasts was about 80 to 90% as determined by the ferricyanide method (Heber and Santarius 1970). The chloroplasts showed the light-dependent oxygen evolution at about 100 μmol mg Chl^{-1} h^{-1} in the presence of 10 mM bicarbonate.

Measurements of oxygen exchange — Evolution and uptake of dioxygen were followed with a Hansatech oxygen electrode. After incubation in the dark for 5 min, the reaction mixture (1 ml) was illuminated by a 300-watt iodine lamp projector (320 W m^{-2}) at 25°C.

Assays of enzymes and Chl — Ascorbate peroxidase activity was determined as described previously (Nakano and Asada 1987) using a reaction mixture (1 ml) containing 50 mM potassium phosphate, pH 7.0, 0.1 mM hydrogen peroxide and 0.5 mM ascorbate. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (2.8 mM^{-1} cm^{-1}). The assay mixture (1 ml) for other electron donors of peroxidase contained 50 mM potassium phosphate, pH 7.0, 0.1 mM hydrogen peroxide and one of the following donors: 0.15 mM NAD(P)H (absorbance at 340 nm, 6.2 mM^{-1} cm^{-1}), 40 μM reduced Cyt *c* (absorbance at 550 nm due to reduced form, 19 mM^{-1} cm^{-1}) or 20 mM pyrogallol (absorbance at 430 nm, 2.47 mM^{-1} cm^{-1}). When GSH was the electron donor, the reaction mixture contained 0.12 mM NADPH and glutathione reductase in order to determine GSSG by the decrease in the absorbance at 340 nm (Little et al. 1970). The hydrogen

peroxide-dependent oxidation of the donors was determined from the absorbance changes at the respective wavelengths and absorbance coefficients cited in parentheses.

MDA reductase was assayed by following a decrease in absorbance at 340 nm due to to the oxidation of NADH or NADPH using an absorbance coefficient of 6.2 mM^{-1} cm^{-1} at 25°C. In this assay, MDA was generated by ascorbate oxidase (Yamazaki and Piette 1961) using a reaction mixture (1 ml) containing 50 mM HEPES-KOH, pH 7.6, 0.1 mM NADH or NADPH, 2.5 mM ascorbate, ascorbate oxidase (0.14 unit, 1 µmol ascorbate oxidized min^{-1} being 1 unit) and enzyme. Under these conditions, the steady-state concentration of MDA was 2.1 µM, as determined from the absorbance at 360 nm assuming an absorbance coefficient of 3.3 mM^{-1} cm^{-1} (Schuler 1977). DHA reductase was assayed as described by Nakano and Asada (1981). The assay mixture contained 50 mM potassium phosphate, pH 7.0, 2.5 mM GSH, 0.2 mM DHA and 0.1 mM EDTA in a final volume of 1 ml. Reaction rates were measured by monitoring an increase in absorbance at 265 nm, 10 to 30 s after the addition of the enzyme. Chlorophyll was determined by the method of Arnon (1949).

Measurement of chlorophyll fluorescence — Modulated chlorophyll fluorescence (Schreiber et al. 1986) was measured with a PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany) with the fiberoptic-cuvette geometry, using the emitter-detector units ED 101. A thermostated (25°C) cuvette (KS 101, Walz) was used, and the mixture was stirred during the measurements. Chlorophyll fluorescence was emitted by excitation with a weak modulated measuring beam (1.9 W m^{-2}), and the yield varied between 1 relative unit (dark-level, F_0) and somewhat more than 4 relative units (maximal level, F_M) upon application of 500-ms pulse of saturating light (500 W m^{-2}). The relative ratio of linear electron flow can be estimated from photochemical quenching: it is assumed that photochemical quenching is selectively suppressed by the saturation pulses, while non-photochemical quenching is not. Hence, to a first approximation, the amplitude of the pulse light-induced spikes represents a relative measure of the rate of

photochemical charge separation at a given intensity of actinic light (Schreiber et al. 1986).

Results and Discussion

Occurrence of thylakoid-bound ascorbate peroxidase — The intact spinach chloroplasts were prepared using an ascorbate-containing media and disrupted by osmotic shock in an ascorbate-containing hypotonoic medium. The thylakoids and stroma were separated by centrifugation, and the thylakoids were suspended in the ascorbate-containing assay medium. The thylakoids showed a nearly equal activity of ascorbate peroxidase to that in the stroma of spinach chloroplasts (Table II-1). The ascorbate peroxidase activity was lost by boiling the thylakoids for 3 min. Activity of ascorbate peroxidase was detectable in the thylakoids only when the ascorbate-containing media were used throughout the isolation of chloroplasts and thylakoids, and the omission of ascorbate at any step caused the inactivation of the enzyme.

Table II-1. *Distribution of ascorbate peroxidase, MDA reductase and DHA reductase in stroma and thylakoid fractions of spinach chloroplasts.* The stroma and thylakoids were separated by centrifugation after osmotic shock of intact chloroplasts, all using ascorbate-containing media, and their enzyme activities were determined as described in Materials and Methods. The detection limits of ascorbate peroxidase, MDA reductase and DHA reductase were 0.1 (ascorbate), 0.2 (NAD(P)H) and 0.01 (ascorbate) µmol oxidized or reduced mg Chl^{-1} min^{-1}, respectively.

Enzyme activity	Stroma (µmol substrate oxidized or reduced mg Chl^{-1} min^{-1})	Thylakoids	
Ascorbate peroxidase	12.9 ± 1.1	11.6 ± 2.3	(n=7)
MDA reductase (NADH)	10.1 ± 0.8	0.0	(n=3)
(NADPH)	9.2 ± 0.9	0.0	(n=3)
DHA reductase	1.51 ± 0.08	0.00	(n=3)

Groden and Beck (1979) showed the occurrence of ascorbate peroxidase in spinach thylakoids. However, Nakano and Asada (1981) have failed to show the enzyme activity in the thylakoids, and found the activity only in the stroma. As shown later, the thylakoid-bound ascorbate peroxidase is rapidly inactivated in the absence of ascorbate, and this would be a reason why Nakano and Asada (1981) could not find the activity in the thylakoids which had been washed with an ascorbate-depleted medium. In contrast to ascorbate peroxidase, both NADH- and NADPH-dependent activities of MDA reductase, and DHA reductase were localized only in the stroma (Table II-1), as previously reported (Hossain and Asada 1984, Nakano and Asada 1981).

Thylakoid membranes were fractionated into grana thylakoids and stroma thylakoids using a Yeda pressure cell. The fraction of stroma thylakoids showed a high ratio of Chl *a*/*b* reflecting its enrichment in PS I complex. Although ascorbate peroxidase

Table II-2. *Distribution of ascorbate peroxidase in the stroma thylakoids and grana thylakoids of spinach chloroplasts.* The thylakoids isolated from intact chloroplasts (40 μg Chl) were fractionated into stroma thylakoids and grana thylakoids using an Yeda pressure cell and centrifugation in an ascorbate-containing assay medium, as described in Materials and Methods. Ascorbate peroxidase was assayed by the standard method.

	Grana Thylakoids	Stroma Thylakoids
Total Chl (mg)	28	8
Chl *a*/Chl *b*	2.1	4.7
Ascorbate peroxidase (Ascorbate oxidized μmol min^{-1})	157	113
Ascorbate peroxidase/mg Chl	5.6	14.1
P700/Ascorbate peroxidase[a)]	1.4	5.7

a) The molar ratio of P700 to ascorbate peroxidase is estimated as described in the text.

was equally found in the stroma thylakoids and grana thylakoids, the activity on the basis of chlorophyll in the stroma thylakoids

is 2.5-fold higher than that in grana thylakoids (Table II-2). If I assume that the molar ratio of Chl to P700 is 262 in the stroma thylakoids and 2600 in the inside-out grana thylakoids of spinach chloroplasts (Anderson and Melis 1983), the molecular weight of ascorbate peroxidase is 32,000 (Nakano and Asada 1987) and its specific activity is 580 μmol AsA oxidized mg protein^{-1} min^{-1} (Chen and Asada 1989), the molar ratios of P700 to ascorbate peroxidase in the stroma thylakoids and the grana thylakoid are estimated to be 5.6 and 1.4, respectively, from the activities in Table I-2.

To understand the binding state of ascorbate peroxidase to the thylakoid membranes, extraction of the enzyme with several compounds was tested. The thylakoids (0.43 mg Chl) was suspended in 2 ml of the following 1 mM ascorbate-containing media: a) 2 mM EDTA and 2 mM HEPES-KOH, pH 7.0, b) 1 M KCl and 50 mM potassium phosphate, pH 7.0, c) 2 M NaBr and 10 mM HEPES-KOH, pH 7.0, and d) 2 M NaSCN and 10 mM HEPES-KOH, pH 7.0, for 60 min at 0°C. Subsequently, the mixture was centrifuged at 100,000 x g for 60 min. Over 95% activity of ascorbate peroxidase was retained in the sedimented thylakoids in all media. Thus, metal cations, electrostatic interaction and hydrophobic interaction with thylakoid proteins would not participate in the binding of ascorbate peroxidase to the thylakoids. On the contrary, thylakoid-bound ascorbate peroxidase could be solubilized by detergents. When the thylakoids (13 μg Chl) were dissolved in 1 ml of either 1.0% heptyl-thioglucoside, 1.0% CHAPSO or 1.0 and 1.5% octyl-glucoside in 50 mM potassium phosphate, pH 7.0, and 1 mM ascorbate, the enzyme was nearly all recovered in the supernatant after centrifugation at 100,000 x g for 60 min, and little activity was found in the sediments. The activity in the supernatants was not lost for, at least, three days at 5°C. However, when the thylakoids were dissolved in 1 ml of either 0.1% polyethyleneglycol-*p*-isooctylphenyl ether (NP-40), 0.1% Triton X-100 or 0.1% lauryldimethylamine oxide (LDAO), the activity was lost within 5 min.

Properties of thylakoid-bound ascorbate peroxidase —— Properties of the thylakoid-bound enzyme were studied using

either the thylakoids isolated from intact chloroplasts (thylakoid enzyme) or the supernatant obtained by centrifugation of the 1.5% octyl-glucoside-solubilized thylakoids (solubilized enzyme).

Specificity of the electron donor — Both the thylakoid and solubilized ascorbate peroxidases were able to oxidize ascorbate by hydrogen peroxide, but not GSH, Cyt *c*, NADH and NADPH. Pyrogallol was oxidized at a rate of about 10% of ascorbate (Table II-3). A high specificity of this peroxidase to ascorbate is similar to that of ascorbate peroxidase from the stroma of spinach chloroplasts (Nakano and Asada 1987) and of chloroplastic isozyme from tea (Chen and Asada 1989).

Table II-3. *Specificity of electron donors of ascorbate peroxidase from spinach thylakoids in the thylakoid-bound and solubilized forms.* The thylakoid ascorbate peroxidase (5 μg Chl) or the solubilized enzyme was assayed from the initial oxidation rate (5-10 s) of the respective donors as described in the Materials and Methods, starting the reaction by the addition of the enzyme (5 μl) containing 1 mM ascorbate. In the case of pyrogallol, the maximum rate attained after a lag period was recorded. The thylakoid and solubilized ascorbate peroxidases gave the oxidation rates of ascorbate of 12.1 and 12.7 μmol mg Chl^{-1} min^{-1}, respectively, in the control.

	Relative Activity (%)	
Electron donor	Thylakoid ascorbate peroxidase	Solubilized ascorbate peroxidase
Ascorbate (0.5 mM)	100	100
NADPH (0.15 mM)	0.0	0.0
NADH (0.15 mM)	0.0	0.0
GSH (1 mM)	0.0	0.0
Pyrogallol (20 mM)	11.9	8.5
Cyt *c* (40 μM)	0.0	0.0

Kinetic properties — Thylakoid and solubilized ascorbate peroxidases showed a pH optimum at 6.50 and 6.75, respectively, in 50 mM potassium phosphate buffer, which are a little lower than that of stromal ascorbate peroxidase (Fig. II-1). The

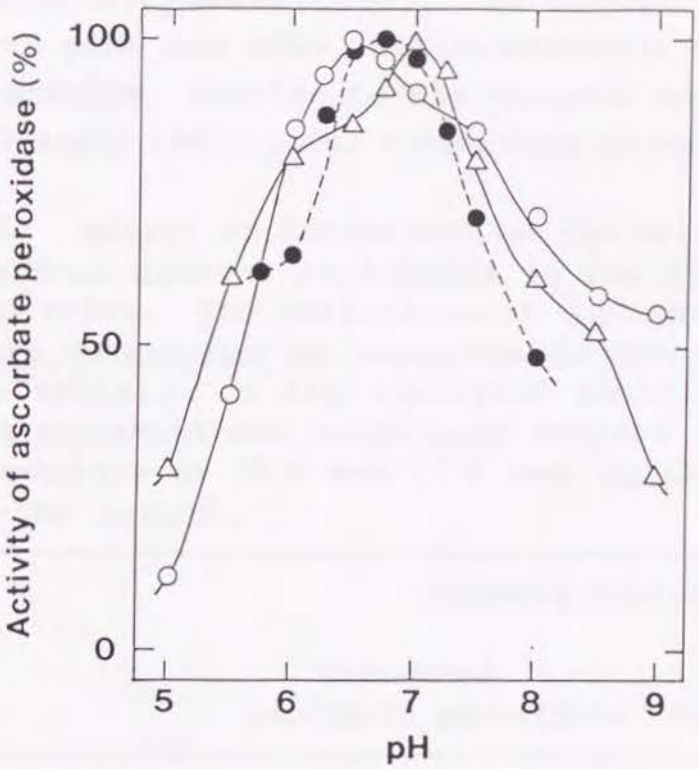


Fig. II-1 *The effect of pH on the activities of ascorbate peroxidase from spinach chloroplasts in the thylakoid-bound form, solubilized form from the thylakoids and the stroma.* The reaction mixture (1 ml) contained 0.33 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 0.5 mM KH_2PO_4, 0.5 mM ascorbate, the enzyme and the following buffers; pH 5.0-5.5, 50 mM MES-KOH; pH 6.0-7.5, 50 mM potassium phosphate; pH 8.0-9.0, 50 mM Tricin-KOH. The enzyme activities were assayed by the addition of 0.1 mM hydrogen peroxide as described in Materials and Methods. The enzyme used was the thylakoid fraction (open circle), 1.5% octyl-glucoside solubilized enzyme (closed circle) or the stroma fraction (triangle) which gave the oxidation rate of ascorbate of 9.7, 9.1 or 7.3 μmol mg Chl^{-1} min^{-1}, respectively, at pH 7.0.

apparent K_m values for ascorbate and hydrogen peroxide of the thylakoid enzyme were determined to be 480 μM and 26 μM, and those of the solubilized enzyme were 490 μM and 23 μM, respectively, from the non-linear regression analysis using the Michaelis-Menten's equation (Sakoda and Hiromi 1976). Affinities of thylakoid and solubilized enzymes to ascorbate are lower than that of the stromal ascorbate peroxidase (K_m; 300 μM, Nakano and Asada 1987).

Inhibitors — Both the thylakoid and solubilized ascorbate

peroxidases were inhibited by cyanide and azide, similar to other heme peroxidases (Table II-4). In addition, the enzyme was sensitive to pCMB and DTNB, but insensitive to monoiodoacetate and iodoacetamide, similar to the stromal ascorbate peroxidase (Nakano and Asada 1987). The inhibition by the thiol reagents is

Table II-4. *Effect of inhibitors on the activity of ascorbate peroxidase from spinach thylakoids in the thylakoid-bound and solubilized forms.* The activities of thylakoid and solubilized enzymes were determined as described in Materials and Methods, except the addition of the indicated inhibitors prior to the assay. The thylakoid and solubilized enzymes gave the oxidation rates of ascorbate of 12.1 and 12.9 μmol mg Chl^{-1} min^{-1}, respectively, in the control.

	Relative Activity (%)	
Inhibitor	Thylakoid ascorbate peroxidase	Solubilized ascorbate peroxidase
None	100	100
KCN (1 mM)	0.0	0.0
NaN_3 (2.5 mM)	62	58
(5 mM)	39	31
pCMB (50 μM)	0.0	0.0
Iodoacetamide		
(2.5 mM)	100	97
(5 mM)	92	94
Iodoacetic acid		
(2.5 mM)	100	100
(5 mM)	89	86
DTNB (0.1 mM)	27	33

one of the characteristic properties of ascorbate peroxidase, distinguished from guaiacol peroxidase, which can be inferred by their amino acid sequences (Chen et al. 1992b).

Inactivation in ascorbate-depleted medium — One of the specific properties of ascorbate peroxidase is rapid inactivation in an ascorbate-depleted medium. This is also the case of the thylakoid ascorbate peroxidase in the thylakoid-bound and solubi-

lized forms. When the solubilized (data not shown) or thylakoid enzyme was diluted with the ascorbate-depleted medium, the peroxidase activity was lost. The inactivation was the first order with respect to the remaining activity with a half time of about 15 s (Fig. II-2). The enzyme in the stroma also was inactivated

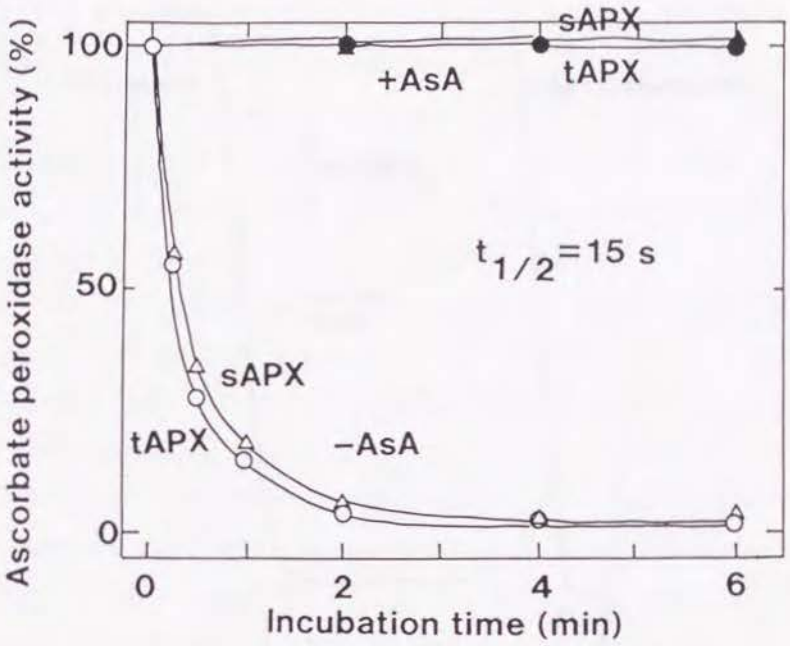


Fig. II-2 *Inactivation of thylakoid-bound and stromal ascorbate (AsA) peroxidases from spinach chloroplasts in ascorbate-depleted medium.* The thylakoids and stroma were separated from intact spinach chloroplasts as described in Materials and Methods. The thylakoid (5 µl, 1.1 µg Chl, tAPX, open circle) and the stroma (5 µl, sAPX, open triangle) fractions in the assay medium containing 1 mM ascorbate were diluted with 1 ml of 50 mM potassium phosphate, pH 7.0. At the indicated times after the dilution, 0.5 mM ascorbate was added, and the ascorbate peroxidase activity was assayed by the addition of 0.1 mM hydrogen peroxide. Where indicated (+AsA), the dilution was done with the buffer containing 0.5 mM ascorbate (tAPX, closed circle and sAPX, closed triangle).

at the similar rate, in accordance to that of the soluble ascorbate peroxidases (Nakano and Asada 1987, Chen and Asada 1989).

These results indicate little difference in properties between thylakoid and solubilized ascorbate peroxidases except for a small difference in the pH-activity curves. Thus, ascorbate

peroxidase seems to be bound to the thylakoids in such a form that the active site of the enzyme is exposed to the stroma for the access of the substrates.

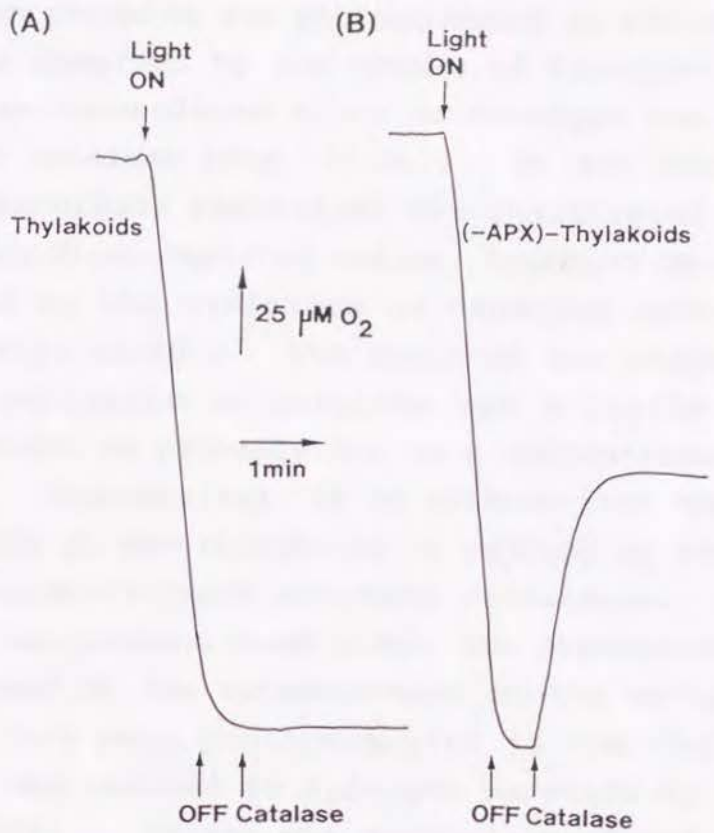


Fig. II-3 *Reduction of the hydrogen peroxide generated in illuminated thylakoids by ascorbate catalyzed with the thylakoid-bound ascorbate peroxidase (APX).* In trace (A), the reaction mixture (1 ml) contained the spinach thylakoids (30 μg Chl) which had been prepared from intact chloroplast as described in Materials and Methods, 1 mM methyl viologen and 0.1 μM nigericin in the assay medium containing 1 mM ascorbate. In trace (B), the thylakoids (30 μg Chl) whose APX activity had been inactivated by the treatment with an ascorbate-depletion assay medium. For the treatment, thylakoids (7.5 mg Chl) in 5 ml of the 1 mM ascorbate containing assay medium were diluted with 250 ml of the ascorbate-depleted assay medium. After 10 min the diluted thylakoids were centrifuged at 5,500 x g for 10 min, and the sedimented thylakoids suspended in the ascorbate-depleted assay medium were centrifuged again under the same conditions. The sedimented thylakoids were suspended in 4 ml of the 1 mM ascorbate-containing assay medium for the measurememt. After the light was turned off, catalase (280 units) was added. The oxygen uptake under the illumination (320 W m^{-2}) and the catalase-dependent oxygen evolution were measured using an oxygen electrode.

Reduction of hydrogen peroxide by ascorbate catalyzed with thylakoid-bound ascorbate peroxidase — If the thylakoid-bound ascorbate peroxidase operates for the scavenging of the hydrogen peroxide photoproduced in the thylakoids, no hydrogen peroxide would be accumulated in the presence of ascorbate in the light. The hydrogen peroxide was photoproduced in the presence of methyl viologen as observed by the uptake of dioxygen, but no hydrogen peroxide was accumulated since no dioxygen was evolved upon the addition of catalase (Fig. II-3A). On the other hand, when the thylakoid-ascorbate peroxidase was inactivated by the treatment with the ascorbate-depleted medium, hydrogen peroxide accumulated as observed by the evolution of dioxygen upon the addition of catalase (Fig. II-3B). The ratio of the oxygen uptake to that of oxygen evolution by catalase was a little lower than that expected, which is probably due to a contaminated catalase in the thylakoids. Nevertheless, it is evident that the hydrogen peroxide generated in the thylakoids is reduced by ascorbate catalyzed with the thylakoid-bound ascorbate peroxidase.

Under the present conditions, the superoxide was the primary product formed by the autooxidation of the methyl viologen radicals which had been photogenerated in the thylakoids, and the superoxide was reduced to hydrogen peroxide by ascorbate (Allen and Hall 1974). If the MDA radicals produced in the reduction of the superoxide radicals and also in the reaction of ascorbate peroxidase are spontaneously disproportionated to ascorbate (AsA) and DHA, the molar ratio of the ascorbate oxidized (to DHA) to the apparent oxygen uptake is expected to be 2, as follows:

$$2\ H_2O \longrightarrow O_2 + 4\ H^+ + 4\ [e^-] \qquad \text{(PS II)}$$

$$4\ [e^-] + 4\ O_2 \longrightarrow 4\ O_2^-$$

(Methyl viologen-mediated eaction in PSI)

$$4\ O_2^- + 4\ AsA \longrightarrow 4\ H_2O_2 + 4\ MDA \qquad \text{(Spontaneous reaction)}$$

$$4\ H_2O_2 + 8\ AsA \longrightarrow 8\ MDA + 8\ H_2O \qquad \text{(Ascorbate peroxidase)}$$

$$12\ MDA \longrightarrow 6\ AsA + 6\ DHA$$

(Spontaneous disproportionation)

$$3\ O_2 + 6\ AsA \longrightarrow 6\ H_2O + 6\ DHA \qquad \text{(Sum)}$$

I determined the decrease of ascorbate during the illumination, and found that it was lower than that expected from the oxygen uptake (Fig. II-4). These observations suggest that ascorbate

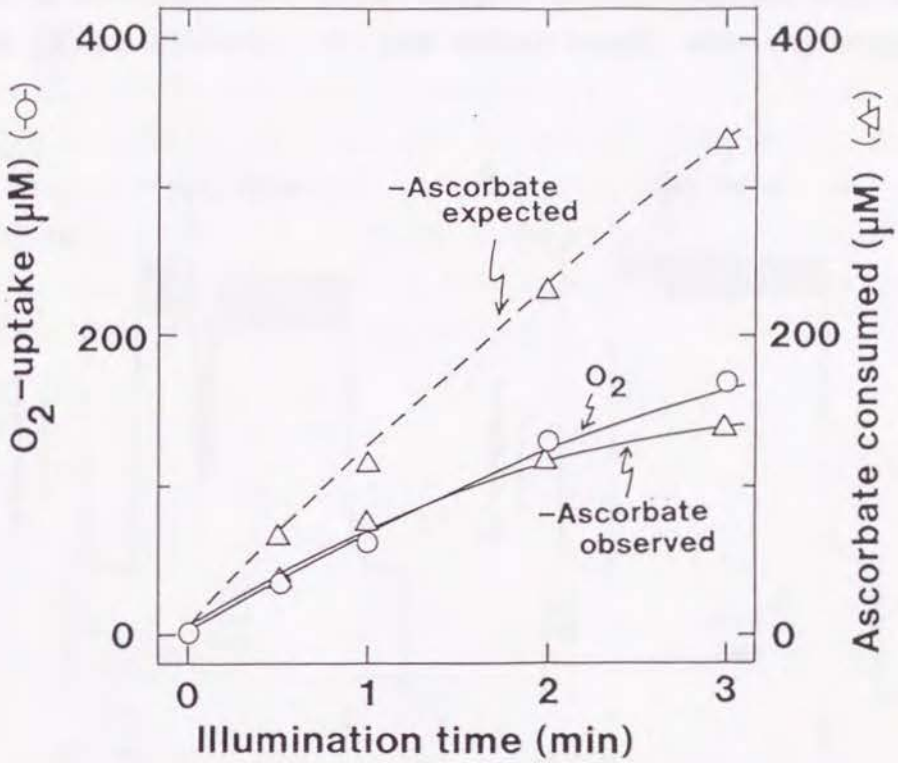


Fig. II-4 *Stoichiometry of ascorbate oxidation coupled to the reduction of superoxide and hydrogen peroxide produced in the thylakoids.* The reaction mixture (1 ml) in the oxygen electrode vessel contained the thylakoids (30 μg Chl), 1 mM methyl viologen and 0.1 μM nigericin in the assay medium containing 1 mM ascorbate. After the light was turned on, the uptake of dioxygen was monitored and at indicated times aliquots of the reaction mixture was sampled. The concentration of ascorbate in the sampled aliquots was determined by the decrease in absorbance at 265 nm upon the addition of ascorbate oxidase in 0.1 M potassium phosphate, pH 5.6 (Foyer et al. 1983). The expected decrease of ascorbate is estimated from the oxygen uptake, assuming that no photoregeneration of ascorbate occurred in the thylakoids.

is photoregenerated from either MDA or DHA by the electron transport system of the thylakoids. I examined such a reaction by the quenching of chlorophyll fluorecence.

Hydrogen peroxide induces the quenching of chlorophyll

fluorescence in intact chloroplasts (Neubauer et al. 1989) and algal cells (Chapter V), which has been inferred by the photoreduction of hydrogen peroxide with the peroxidase system using a photoreductant as the electron donor. When hydrogen peroxide was added to the thylakoids which had been prepared in the ascorbate-containing medium, the chlorophyll fluorescence was transiently quenched (Fig. II-5A). On the other hand, when hydrogen peroxide

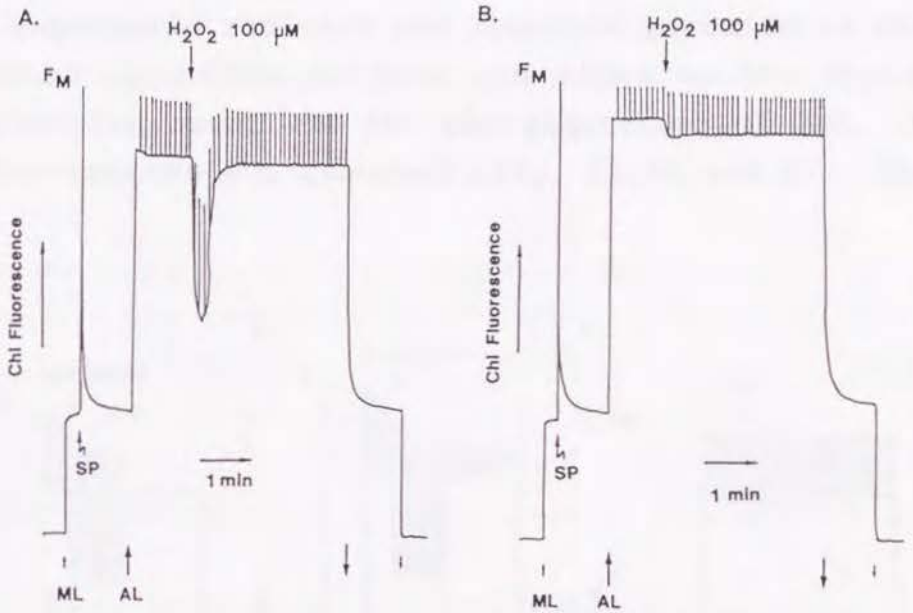


Fig. II-5 *Effect of hydrogen peroxide on chlorophyll fluorescence in thylakoid membranes.* Fluorescence yield was monitored with a weak, modulated measuring light (ML) and the maximum yield (F_M) was induced by 500-ms pulses of saturaing white light (SP). Illumination of continuous actinic light (AL, red light) was started by the AL arrow. (A) The reaction mixture (1.0 ml) contained thylakoid membranes (60 μg Chl), 0.1 μM nigericin in the assay medium containing 1 mM ascorbate. Hydrogen peroxide (100 μM) was added 60 s after the actinic light was turned on. (B) The reaction mixture was the same as in (A), but thylakoid membranes had been treated with the ascorbate-depleted assay medium as in Fig. II-3.

was added to thylakoid membranes whose peroxidase was inactivated by either 1 mM KCN or 50 μM pCMB, no transient quenching of chlorophyll fluorescence was observed (data not shown). This is also the case when the thylakoid-bound peroxidase was inactivated by the treatment with the ascorbate-depleted medium and the treated

thyalkoids were tested in the ascorbate-containing medium (Fig. II-5B). Most of the transient quenching of chlorophyll fluorescence by hydrogen peroxide was accounted for by the photochemical quenching. These findings indicate that the thylakoids can photoreduce the oxidation product of ascorbate, MDA or DHA, in a reaction of the thylakoid-bound ascorbate peroxidase.

Photoreduction of monodehydroascorbate radicals by thylakoids — Ascorbate oxidase catalyzes the four electron oxidation of ascorbate producing four MDA radicals and water, but dose not release superoxide radicals and hydrogen peroxide as the intermediate. When ascorbate oxidase was added to the thylakoid membranes containg ascorbate for the generation of MDA, the chlorophyll fluorescence was quenched (Fig. II-6A and B). The quench-

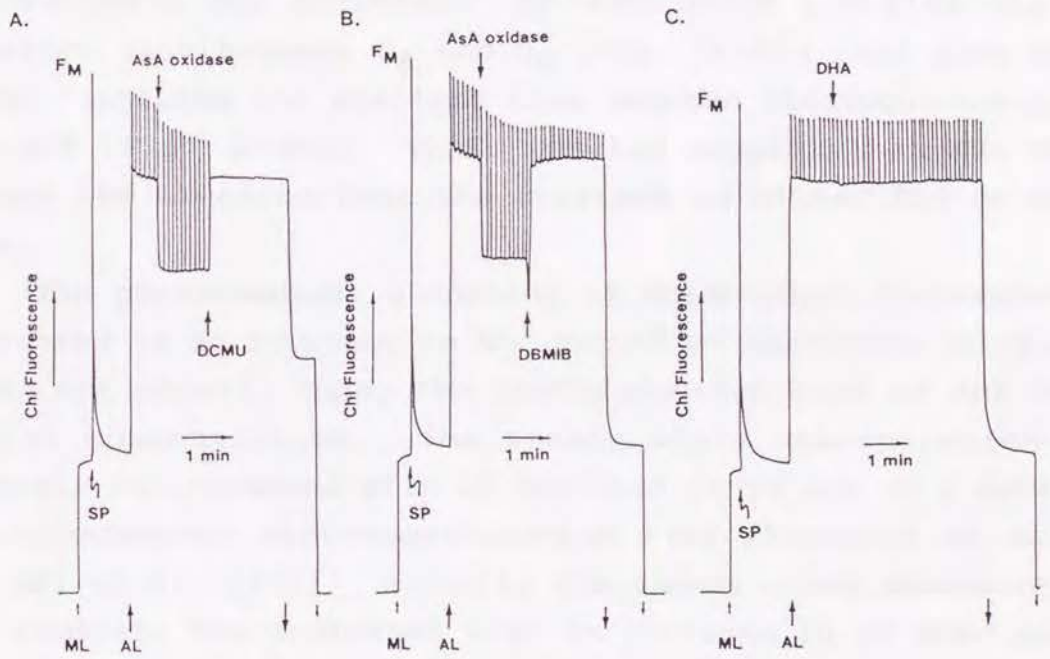


Fig. II-6 *Quenching of chlorophyll fluorescence by MDA radical in thylakoid membranes.* Fluorescence was measured as in Fig. II-5. The reaction mixture (1 ml) contained thylakoid membranes (60 µg Chl) in the assay medium, in which the concentration of ascorbate was 0.5 mM. Ascorbate oxidase (0.35 unit, A and B) which gave a steady state concentration of MDA radical of 2.1 µM or 2 mM DHA (C) was added 60 s after the actinic light was turned on. DCMU (10 µM, A) and DBMIB (0.5 µM, B) were added at a time, where indicated.

ing by MDA radicals was also observed with either the thylakoid membranes which had been washed in an ascorbate-depleted medium or the membranes which had been treated with pCMB (data not shown). Thus, the inactivation of thylakoid-bound ascorbate peroxidase did not affect the MDA-induced quenching. Further, pCMB is an inhibitor of MDA reductase (Hossain and Asada 1985), which excludes the participation of the thylakoid-bound MDA reductase, if any, in the quenching, although I could not detect MDA reductase in the thylakoids (Table II-1). In contrast to MDA radicals, DHA did not induce any quenching of chlorophyll fluorescence (Fig. II-6). These findings indicate that the primary product in a reaction of ascorbate peroxidase, MDA (Hossain et al. 1984), could be directly photoreduced by the thylakoids, but DHA not.

The ascorbate oxidase-induced quenching of chlorophyll fluorescence was inhibited by DCMU which inhibits the linear electron flow between Q_A and Q_B (Fig. II-6A), and also by DBMIB which inhibits the electron flow between plastoquinone-pool and Cyt *b/f* (Fig. II-6B). These results suggest that MDA radicals accept the electron from the carriers at either PSI or *b/f* complex.

The photochemical quenching of chlorophyll fluorescence was increased as an increase in the amount of ascorbate oxidase added (data not shown), thus, the photoreduction rate of MDA depended on its concentration. The steady state concentration of MDA radicals is increased with an increase in pH due to a decrease of its spontaneous disproportionation rate (Yamazaki et al. 1960, Bielski et al. 1971). Actually the steady state concentration of MDA radicals was increased with an increase in pH when generated by ascorbate oxidase. In parallel with an increase in the concentration of MDA, the degree of the quenching was increased when MDA radicals were generated by either ascorbate oxidase or the thylakoid-bound ascorbate peroxidase plus hydrogen peroxide (Fig. II-7). Even at high pH, the concentration of MDA was below 10 μM, which is only approximate to the K_m value for its photoreduction as determined in Fig. II-8. Therefore, theresults of Fig.

II-7 shows the dependence of the photoreduction of MDA on its concentrations rather than an pH. Difficulty to generate the MDA radicals at high concentrations did not allow to estimate the optimum pH for the photoreduction.

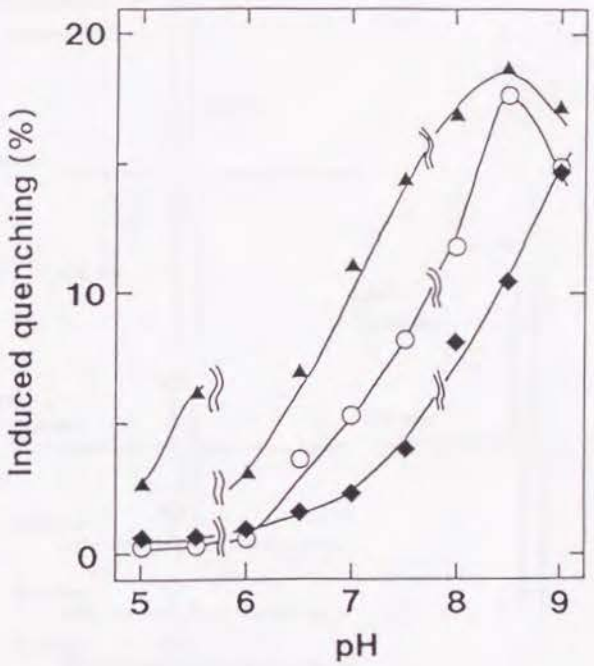


Fig. II-7 *The effect of pH on MDA-dependent and hydrogen peroxide-dependent quenchings of chlorophyll fluorescence in the thylakoid membranes from spinach intact chloroplasts.* The reaction mixture (1 ml) contained thylakoid membranes (60 µg Chl) and 0.1 µM nigericin in the buffers as in Fig. II-1. Hydrogen peroxide-dependent quenching (closed triangle) was determined after the addition of 100 µM hydrogen peroxide, as in Fig. II-5. MDA radical-dependent quenching (open circle) was determined by the addition of ascorbate oxidase (0.21 unit) which gave a steady state concentration of MDA of 1.2 µM, pH 7.0, as in Fig. II-6. Under the same conditions, the steady state concentration of MDA radical (closed diamond) was determined from the absorbance at 360 nm using an absorption coefficient of 3.3 mM^{-1} cm^{-1}. The hydrogen peroxide or MDA-dependent photochemical quenching is shown by the difference between the quenching before and after the addition of hydrogen peroxide or ascorbate oxidase. The maximum values of quenching after the addition were used.

Further evidence for the photoreduction of MDA radicals is the disappearance of EPR signal of the radical generated by

ascorbate oxidase in the thylakoids on illumination (Fig. II-8). When the linear electron flow was blocked by DCMU, no decrease of MDA radical was found. In the dark little decrease of the EPR signal of MDA radicals was observed (data not shown).

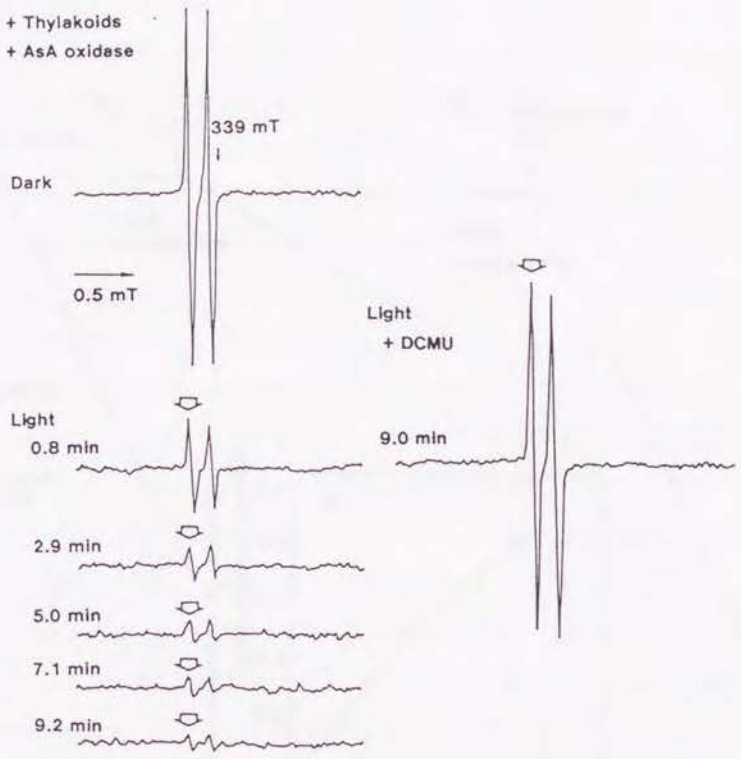


Fig. II-8 *Photoreduction of MDA radical generated by ascorbate oxidase in thylakoids as measured by EPR spectrum.* The reaction mixture (1.0 ml) contained the 1 mM ascorbate-containing assay medium, thylakoid membranes (60 μg Chl) and ascorbate oxidase (1.27 unit) which produced 4 μM MDA radical. Where indicated, 10 μM DCMU was added before the light was turned on. More than 80% of MDA signal was kept for 20 min in the dark. EPR conditions were set as follows: Microwave power, 3 mW; Sweep time, 2 min; Receiver gain, 1.6 x 100; Time constant, 0.3 s; Modulation amplitude, 0.032 mT. Time shown at the left side of EPR signals is the time when EPR signal with open arrow was recorded after the light was turned on.

The photoreduction of MDA radicals in the thylakoids should be accompanied by the evolution of dioxygen in PS II. To confirm the MDA-dependent evolution of dioxygen, oxygen uptakes by the

oxidation of ascorbate in a reaction catalyzed with ascorbate oxidase (Fig. II-9A) and by the photoreduction of dioxygen in the thylakoids (Fig. II-9B) were separately determined. The photoreduction of dioxygen was determined in the presence of ascorbate, but pCMB was added to inhibit the thylakoid-bound peroxidase.

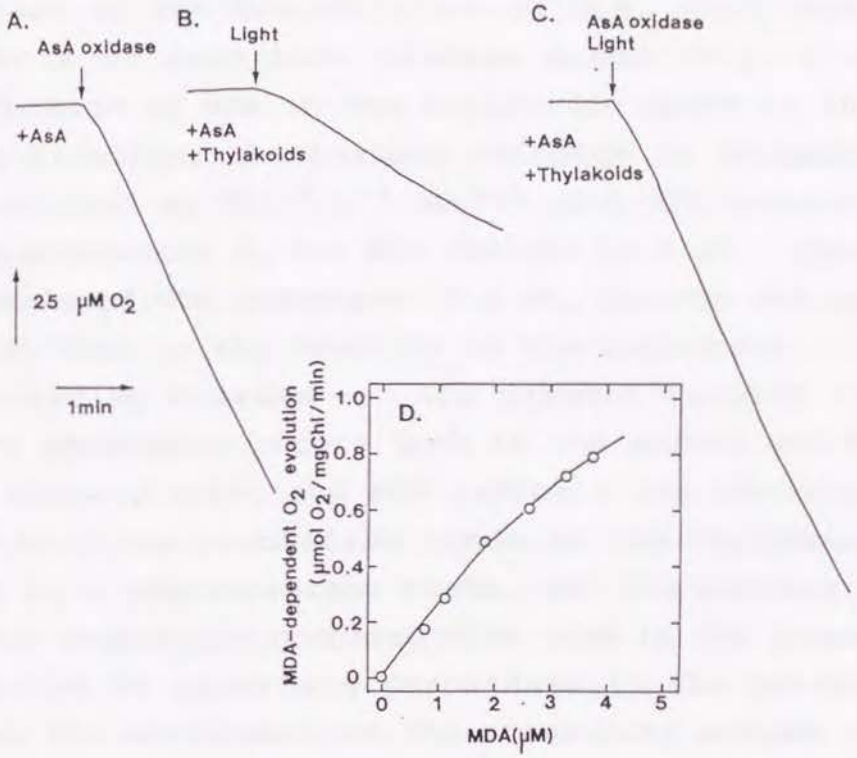


Fig. II-9 *MDA radical-dependent oxygen evolution in illuminated thylakoids from spinach intact chloroplasts.* (A) The reaction mixture (1 ml) contained the assay medium containing 1 mM ascorbate (AsA) and ascorbate oxidase (0.58 unit), and the oxygen uptake due to the oxidase activity was measured in the dark. (B) The reaction mixture (1 ml) contained the assay medium, thylakoid membranes (60 μg Chl) and 50 μM pCMB, and the photoreduction of dioxygen was measured under illumination at 320 W m^{-2}. (C) The reaction mixture was the same as in (B), but the same amounts of ascorbate oxidase as in (A) was added. The oxygen uptake was measured under illumination at the same intensity as in (B). (D) The differences of O_2 uptake rates [(A) + (B)-(C)], corresponding to the MDA radical-dependent O_2 evolution, are plotted against the steady state concentrations of MDA radical, which were adjusted by the various amounts of ascorbate oxidase.

Subsequently, the oxygen uptake by illuminated thylakoids was determined in the presence of the same amounts of ascorbate oxidase as in A (Fig. II-9C). The rate of oxygen uptake in C was lower than the sum of those determinated separately in A and B. The difference [(A + B) - C] would represent the oxygen evolution for the photoreduction of MDA generated by ascorbate oxidase.

The MDA-dependent evolution of dioxygen was increased with an increase in the concentration of MDA, which was adjusted by the amounts of ascorbate oxidase added (Fig. II-9). If the photoreduction of MDA in the thylakoids obeys to the Michaelis-Menten's kinetics, the maximum velocity is estimated to be 148 μmol O_2 evolved mg Chl^{-1} h^{-1} or 592 μmol MDA reduced mg Chl^{-1} h^{-1}, and the apparent K_m for MDA radical is 8 μM. The K_m value for MDA radicals of MDA reductase (1.4 μM, Hossain and Asada 1985) is lower than that in the reaction in the thylakoids.

Concluding remarks — The present results indicate that ascorbate peroxidase occurs both in the stroma and thylakoids of spinach chloroplasts, and MDA radicals are photoreduced in the thylakoids. The peroxidase binds to the thylakoid membranes, probably in a transmembrane state, and its content is higher in the stroma thylakoids compared with that in the grana thylakoids. Localization of ascorbate peroxidase in the stroma thylakoids indicates the enrichment of the scavenging enzyme in PS I where superoxide radicals and hydrogen peroxide are photogenerated. The thylakoid-bound ascorbate peroxidase shares the enzymatic properties with the stromal enzyme, which are distinguished from those of guiaiacol peroxidase localized in cell walls and cytosol (Chen et al. 1992, Asada 1992). Although the similarity between the thylakoid-bound and stromal ascorbate peroxidases in their enzymatic properties, the bound enzyme showed a higher molecular weight than that of the stromal enzyme (Chapter III). Thus, the molecular species of the thylakoid-bound ascorbate peroxidase appears to be different from that of the chloroplastic ascorbate peroxidase purified so far from spinach (Nakano and Asada 1987, Tanaka et al. 1991) and tea (Chen and Asada 1989).

MDA radical-induced quenching of chlorophyll fluorescence (Fig. II-6 and II-7) and oxygen evolution (Fig. II-9), and the disappearance of EPR signals of MDA radicals in illuminated thylakoids (Fig. II-8), all indicate the photoreduction of MDA radicals in the thylakoids. No evidence for the participation of the stromal MDA reductase in the photoreduction is obtained. The inhibition by DCMU and DBMIB shows that PS II is not the site for the reduction of MDA (Fig. II-6 and II-8). No reduction of MDA by reduced ferredoxin generated with the NADPH-ferredoxin NADP reductase system (Chapter V) indicate that ferredoxin is not an electron carrier for the photoreduction of MDA radicals. At present PS I seems to be a possible site of the reduction, but, Cyt *b* 563 in the *b/f* complex is another possible site. It has been shown that in mitochondria Cyt *b* in the outer membranes catalyzes the reduction of MDA (Nishino and Itoh 1986). In chromatic granule membranes from bovine adrenal medulla, Cyt *b* 561 mediates the transmembrane transfer of one electron from ascorbate outside the membranes to MDA radical inside the membranes (Kelly et al. 1990, Dhariwal et al. 1991).

The reduction of the hydrogen peroxide produced in PS I by the thylakoid-bound ascorbate peroxidase, and the photoreduction of the primary oxidation product of the peroxidase reaction in the thylakoids allow to scavenge the hydrogen peroxide with catalytic amounts of ascorbate in thylakoids.

$$H_2O_2 + 2\ \text{Ascorbate} \xrightarrow{\text{(Thylakoid-bound ascorbate peroxidase)}} 2\ H_2O + 2\ \text{MDA}$$

$$2\ \text{MDA} + 2\ [e^-] \longrightarrow 2\ \text{Ascorbate}$$

($[e^-]$ represents the electron generated in thylakoids)

I propose that this short cycle is the primary scavenging system of hydrogen peroxide in chloroplasts (Fig. II-10). Even when the photoproduction rate of hydrogen peroxide is enhanced by methyl viologen, the thylakoids can reduce all of the hydrogen peroxide in the presence of ascorbate (Fig. II-3). Under the conditions of Fig. II-4, about a half of MDA radicals is failed

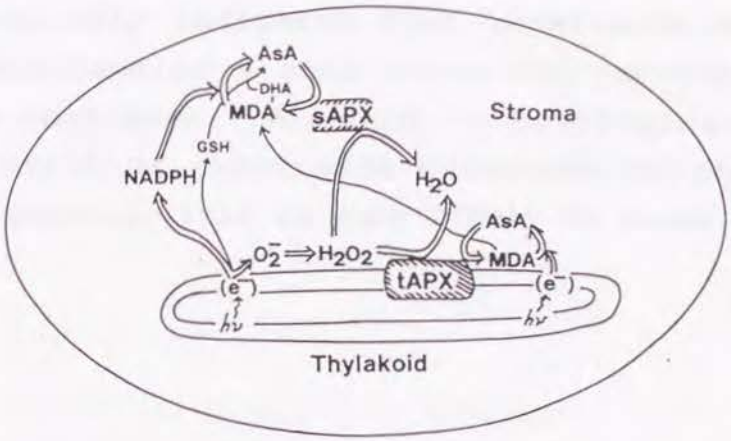


Fig. II-10 *Microlocalization of hydrogen peroxide-scavenging systems in chloroplasts.* Dioxygen is univalently photoreduced in PS I of thylakoids, and the superoxide anion radicals are disproportionated to hydrogen peroxide and dioxygen by superoxide dismutase. The hydrogen peroxide is reduced to water by the thylakoid-bound ascorbate peroxidase (tAPX), and ascorbate (AsA) is regenerated from the primary oxidation product in a reaction of ascorbate peroxidase, MDA, by its photoreduction in the thylakoids. The hydrogen peroxide and MDA, which are escaped from the interaction in the thylakoids, are reduced by stroma ascorbate peroxidase (sAPX) and MDA reductase in the stroma, respectively. DHA reductase is localized in the stroma, which would regenerate ascorbate from the DHA produced from MDA through its disproportionation.

to be photoreduced to ascorbate. In chloroplasts, however, its photoreduction ratio should be higher, because of a slow diffusion of the MDA radicals from the surface of the membranes due to a high viscosity of the stroma and, further, of a high maximum photoreducing activity of MDA of about 600 µmol mg Chl^{-1} h^{-1} (Fig. II-9).

The scavenging system of hydrogen peroxide in the stroma is composed of ascorbate peroxidase and the regeneration system of ascorbate by MDA reductase, DHA reductase and glutathione reductase. All of the participating enzymes have been found in the stroma and characterized (Asada and Takahashi 1987). The stromal system would be the secondary scavenging system for the hydrogen peroxide which is failed to be reduced by the primary, thylakoid

system and is produced in the stroma, if any (Fig. II-10). Occurrence of the thylakoid system for the scavenging of hydrogen peroxide inevitably indicates that superoxide dismutase also should be localized at a site where the ascorbate peroxidase binds to the membranes. Although no cytological evidence for microlocalization of superoxide dismutase in chloroplasts is available at present, this is very likely to occur.

Chapter III

Purification and Molecular Properties of the Thylakoid-Bound Ascorbate Peroxidase in Spinach Chloroplasts

Ascorbate peroxidase is a hydrogen peroxide-scavenging peroxidase which uses ascorbate as the electron donor. It has been found in photosynthetic organisms that include eukaryotic algae and protozoa (Asada 1992). In higher plants, two isozymes of APX with different cellular locations have been found, namely, chloroplastic and cytosolic APXs (Nakano and Asada 1987, Chen and Asada 1989, Mittler and Zilinskas 1991a, Tanaka et al. 1991, Elia et al. 1992). Recently an APX bound to chloroplast thylakoid has been found in addition to the APX that is localized in the stroma (Chapter II). The thylakoid-bound APX (tAPX) and the stromal, soluble APX (sAPX) scavenge the hydrogen peroxide that is produced by the superoxide dismutase-catalyzed disproportionation of superoxide anion radicals, which are produced by the univalent photoreduction of dioxygen in PS I (Asada et al. 1974). tAPX binds preferentially to the stromal thylakoids on which the PS I complex is localized (Chapter II). By contrast, cAPX is localized in cell compartments other than chloroplasts and also in non-photosynthetic tissues.

Although both APX and guaiacol peroxidases, such as horseradish peroxidase, are heme peroxidases, they are distinguishable in terms of their amino acid sequences and enzymatic properties (Asada 1992). The stromal and cytosolic APXs share similar molecular properties but they differ from each other in terms of several characteristics. As compared to cAPX, sAPX is rapidly inactivated in ascorbate-depleted medium. Moreover the sensitivity of sAPX to thiol-modifying reagents and to suicide inhibitors

is higher than that of cAPX. Finally sAPX is more specific for ascorbate as the electron donor than is cAPX.

In addition to the binding of APX to the thylakoid, the primary product of oxidation in the reaction catalyzed by tAPX, namely, the monodehydroascorbate radical, has been shown to be photoreduced in PS I (Chapter II). This photoreduction of the radical is mediated by ferredoxin (Chapter V). Thus, the tAPX and photoreduced ferredoxin in PS I form the primary system for scavenging hydrogen peroxide generated in the chloroplasts.

I report herein the solubilization of APX from spinach thylakoids by treatment with detergents and the purification of the enzyme to homogeneity. While the molecular weights of the two enzymes are different, the absorption spectrum, amino acid sequence of the amino-terminal region, and enzymatic properties of the purified thylakoid-bound APX are very similar to those of the stromal APX, but these features are clearly different from those of the cytosolic APX.

Materials and Methods

Assay of peroxidases — Ascorbate peroxidase activity was determined as described previously (Nakano and Asada 1981) in a reaction mixture (1 ml) that contained 50 mM potassium phosphate (pH 7.0), 0.1 mM hydrogen peroxide and 0.5 mM ascorbate. In this assay, no detergents were added, because no inactivation of tAPX due to its aggregation occurs within the time required for measurement of the initial velocity of the tAPX-catalyzed reaction. The hydrogen peroxide-dependent oxidation of ascorbate was followed by monitoring the decrease in absorbance at 290 nm assuming an absorption coefficient of 2.8 mM^{-1} cm^{-1}. One unit of ascorbate peroxidase is defined as the amount of enzyme that oxidizes 1 μmol of ascorbate per min at room temperature under the above conditions. The assay mixture (1 ml) for examination of other electron donors to the peroxidase contained 50 mM potassium phosphate, pH 7.0, 0.1 mM hydrogen peroxide and one of the

following electron donors: NAD(P)H (0.15 mM, absorbance at 340 nm, 6.2 mM^{-1} cm^{-1}), reduced Cyt *c* (40 µM, absorbance at 550 nm due to the reduced form, 19 mM^{-1} cm^{-1}) or pyrogallol (20 mM, absorbance at 430 nm, 2.47 mM^{-1} cm^{-1}). When GSH was the electron donor, the reaction mixture contained 0.14 mM NADPH and glutathione reductase so that I could monitor the production of GSSG by following the decrease in the absorbance at 340 nm (Little et al. 1970). The extent of hydrogen peroxide-dependent oxidation of the donors was determined from the absorption coefficients cited in parentheses.

Purification of thylakoid-bound ascorbate peroxidase -- Purification of the enzyme was carried out at 0-4°C, unless otherwise specified. Spinach leaves (1.1 kg) obtained from a local market, were homogenized with 2 liters of 50 mM potassium phosphate (pH 7.5)/0.1 mM EDTA/1 mM PMSF/5 mM ascorbate. After filtration through 12 layers of cheesecloth, the homogenate was centrifuged at 5,500 x g for 10 min. The pelleted thylakoids were suspended in 1 liter of 10 mM potassium phosphate (pH 7.5)/0.1 mM EDTA/1 mM PMSF/5 mM ascorbate (buffer A), and the suspension was centrifuged at 5,500 x g for 10 min. The pellets were washed again by the same procedure. The thylakoid-bound enzyme was solubilized from the washed thylakoids by the addition of 1.8% (w/v) octylglucoside (Dojin) in buffer A, at a thylakoid concentration of 1.5 mg Chl ml^{-1}, with stirring for 1 h. In the following purification steps, 1% (w/v) CHAPSO (Dojin) was added to buffers A. After centrifugation at 100,000 x g for 60 min, the supernatant was loaded onto a column of DEAE-Sephacel (5 cm i.d. x 18 cm) (Pharmacia) which had been equilibrated with buffer A. The column was washed with 1 liter of buffer A and the adsorbed enzyme was eluted with 2 liters of a linear gradient of KCl (0-0.2 M) in buffer A. The enzyme eluted at 0.1-0.15 M KCl was pooled and concentrated through an Amicon PM-5 membrane. The concentrated enzyme (10 ml) was loaded onto a column of Superdex-200 (2.6 cm i.d. x 60 cm) (Pharmacia) that had been equilibrated with 50 mM potassium phosphate (pH 7.5)/0.1 mM EDTA/1 mM PMSF/5 mM ascorbate/0.1 M KCl/1% (w/v) CHAPSO (buffer B) and was eluted

with the same buffer. The active fractions were collected and the concentration of KCl in the pooled fractions was lowered to below 0.1 mM by ues of an Amicon PM-5 membrane and buffer A.

The enzyme was then loaded onto a column (2.5 cm i.d. x 15 cm) of QAE-Toyopearl (TOSOH) that had been equilibrated with buffer A. The column was washed with 500 ml of the same buffer, and the enzyme was eluted with 1 liter of a linear gradient of KCl (0-0.2 M) in the same buffer. Ascorbate peroxidase eluted by 0.1-0.15 M KCl was pooled and concentrated by use of an Amicon PM-5 membrane. The concentrated enzyme was loaded onto a column (2.6 cm i.d. x 60 cm) of Superdex G-75 (Pharmacia) that had been equilibrated with buffer B. The concentration of KCl in the pooled enzyme was lowered to below 0.1 mM by use of an Amicon PM-5 membrane and buffer A. The enzyme was then loaded onto a column (1.6 cm i.d. x 10 cm) of Q-Sepharose (Pharmacia) that had been equilibrated with buffer A, and the column was washed with 200 ml of the same buffer. The adsorbed enzyme was eluted with 400 ml of a linear gradient of KCl (0-0.2 M) in buffer A, and the activity was found in the eluate at 0.15 M KCl. The enzyme was concentrated by ultrafiltration through an Amicon PM-5 membrane and stored at -80°C. Under these conditions, the enzyme was stable for at least 10 months.

Electrophoresis -- SDS-PAGE of the purified enzyme was performed by the method of Laemmli (1970) in 6-26% gradient polyacrylamide gels.

Quantitation of protein -- Protein was quantitated by the method of Bradford (1976) with bovine serum albumin as the standard. For detergent-containing samples, however, the protein was precipitated by addition of 6% (w/v) trichloroacetic acid, and the pelleted protein was washed once with the same solution. The precipitated protein was suspended in water, and the suspension was used for the quantitation of protein by Bradford's method.

Amino acid sequencing -- The purified enzyme was subjected to SDS-PAGE, and the proteins, after transfer from the gel to a polyvinylidene difluoride membrane (Immobilon; Millipore), were visualized by staining with 0.1% (w/v) Ponceau S (Nacalai) in 3%

(w/v) acetic acid. The red band corresponding to a protein of molecular weight 40,000 was cut out and washed thoroughly with water. The dried membrane was then cut into 1 x 2 mm segments and subjected directly to peptide sequencing (model 477A gas phase sequencer; Applied Biosystem).

Immunoblotting -- After SDS-PAGE of the enzyme, Western blotting was performed by the method of Hawkes et al. (1982) with skimmed milk to eliminate background interference. Antibody against rabbit IgG, conjugated with horseradish peroxidase, was used as the second antibody. The antibody against the sAPX from tea leaves was prepared as described previously (Chen and Asada 1989).

Preparation of intact chloroplasts and the stromal fraction — Intact chloroplasts were prepared from spinach leaves by Percoll density centrifugation, and the stromal fraction was isolated as described in chapter II.

Results and Discussion

Purification of thylakoid-bound ascorbate peroxidase -- Since thylakoid-bound ascorbate peroxidase (tAPX) from spinach chloroplasts is labile in the absence of ascorbate, as is the stromal enzyme (Chapter II), I added 5 mM ascorbate to the various buffers used in the purification. For the purification of the soluble, stromal and cytosolic ascorbate peroxidases is necessary to include sorbitol in all buffers to stabilize the enzyme (Nakano and Asada 1987, Chen and Asada 1989). However, sorbitol was not required for stabilization of tAPX in the presence of detergents (data not shown), and I were able to purify the enzyme without the addition of sorbitol. APX binds to the thylakoid membranes, and the bound APX is not solubilized by chelators, salts or chaotropic reagents but it can be solubilized by heptyl-thioglucoside, CHAPSO or octylglucoside (Chapter II). Among these detergents, octylglucoside was the most effective and it was used for the solubilization of the enzyme from the thyla-

koids. It should be noted here that other detergents, such as Triton X-100, polyethyleneglycol-*p*-isooctylphenyl ether and lauryldimethylamine oxide, inactivate tAPX (Chapter II). After solubilization from the thylakoids, CHAPSO and octylglucoside showed little difference in terms of stabilization of tAPX during purification. Since octylglucoside is not stable for long periods (Tsuchiya 1990), I purified the enzyme in the presence of CHAPSO, which was included in all the buffers.

Purification of tAPX from spinach thylakoids obtained from 1.1 kg of leaves is summarized in Table III-1. There was a 421-fold increase in specific activity with a yield of 9.4%. The specific activity of the purified enzyme was about 2-fold higher than those of sAPXs from spinach and tea leaves (Nakano and Asada 1987, Chen and Asada 1989).

Table III-1. *Purification of thylakoid-bound APX from spinach thylakoids.*

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (unit mg protein^{-1})	Yield (%)	Purification (-fold)
Octyl-glucoside	2,466	6,190	2.51	100	1
DEAE-Sephacel	151	2,912	19.3	47.0	7.7
Superdex-200	35.8	1,728	48.3	27.9	19
QAE-Toyopearl	14.8	1,375	92.9	22.2	37
Superdex-75	3.40	1,242	365	20.0	146
Q-Sepharose	0.55	581	1056	9.4	421

Purity and molecular weight — Staining with Coomassie blue of the gel after SDS-PAGE of the purified tAPX generated a single band of protein that corresponded to a molecular weight of 40,000 (Fig. III-1A). The molecular weight of purified tAPX was also estimated to be 40,000 $\pm$ 2,000 by gel-filtration on a column of TSKgel G3000SW$_{XL}$ (TOSOH). Thus, the tAPX was purified as monomers. Under the same conditions, but in the absence of CHAPSO, the APX in the stromal fraction prepared from intact chloroplasts

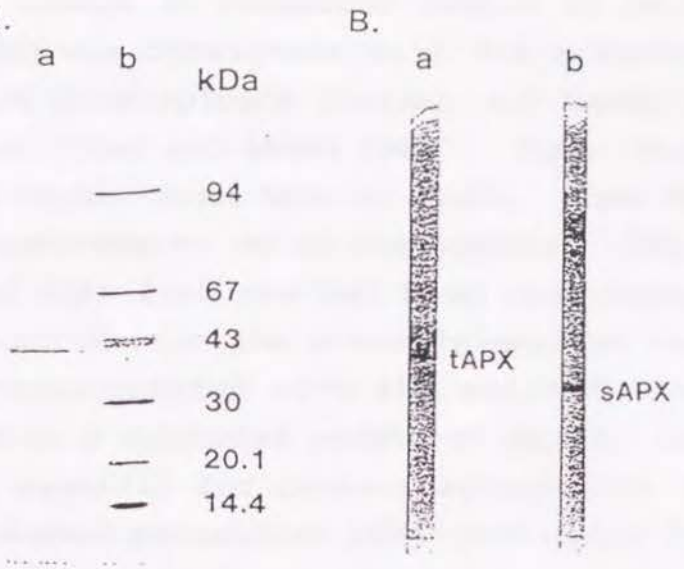


Fig. III-1 (A) *SDS-Polyacrylamide gel electrophoresis of the purified tAPX from spinach.* Lane a, purified tAPX (4 µg). Lane b, molecular-mass standard proteins (kDa, 30 µg total): phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), trypsin inhibitor (20.1), α-lactoalbumin (14.4). (B) Immunoblotting of purified tAPX from spinach and purified sAPX from tea (Chen and Asada 1989) with the antibody against the tea sAPX after SDS-PAGE. Lane a, spinach tAPX (4 µg). Lane b, tea sAPX (4 µg).

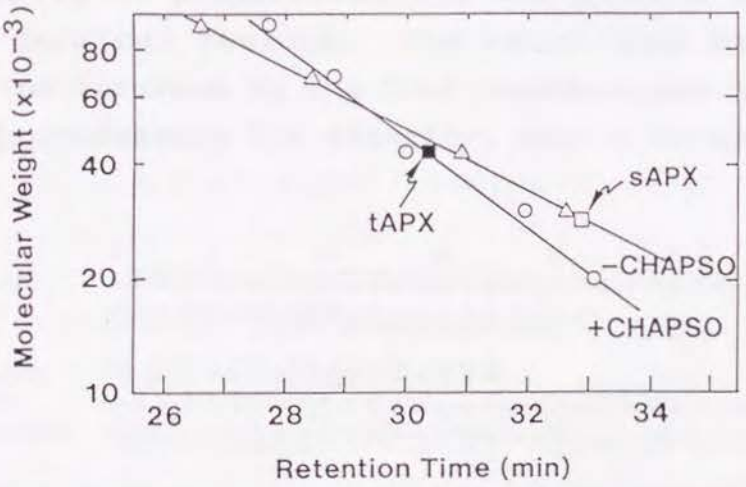


Fig. III-2 *Determination of the molecular weight of purified tAPX from spinach thylakoids and APX in the stromal fraction of intact spinach chloroplasts by gel filtration on a column of TSK gel G3000SW$_{XL}$.* For the determination of molecular weight of tAPX, buffer B containing 1% (w/v) CHAPSO was used, and for that of sAPX, buffer B without CHAPSO was used. The molecular weight of each APX was determined from the retention time of the peak of activity of APX. The column was calibrated with the same molecular-weight standard proteins as indicated in the legend to Fig. III-1. [open circles (+CHAPSO) and triangles (-CHAPSO)].

of spinach, showed an molecular weight of 30,000 ± 2,000 (Fig. III-2), which was consistent with the molecular weight of the purified sAPX from spinach (Nakano and Asada 1987) and also of that from tea (Chen and Asada 1989). Thus, the molecular weight of tAPX was higher than that of sAPX. When the polyvinylidene difluoride membrane to which the purified tAPX from spinach and the purified sAPX from tea had been transferred after SDS-PAGE was immunoblotted with the antibody against tea sAPX, the purified tAPX cross-reacted with the antibody at a position that corresponded to a molecular weight of 40,000 (Fig. III-1B). The antibody is specific for cross-reaction with sAPX and cAPX but not with guaiacol peroxidase (Chen and Asada 1989). Thus, tAPX from spinach and sAPX from tea share common epitopes. The results of the Western blotting also support the conclusion that tAPX from spinach leaves has a higher molecular weight than that of sAPX from tea leaves.

Amino acid sequence of the amino-terminal region — Purified thylakoid-bound ascorbate peroxidase was subjected to Edman degradation. The yield of the first residue was 98%, indicating the high purity of preparation and the absence of acylation of the amino- terminal residue. The amino acid sequence of tAPX from the amino terminus to the 32nd residues was determined (Fig. III-3). Approximately 71% identity, over a 21-amino-acid over-

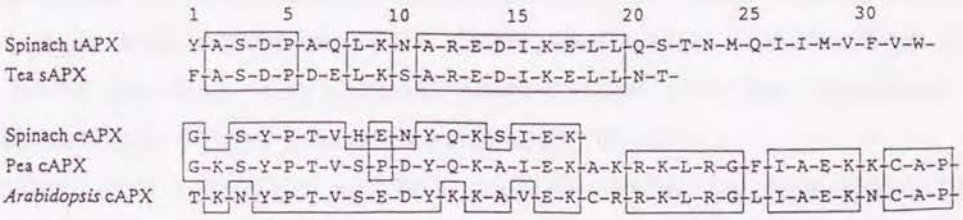


Fig. III-3 *Amino acid sequences of the amino-terminal region of spinach tAPX (this study), tea sAPX (Chen et al. 1992), spinach cAPX (Tanaka et al. 1991), pea cAPX (Mittler and Zilinskas 1991b) and Arabidopsis cAPX (Kubo et al. 1992).*

lap, was found between the amino terminus of tAPX from spinach and that of sAPX from tea (Fig. III-3). However, only a low

degree of homology was found between the tAPX and the cytosolic APXs from spinach (Tanaka et al. 1991), pea (Mittler and Zilinskas 1991a) and *Arabidopsis thaliana* (Kubo et al. 1992).

Ascorbate peroxidase has been classified as a member of the Class I family of heme peroxidases from its amino acid sequence, where Cyt *c* peroxidase is the core member of the family and is part of the lineage of prokaryotic peroxidases (Welinder 1992). The partial amino acid sequence of sAPX from tea showed a higher degree of homology to Cyt *c* peroxidase from yeast than to guaiacol peroxidases from plants, such as horseradish peroxidase (Chen et al. 1992b), as is also the case for cAPXs from pea (Mittler and Zilinskas 1991b) and *Arabidopsis thaliana* (Kubo et al. 1992). Although the amino acid sequence of the amino-terminal region differs between two chloroplastic APXs (tAPX and sAPX) and cytosolic APX (Fig. III-3), the sequences of the proximal and distal histidine-containing regions are conserved in sAPX, cAPX and Cyt *c* peroxidase (Chen et al. 1992b, Welinder 1992). While the amino acid sequence at the active site of tAPX has not yet been determined, the similarity between the enzymatic properties of tAPX and those of cAPX and sAPX, as detailed below, suggest that tAPX also belongs to the Class I family of heme peroxidases.

The higher molecular weight of tAPX, as compared with that of sAPX, might be anticipated from its binding to the thylakoid membranes. Since a high degree of homology in terms of the amino acid sequence of the amino-terminal region was found between tAPX from spinach and soluble sAPX from tea, the domain for the binding of tAPX to the thylakoid membranes can be assumed to be a region other than the amino-terminal region.

Absorption spectra — The native tAPX in the detergent- and ascorbate-containing buffer gave an absorption spectrum characteristic of a ferric high-spin state. A Soret peak was found at 403 nm, and its peak was shifted to 433 nm by reduction with dithionite, with an α peak at 555 nm (Fig. III-4). In addition, the native enzyme had a peak of absorption at 620 nm. This peak disappeared when the enzyme was reduced by dithionite. The absorption coefficient of the reduced form of tAPX at the Soret

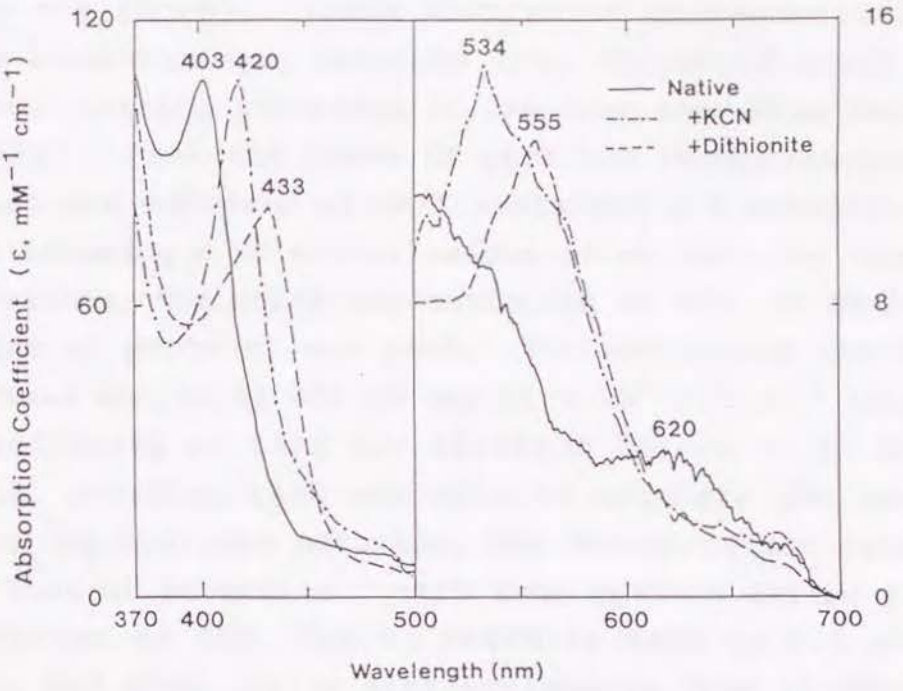


Fig. III-4 *Absorption spectra of purified tAPX from spinach thylakoids.* The absorption spectra of tAPX were recorded from a 1.3 μM solution in 50 mM potassium phosphate (pH 7.5)/0.1 mM EDTA/1 mM PMSF/5 mM ascorbate/0.1 M KCl/1% CHAPSO. Native form (—) , no additions; reduced form (---), dithionite was added; CN-form (-·-), 1 mM KCN was added.

peak was lower than that of native form (Fig. III-4). On the other hand, sAPXs from tea and spinach show little difference in the absorption coefficients at the Soret peaks between their native and reduced forms (Nakano and Asada 1987, Chen and Asada 1989). Further, Soret peak of the reduced form of tAPX was found at 433 nm, as compared to the corresponding peak of the reduced sAPX at 420 nm. These results indicate that the details of the environments of the heme moieties of tAPX and sAPX are different. The cyanide complex of the oxidized tAPX gave peaks at 420 nm and 534 nm, and the Soret peak of the cyanide complex also was shifted towards longer wavelength as compared to that (416 nm) of sAPX from tea.

Pyridine hemochromogen was prepared from the purified tAPX

by the method of Paul et al. (1953), and it gave an α peak at 555 nm, a β peak at 526 nm and a minimum between the two peaks at 538 nm (data not shown). These absorption characteristics of the pyridine hemochromogen indicate that thylakoid-bound ascorbate peroxidase contains protoheme IX, as does sAPX from tea (Chen and Asada 1989). From the level of pyridine hemochromogen, I estimated that one molecule of tAPX contained 0.9 molecule of protoheme IX, assuming a molecular weight of 40,000. In the following determinations, the molar concentration of APX, as estimated from the amount of protein, was used. The absorption coefficient of the oxidized enzyme at 403 nm was 11 x 10^4 M^{-1} cm^{-1} (Fig. III-4).

Specificity of tAPX for electron donors — In addition to ascorbate, purified tAPX was able to catalyze the oxidation of pyrogallol by hydrogen peroxide, but the oxidation rate was only 5.5% of that of ascorbate. tAPX from spinach failed to catalyze the oxidation of GSH, Cyt *c*, NADPH or NADH by 0.1 mM hydrogen peroxide, and thus, it is distinguishable from glutathione peroxidase, Cyt *c* peroxidase and NAD(P)H peroxidase. The high specificity, in terms of electron donor, of the thylakoid-bound APX for ascorbate is similar to that of ascorbate peroxidase from the stroma of spinach chloroplasts (Nakano and Asada 1987) and the chloroplastic isozyme from tea (Chen and Asada 1989). However, the specificity of tAPX is different from that of cytosolic APXs from tea and pea, which are able to catalyze the oxidation of pyrogallol by hydrogen peroxide at a 4-fold higher rate than that of ascorbate (Chen and Asada 1989, Mittler and Zilinskas 1991a). The donor specificity of the purified APX from spinach thylakoids is very similar to that of detergent-solubilized preparations from thylakoids (Chapter II), indicating that APX is the sole peroxidase bound to the thylakoid membranes.

Reaction kinetics — Since the primary product of oxidation in the reaction catalyzed by APX is the monodehydroascorbate radical (Hossain et al. 1984), tAPX seems to catalyze the oxidation of ascorbate by hydrogen peroxide via the two-electron oxidation of the enzyme with two subsequent single-electron reductions, as proposed for guaiacol peroxidase:

$$\text{APX} + H_2O_2 \xrightarrow{k_1} \text{APX-I} + H_2O \quad (1)$$

$$\text{APX-I} + \text{AsA} \xrightarrow{k_2} \text{APX-II} + \text{MDA} \quad (2)$$

$$\text{APX-II} + \text{AsA} \xrightarrow{k_3} \text{APX} + \text{MDA} + H_2O \quad (3)$$

where APX-I and APX-II represent the two- and one-electron oxidized enzymes, namely Compounds I and II; AsA represents ascorbate; and MDA represents the monodehydroascorbate radical. The sum of reactions 1 to 3 is:

$$2\ \text{AsA} + H_2O_2 \longrightarrow 2\ \text{MDA} + 2\ H_2O \quad (4).$$

The above reaction cycle shows a modified type of ping-pong kinetics, which has been referred to as peroxidase ping-pong kinetics (Dunford 1991).

The initial rate of oxidation of AsA (v), catalyzed by APX, can be represented by the following equation;

$$\frac{2\,[\text{APX}]_o}{v} = \frac{(k_2+k_3)}{k_2k_3}\frac{1}{[\text{AsA}]} + \frac{1}{k_1}\frac{1}{[H_2O_2]} \quad (5),$$

where $[\text{APX}]_o$ is total concentration of enzyme. If I assume that, as in the case of horseradish peroxidase (Dunford 1991), k_2 of tAPX is ten times greater than k_3, the equation (5) can be simplified to

$$\frac{2\,[\text{APX}]_o}{v} = \frac{1}{k_3}\frac{1}{[\text{AsA}]} + \frac{1}{k_1}\frac{1}{[H_2O_2]} \quad (6).$$

This equation shows that, under conditions where the concentration of ascorbate is fixed and that of hydrogen peroxide is varied, the reciprocal of the initial rate of oxidation of ascorbate, catalyzed by APX, and the reciprocal of the concentration of hydrogen peroxide are linearly related. The value of k_1 obtained from the slope of the straight line, and k_3 can be obtained from the intercept of the line with the perpendicular axis.

The initial rates of oxidation of ascorbate were determined in the presences of hydrogen peroxide at various concentrations at a range of concentrations of ascorbate between 10 and 400 μM. In the range of concentrations of ascorbate tested, the reciprocal of the initial rate of oxidation of ascorbate, catalyzed by tAPX, and the reciprocal of the concentration of hydrogen peroxide (1.0 - 40 μM) were linearly related. When reciprocal values of the initial rate of oxidation of ascorbate were plotted against those of hydrogen peroxide, parallel straight lines were obtained (Fig. III-5A). These results indicate that APX also

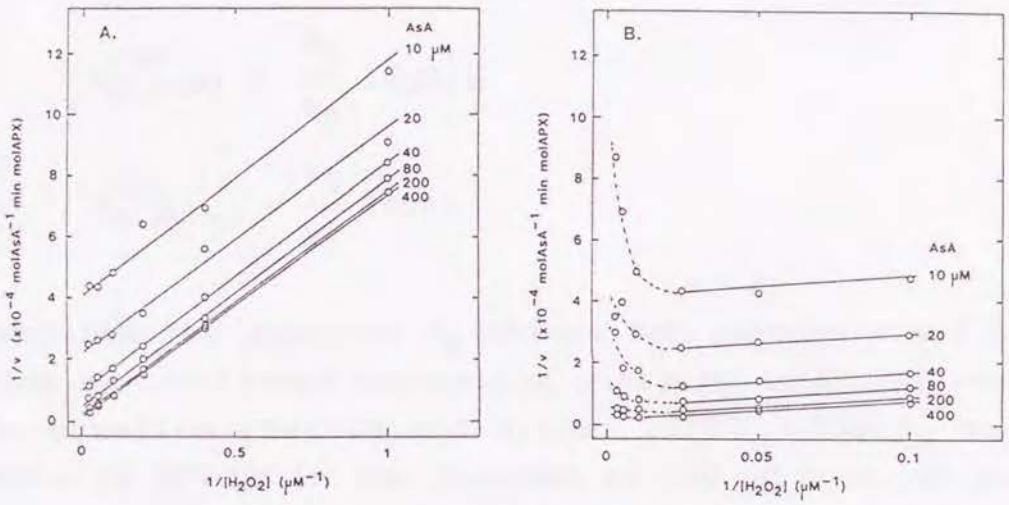


Fig. III-5 *Double-reciprocal plots of the initial velocity of the reaction catalyzed by purified tAPX from spinach thylakoids against the concentration of hydrogen peroxide (A, 1.0 - 40 μM; and B, 10 - 400 μM) at the indicated concentrations of ascorbate.* The initial rates of oxidation of ascorbate catalyzed by tAPX (1 nM) were determined as described in Materials and Methods, with the concentrations of substrate as indicated.

catalyzes the oxidation of ascorbate by the peroxidase ping-pong mechanism. From the slopes of straight lines and the intercepts, the rate constants k_1 and k_3 were calculated to be 1.2×10^7 $M^{-1}s^{-1}$ and $2.1 \pm 0.2 \times 10^6$ $M^{-1}s^{-1}$ at pH 7.0, respectively.

In the range of concentrations of ascorbate tested (10 - 400 μM), the initial rate of oxidation of ascorbate by tAPX was decreased at higher concentrations of hydrogen peroxide than 100 μM

(Fig. III-5B), indicating the inactivation of tAPX by hydrogen peroxide. The lower was the concentration of ascorbate, the higher was the extent of the inactivation of tAPX by hydrogen peroxide. Thus, the kinetic constants k_1 and k_3 must be determined at higher ratios of the concentration of ascorbate to that of hydrogen peroxide.

The values of k_1 and k_3 allow us to estimate apparent K_m values for ascorbate and hydrogen peroxide from the following equations. The estimated K_m for ascorbate is 570 μM when the concentration of hydrogen peroxide is 100 μM, and that for hydrogen peroxide is 80 μM when the concentration of ascorbate is 500 μM.

$$K_{m(AsA)}^{app} = \frac{k_1}{k_3} [H_2O_2] \quad (7)$$

$$K_{m(H_2O_2)}^{app} = \frac{k_3}{k_1} [AsA] \quad (8).$$

I determined the apparent K_m values for ascorbate and hydrogen peroxide by non-linear regression analysis using the Michaelis-Menten equation (Sakota and Hiromi 1976). The K_m value for ascorbate is 500 μM in the presence of 100 μM hydrogen peroxide, in the range of concentration of ascorbate between 200 and 700 μM, and that for hydrogen peroxide is 87 μM in the presence of 500 μM ascorbate, in the range of concentration of hydrogen peroxide between 10 and 60 μM. When the K_m value for hydrogen peroxide in the presence of 500 μM ascorbate was determined at concentrations of hydrogen peroxide above 100 μM, the K_m value decreased to 22 μM. It is possible that the apparent K_m value determined for hydrogen peroxide was lower than the value estimated from equation (8) because of the inactivation of tAPX by hydrogen peroxide at higher concentrations, as discussed above. The apparent K_m value of tAPX for ascorbate is larger than that of the sAPX from spinach (K_m, 300 μM; Nakano and Asada 1987) and tea (K_m, 220 μM; Chen and Asada 1989), suggesting that the respective values of k_1 and k_3 differ between tAPX and sAPX.

Inhibitors — The purified tAPX was inhibited by cyanide and azide, as are other APXs. Furthermore, the enzyme was sensitive to the thiol-modifying reagents pCMB and 5,5'-dithiobis-(2-nitrobenzoic acid), but it was insensitive to monoiodoacetate and iodoacetamide, as are sAPX from spinach (Nakano and Asada 1987) and cAPXs from tea (Chen and Asada 1989) and pea (Mittler and Zilinskas 1991a). When pCMB was added at 50 μM to oxidized

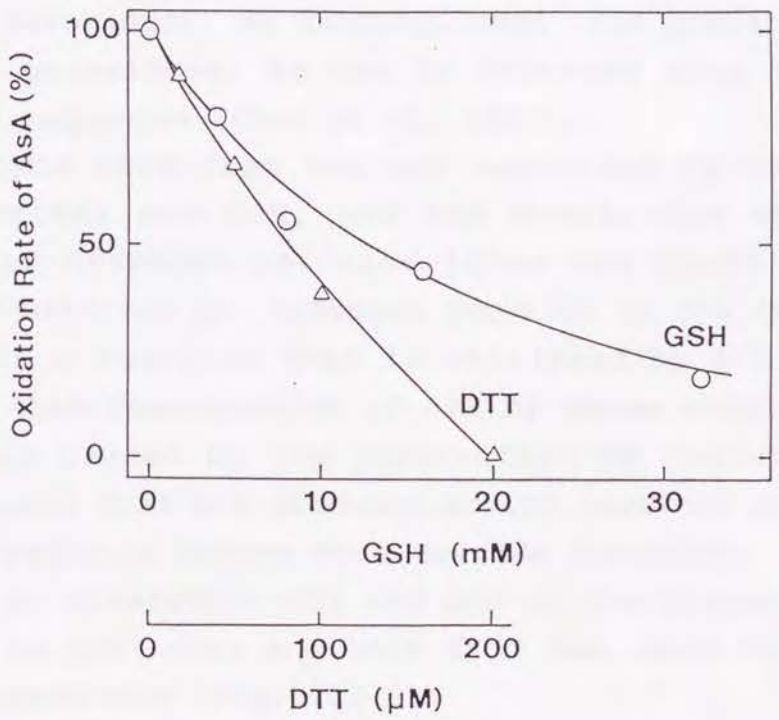


Fig. III-6 *Inhibition of tAPX from spinach thylakoids by thiols, as determined from the rate of oxidation of ascorbate.* The reaction mixture contained 50 mM potassium phosphate (pH 7.0)/0.5 mM ascorbate/tAPX (0.04 unit)/0.1 mM hydrogen peroxide/dithiothreitol (DTT) (triangles) or GSH (circles) at the indicated concentrations. The initial rate of oxidation of AsA was determined from the decrease in the absorbance at 290 nm which was recorded for 5 s from 1.5 s after the start of the reaction by the addition of hydrogen peroxide. The rate in the absence of thiols is taken as 100%.

tAPX, the absorption spectrum in the visible range was not affected, indicating that little direct interaction occurred between the functional thiol group and the heme. However, the addition of 1 mM hydrogen peroxide to tAPX in 50 mM potassium phosphate (pH 7.0) that contained 1 mM ascorbate did not resulted in the characteristic spectrum of Compound I (data not shown). These results suggest either that the thiol group participates in the oxidation of the heme by hydrogen peroxide or that the conformational change caused by the thiol-modification with pCMB interrupts the formation of Compound I. Inhibition by thiol-modifying reagents is one of the characteristic properties of ascorbate peroxidase, as distinguished from guaiacol peroxidase and Cyt *c* peroxidase, as can be inferred from the respective amino acid sequences (Chen et al. 1992b).

sAPX and cAPX from tea are inhibited by thiols, such as dithiothreitol and GSH, and the inhibition depends on the presence of hydrogen peroxide (Chen and Asada 1992). These thiols are oxidized by hydrogen peroxide to the respective thiyl radicals in a reaction that is catalyzed by APX. It has been suggested that inactivation of APX by these thiols and hydrogen peroxide is caused by the interaction of the enzyme with the thiyl radicals that are produced at its reaction center such that the thiyl radicals behave as a suicide inhibitor. tAPX also was inhibited by dithiothreitol and GSH in the presence of hydrogen peroxide, as are sAPX and cAPX from tea, with 50% at 80 μM and 12 mM, respectively (Fig. III-6).

tAPX was also inhibited by the following suicide inhibitors: hydroxylamine, hydroxyurea and *p*-aminophenol (Table III-2), as are sAPX and cAPX from tea (Chen and Asada 1990). These suicide inhibitors are oxidized to the respective radicals by hydrogen peroxide in a reaction catalyzed by APX, and these radicals seem to inactivate APX. In fact, in the case of tAPX, inactivation was observed only when the enzyme was preincubated with the inhibitors and hydrogen peroxide.

Table III-2. *Effects of preincubation of the purified tAPX from spinach thylakoids with p-aminophenol (AP), hydroxylamine (HA), and hydroxyurea (HU) on the initial rate of oxidation of ascorbate by hydrogen peroxide.* tAPX (0.086 unit) was preincubated at 25°C for 5 min in 0.1 ml of 50 mM potassium phosphate, pH 7.0/0.5 mM ascorbate, in the presence of 100 μM hydrogen peroxide and/or AP, HA or HU at the indicated concentrations. After the preincubation, each mixture was diluted by the addition of 0.9 ml of 50 mM potassium phosphate, pH 7.0, that contained 0.5 mM ascorbate, and the reaction was started by the addition of 100 μM hydrogen peroxide. The initial (1.5 - 9 s) rate of oxidation of ascorbate was recorded.

Preincubation conditions	tAPX activity (%)
No additions	100
100 μM H_2O_2	98
1 mM AP, no preincubation	100
1 mM AP	94
1 mM AP and 100 μM H_2O_2	0
1 mM HA, no preincubation	90
1 mM HA	94
1 mM HA and 100 μM H_2O_2	0
40 mM HU, no preincubation	98
40 mM HU	96
40 mM HU and 100 μM H_2O_2	0

Concluding remarks — The enzymatic properties of the purified tAPX were consistent with those of the enzyme in intact thylakoids and in detergent-solubilized thylakoids (Chapter II). Thus, the purified tAPX seems to have the same properties as the enzyme in its membrane-bound state. These results suggest that the site of reduction of hydrogen peroxide by tAPX is exposed to the stromal side of the thylakoid membranes where the ferredoxin-dependent regeneration of ascorbate occurs.

The present results indicate the thylakoid-bound APX from

spinach and the stromal APXs from spinach (Nakano and Asada 1987) and tea (Chen and Asada 1989) share the molecular and enzymatic properties. A characteristic property of sAPX is its rapid inactivation in ascorbate-depleted medium (Nakano and Asada 1987, Chen and Asada 1989), which is also a property of the tAPX in a thylakoid-bound state (Chapter II). The purified tAPX was also rapidly inactivated in ascorbate-depleted medium (data not shown), and I have found that this inactivation is caused by the extreme lability of the Compound I of tAPX (Chapter IV).

The sole difference in terms of molecular properties between sAPX and tAPX is the higher molecular weight of the membrane-bound enzyme as compared to the soluble enzyme. Since the amino-terminal sequence of tAPX shows a high degree of homology to that of sAPX, this region does not seem to be a domain required for the binding to the thylakoid membranes. Even though tAPX and sAPX in chloroplasts have similar molecular properties, these chloroplastic APXs have different properties and amino acid sequences from the cytosolic APXs purified to date. From the present results, it seems very likely that the ancestor of APX first diverged to give cytosolic and chloroplastic isozymes, and the ancestral chloroplastic APX further diverged further to give the stromal and thylakoid-bound isozymes. Such divergence to cytosolic and chloroplastic isozymes has already been postulated for CuZn-superoxide dismutase (Kanematsu and Asada 1990).

Chapter IV

Inactivation Mechanism of Ascorbate Peroxidase by Ascorbate Depletion

Ascorbate peroxidase (APX) is a hydrogen peroxide-scavenging peroxidase which uses ascorbate as the electron donor. APX could be divided into two groups with respect to cellular localization. The first group of APX, constituted of the thylakoid-bound APX (tAPX) (Chapter II, III) and stromal APX (sAPX) (Nakano and Asada 1987, Chen and Asada 1989), is localized in chloroplasts and have been found functioning in scavenging of the hydrogen peroxide photoproduced in thylakoids. The second group of APX is localized in the cytosol and its functions would be the scavenging of hydrogen peroxide produced in the cytosol. Further, tAPX and sAPX are characterized by its higher specificity for ascorbate as the electron donor (Nakano and Asada 1987, Chen and Asada 1989, Chapter II), compared to cAPX which catalyzes the oxidation of phenols at higher rate than that of ascorbate (Chen and Asada 1989).

All APX isozymes are inhibited by thiol-modifying reagents such as *p*-chloromercuribenzoate (pCMB) (Nakano and Asada 1987, Chen and Asada 1989, Mittler and Zilinskas 1991a, Chapter II), which has not been found for guaiacol peroxidases (GPX) and cytochrome *c* peroxidase. pCMB does not affect the absorption spectrum of APX in the visible range, indicating little interaction of the functional thiol group with the heme (Chapter III). The characteristic spectrum of Compound I of APX, however, was not observed by addition of hydrogen peroxide in the presence of pCMB, suggesting the participation of thiol group in the oxidation of the heme by hydrogen peroxide. Further, all APX isozymes are inhibited by thiyl radicals of GSH and dithiothreitol and phenoxy or aminoxy radicals of *p*-aminophenol, hydroxyurea and hydroxyamine (Chen and Asada 1990, 1992, Chapter III). These

radicals are generated by hydrogen peroxide at the reaction center of APX, and the enzyme is inactivated by a suicide mechanism. These radicals also are produced with HRP, but do not inhibit its activity, which is also one of the properties of GPX distinguished from those of APX.

Ascorbate peroxidase loses its activity in the absence of electron donor. This would be one of the reasons why APX had not been found for a long time. The half inactivation times of tAPX and sAPX are only 15 s, and that of cAPX is about 60 min in the absence of ascorbate (Nakano and Asada 1987, Chen and Asada 1989, Chapter II). This inactivation is prevented by removal of dioxygen (Nakano and Asada 1987), suggesting that a compound produced by autooxidization inactivates APX. In this Chapter, an inactivation mechanism of APX in the absence of ascorbate is described in detail. The autooxidation product participating in the inactivation of APX is identified to be hydrogen peroxide, and the inactivation is caused by a rapid degradation of Compound I of APX.

Materials and Methods

Thylakoid-bound ascorbate peroxidase — Thylakoid-bound ascorbate peroxidase (tAPX) was purified from spinach thylakoids to homogenity as described in Chapter III. The concentration of tAPX was determined by the absorbance at 403 nm using an absorption coefficient of 110 mM^{-1} cm^{-1} (Chapter III). The activity of tAPX was determined spectrophotometrically, and its unit is defined, as described in Chapter III.

Horseradish peroxidase — Purified horseradish peroxidase (HRP) was obtained from Toyobo, grade I-C, RZ ($A_{403\ nm}/A_{275\ nm}$) $\geq$ 3.0. The enzyme concentration was estimated by the absorbance at 403 nm using an absorption coefficient of 100 mM^{-1} cm^{-1} (Nakajima and Yamazaki 1979).

Results and Discussion

Inactivation of thylakoid-bound ascorbate peroxidase in the absence of ascorbate — One of the specific properties of ascorbate peroxidase is its rapid inactivation in an ascorbate-depleted medium (Hossain and Asada 1984, Nakano and Asada 1987, Chen and Asada 1989). This is also the case of thylakoid-bound ascorbate peroxidase (tAPX) purified from spinach thylakoids (Chapter II). When tAPX was diluted with an ascorbate-depleted medium to

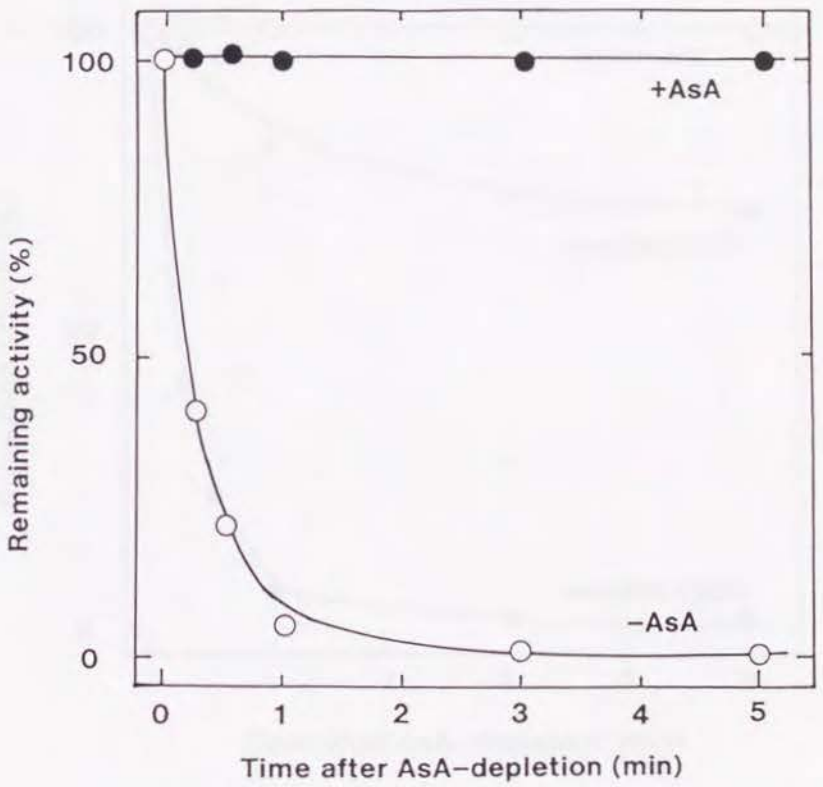


Fig. IV-1 *Inactivation of tAPX purified from spinach thylakoids in ascorbate-depleted medium.* tAPX (2 µl, 500 nM) containing 1 mM ascorbate (AsA) was diluted with 1 ml of 50 mM potassium phosphate, pH 7.0. At the indicated times after the dilution, 0.5 mM ascorbate was added to the diluted mixture to quench the inactivation, and the ascorbate peroxidase activity was assayed by the addition of 0.1 mM hydrogen peroxide as described in Materials and Methdods. Where indicated (+AsA), the dilution was done with the buffer containing 0.5 mM ascorbate. Activity of tAPX in the presence of ascorbate at 0 min was 0.04 unit and is taken as 100%.

lower the concentration of ascorbate below 2 μM, the peroxidase activity was lost with a first order kinetics with respect to the remaining activity at a half time of about 15 s (Fig IV-1). The inactivation of tAPX in the absence of ascorbate was suppressed under anaerobic conditions, which was consistent with the results of Nakano and Asada (1987). We found further the suppression of the inactivation of tAPX by the addition of catalase to an extent of 80%. However, SOD did not show any effect on the inactivation of tAPX in the absence of ascorbate (Fig. IV-2). These results

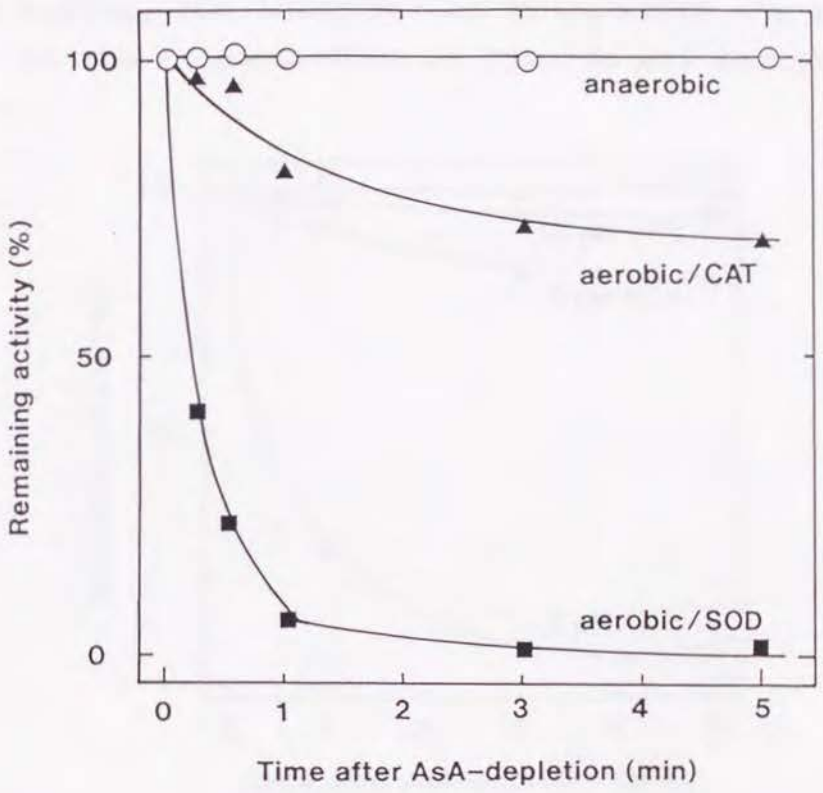


Fig. IV-2 *Effects of dioxygen, catalase and superoxide dismutase on tAPX inactivation in ascorbate-depleted medium.* tAPX (2 μl, 500 nM) containing 1 mM ascorbate (AsA) was diluted with 1 ml of 50 mM potassium phosphate, pH 7.0. At the indicated times after the dilution, 0.5 mM ascorbate was added to the diluted mixture to quench the inactivation, and the ascorbate peroxidase activity was assayed as described in Materials and Methods. Where indicated, the dilutions were done with the following buffers: aerobic/SOD, the buffer was equilibrated with air and contained CuZn-superoxide dismutase (200 units); aerobic/CAT, the buffer was equilibrated with air and contained catalase (1,000 units); anaerobic, the buffer was deaerated with argon gas and the dilution was done under anaerobic conditions. Activity of tAPX in the presence of ascorbate at 0 min was 0.04 unit and is taken as 100%.

suggest that hydrogen peroxide is produced on dilution of tAPX with the ascorbate-depleted medium and inactivates the enzyme. The hydrogen peroxide would be produced via an autooxidation of 2 μM ascorbate derived from a stock solution of tAPX, in which 1 mM ascorbate has been added to prevent the inactivation of tAPX.

If tAPX is inactivated by the hydrogen peroxide produced by autooxidation of a trace amount of ascorbate derived from the APX-stock solution, the inactivation is expected to be inhibited by suppression of the interaction of tAPX with the hydrogen peroxide by cyanide. When tAPX is diluted by the cyanide-containing buffer, the inactivation by dilution was suppressed (Fig. IV-3). As the concentration of cyanide was increased, the inac-

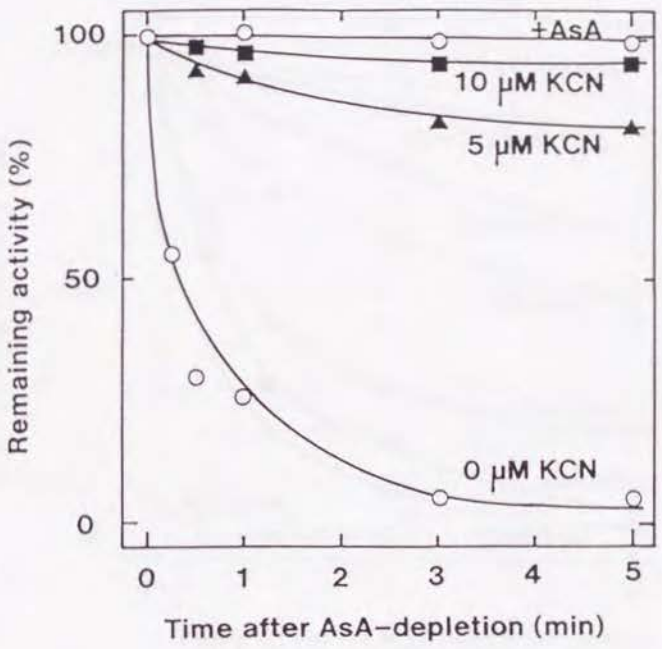


Fig. IV-3 *Suppression of tAPX inactivation in ascorbate-depleted medium by potassium cyanide.* tAPX (2 μl, 500 nM) containing 1 mM ascorbate (AsA) was diluted with 1 ml of 50 mM potassium phosphate, pH 7.0. At the indicated times after the dilution, 0.5 mM ascorbate was added to the diluted mixture to quench the inactivation, and the ascorbate peroxidase activity was assayed by the addition of 0.1 mM hydrogen peroxide as described in Materials and Methods. Where indicated, the dilutions were done with the buffer containing 0.5 mM ascorbate (AsA) and KCN at indicated concentrations. Activity of tAPX in the presence of ascorbate at 0 min was 0.04 unit and is taken as 100%. In the presences of 5 and 10 μM cyanide, activities of tAPX in the presence of ascorbate at 0 min was 60% and 24% of that in the absence of cyanide, respectively, and these activities are taken as 100%.

tivation of APX was more suppressed. In the presence of cyanide, tAPX formed the cyanide complex and did not interact with the hydrogen peroxide. After the quenching of the inactivation by ascorbate, the assay medium displaced the ligated cyanide of tAPX by hydrogen peroxide and allowed to assay the activity of tAPX. The suppression by cyanide of the inactivation in the absence of ascorbate also sustains that the interaction of tAPX with hydrogen peroxide causes the inactivation.

Inactivation of tAPX by the addition of hydrogen peroxide under anaerobic condition — In order to confirm the participation of hydrogen peroxide in the inactivation of APX, the effect

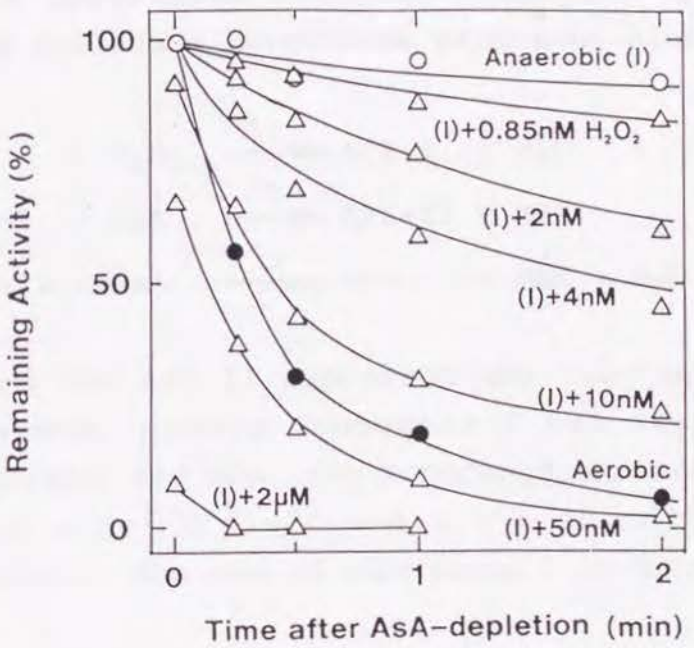


Fig. IV-4 *Inactivation of tAPX by hydrpogen peroxide in ascorbate (AsA)-depleted medium under anaerobic conditions.* tAPX (2 μl, 425 nM) in 1 mM ascorbate was diluted with 1 ml of 50 mM potassium phosphate, pH 7.0, containing hydrogen peroxide at the indicated concentrations under anaerobic conditions, unless otherwise specified. The final concentration of tAPX was 0.85 nM. At the indicated times, the inactivation was quenched by the addition of 0.5 mM ascorbate to the incubation mixture. Subsequently, APX activity was determined under the standard assay conditions as described in Materials and Methods. Activity of tAPX in the presence of ascorbate at 0 min was 0.034 unit and is taken as 100%.

of hydrogen peroxide on the activity of APX was tested. tAPX was inactivated by the addition of hydrogen peroxide under anaerobic conditions, with the first order kinetics with respect to the remaining activity (Fig. IV-4). The inactivation rate of tAPX was increased with an increase in the concentration of hydrogen peroxide. The kinetics of the inactivation of APX suggests that the inactivation of APX is inferred by the decay of one component produced in an interaction of APX with hydrogen peroxide. The two-electron oxidized intermediate of APX by hydrogen peroxide, Compound-I, is a candidate for the labile component, unless the Compound-I is reduced by ascorbate to the native form of the enzyme via the Compound-II.

Degradation of Compound I of tAPX in the absence of ascorbate — A peroxidase reaction catalyzed by tAPX from spinach thylakoids follows a peroxidase ping-pong kinetics (Chapter III).

$$\text{APX} + H_2O_2 \xrightarrow{k_1} \text{APX-I} + H_2O \qquad (1)$$

$$\text{APX-I} + \text{AsA} \xrightarrow{k_2} \text{APX-II} + \text{MDA} \qquad (2)$$

$$\text{APX-II} + \text{AsA} \xrightarrow{k_3} \text{APX} + \text{MDA} + H_2O \qquad (3)$$

where APX-I and APX-II represent the two- and one-electron oxidized enzymes, namely Compounds I and II; AsA, ascorbate as electron donor; and MDA, the monodehydroascorbate radical; k_1 and k_3 are 1.2×10^7 M^{-1} s^{-1} and 2.1×10^6 M^{-1} s^{-1}, respectively (Chapter III). The sum of reactions 1 to 3 is:

$$2\ \text{AsA} + H_2O_2 \longrightarrow 2\ \text{MDA} + H_2O \qquad (4).$$

The native form of tAPX gave an absorption spectrum characteristic of a ferric high-spin state, with a Soret peak at 403 nm (Fig. III-4, Fig. IV-5A). When hydrogen peroxide was added to native tAPX, hydrogen peroxide was bound to tAPX and Compound I of tAPX was formed, where the Soret peak of native tAPX was shifted to 415 nm, an Soret peak of the Compound I of tAPX (Fig. IV-5A). Under the present conditions, 1 mM ascorbate in the

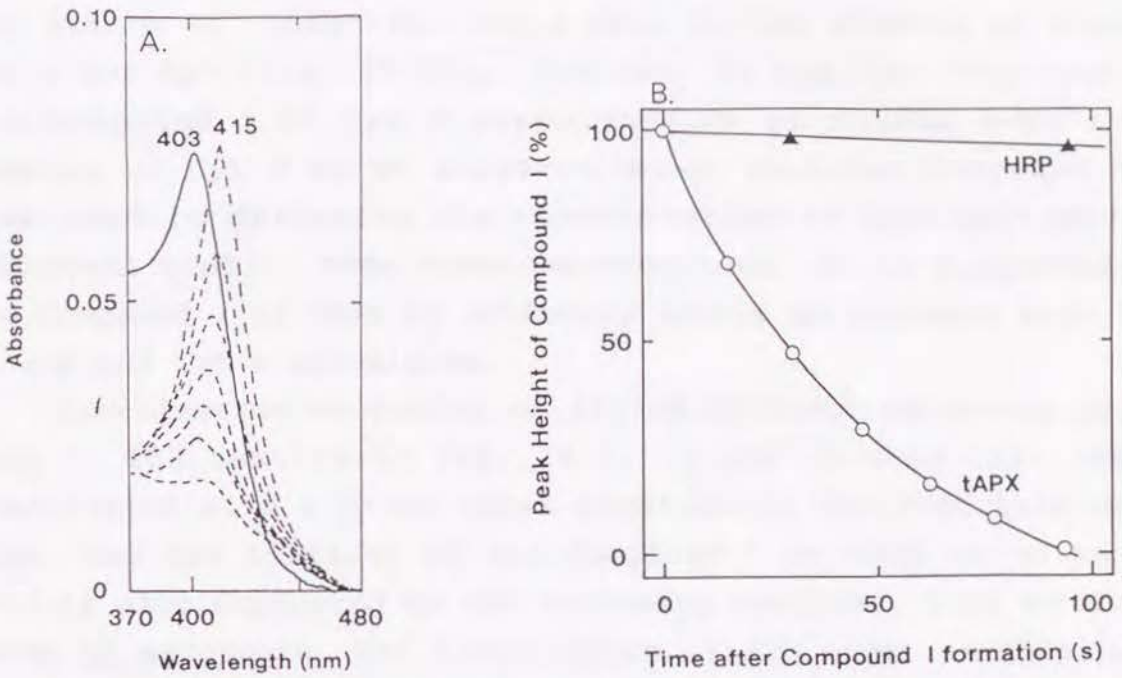
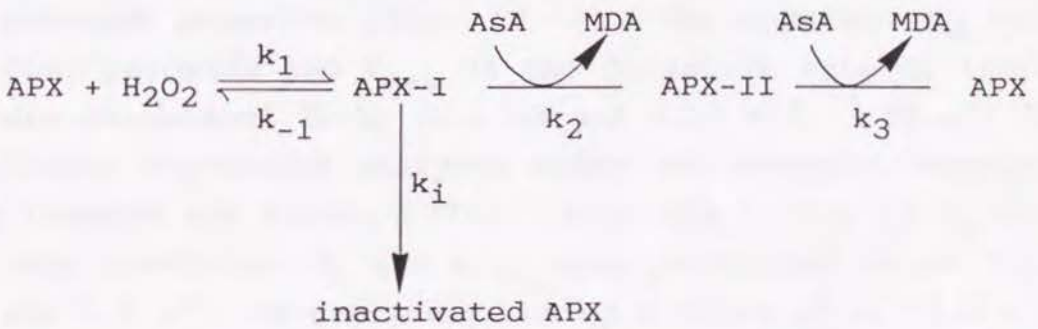


Fig. IV-5 (A) *Degradation of Compound I of tAPX in the absence of ascorbate.* The absorption spectrum of native tAPX (0.9 µM) was recorded in 50 mM potassium phosphate, pH 7.0, 1 mM ascorbate and 1% CHAPSO (—). Subsequently, 1 mM hydrogen peroxide was added, and the recording of the absorption of the Compound I was started from 480 nm at a scanning rate of 7.1 nm s^{-1} (---). The absorbances at 415 nm of APX-I were recorded at 8.8, 23.94, 39.06, 54.18, 69.3, 84.42 and 99.54 s after the addition of hydrogen peroxide from the top to the bottom spectra. (B) *Time-dependent decreases of Compounds I of tAPX and horseradish peroxidase (HRP).* Native form of 1 µM HRP in 50 mM potassium phosphate, pH 7.0, gave an absorption spectrum characteristic of a ferric high-spin state with a Soret peak at 403 nm (data not shown). The Soret peak of native HRP was shifted to 415 nm to form the two-electron oxidized enzyme, Compound I, by the addition of 2 mM hydrogen peroxide. The absorbances at 415 nm of Compound I of HRP were recorded at 8.8, 39.06 and 99.54 s after the addition of hydrogen peroxide. Absorbance changes at 415 nm of APX-Compound I and HRP Compound I are plotted against times after hydroge peroxide was added. Absorbances of Compounds I of APX and HRP at 415 nm at 8.8 s, after the addition of hydrogen peroxide, are taken as 100% at 0 s.

enzyme was oxidized by 1.1 mM hydrogen peroxide within 2 s, and the remaining hydrogen peroxide oxidized tAPX to its Compound I. Subsequently, the compound I disappeared as observed in a decrease in the absorbance at 415 nm, with a half time of about

25s. The intermediate compound at 407 nm appeared and further degradated (Fig. IV-5A,B). In contrast, the Compound I of HRP was stable at least for 100 s even in the absence of electron donor for HRP (Fig. IV-5B). Further, it has been reported that the Compound I of Cyt *c* peroxidase is so stable even in the absence of Cyt *c* as an electron donor that the Compound I has been used to determine the concentration of hydrogen peroxide (Yonetani 1965). From these observations, it is suggested that the Compound I of tAPX is extremely labile as compared with those of HRP and Cyt *c* peroxidase.

Inactivation mechanism of thylakoid-bound ascorbate peroxidase — The results in Fig. IV-1, -2 and -3 show that tAPX is inactivated with a first order kinetics by the ascorbate depletion, and the lability of the Compound I of tAPX shown in Fig. IV-5 is also supported by the following results. Even in the absence of ascorbate, the inactivation of APX under aerobic conditions is suppressed by pyrogallol that works as the electron donor (Nakano and Asada 1984). This result shows that pyrogallol reduces the Compound-I of APX as an electron donor in place of ascorbate, and lowers the steady state concentration of the Compound-I. The extreme lability of Compound I of tAPX would be the cause for APX to be inactivated in the absence of electron donor and make kinetics of APX inactivation a first order. I propose a scheme for inactivation of tAPX by hydrogen peroxide in the absence of ascorbate.



If ascorbate is absent, APX-I is accumulated leading towards its spontaneous degradation at the rate constant, k_i. Under these conditions, the steady-state concentration of APX-I is given by

the following equation.

$$[\text{APX-I}] = \frac{k_1[\text{APX}]_o[H_2O_2]}{k_1[H_2O_2] + k_{-1} + k_i},$$

where $[APX]_o$ is the total concentration of APX. The initial formation rate (v) of inactivated APX would be given as follows;

$$v = k_i[\text{APX-I}] = \frac{k_i[\text{APX}]_o[H_2O_2]}{\frac{k_i + k_{-1}}{k_1} + [H_2O_2]}.$$

Thus, the formation rate of inactivated APX, v, follows the kinetics of Michaelis and Menten against the concentration of hydrogen peroxide, where $k_i[APX]_o$ gives the maximal formation rate of inactivated APX, V_{max}, and $(k_i + k_{-1})/k_1$ gives an apparent K_m value of tAPX for hydrogen peroxide in the formation of inactivated APX.

Using the data in Fig. IV-4, the inactivation rate of APX was estimated from the slope of the logarithmic plots of remaining activity against time after the addition of hydrogen peroxide. The rate increased with an increase in the concentration of hydrogen peroxide (Fig. IV-6). The apparent K_m value for hydrogen peroxide and V_{max} of the formation rate of inactivated APX are calculated to be 93.5 nM and 8.13×10^{-2} nM s^{-1} from the non-linear regression analysis using the Michaelis-Menten' equation (Sakoda and Hiromi 1976). From the values of K_m and V_{max}, the rate constants, k_i and k_{-1}, were calculated to be 9.6×10^{-2} s^{-1} and 1.0 s^{-1}, respectively, using a value of k_1 (1.2×10^7 M^{-1} s^{-1}). The rate constant, k_i, gives a half life of APX-I of 7.2 s in the absence of ascorbate, nearly equal to that of inactivation of tAPX by the depletion treatment of ascorbate, supporting the validity of my proposed scheme.

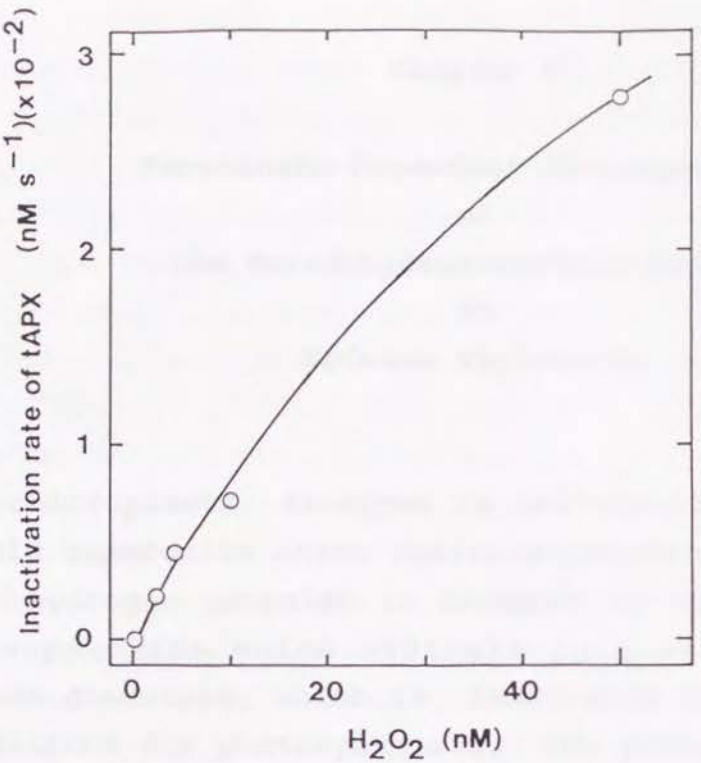


Fig. IV-6 *Effect of hydrogen peroxide concentration on inactivation rate of tAPX under anaerobic conditions.* Inactivation rate of tAPX was calculated from Fig. IV-4 as described in text and is plotted against the concentrations of hydrogen peroxide.

Chapter V

Ferredoxin-Dependent Photoreduction of the Monodehydroascorbate Radical in Spinach Thylakoids

In chloroplasts, dioxygen is univalently photoreduced in PS I to yield superoxide anion radicals (Asada et al. 1974). Subsequently, hydrogen peroxide is produced by the disproportionation of the superoxide anion radicals in a reaction catalyzed by superoxide dismutase, which is inevitable even under the favorable conditions for photosynthesis. The production rate of superoxide anion radicals in chloroplasts is estimated to be 240 μM s^{-1}, which gives a production rate of hydrogen peroxide 120 μM s^{-1} (Asada and Takahashi 1987). The 50% inhibition of photosynthesis by hydrogen peroxide occurred at 10 μM (Kaiser 1976). Thus, if the photoproduced hydrogen peroxide was not promptly scavenged in chloroplasts, the photosynthetic fixation of carbon dioxide would stop within a second, resulting in the wilting of plants. These conjectures suggest the necessity of a system for the immediate scavenging of hydrogen peroxide at its producing site.

Thylakoid-bound ascorbate peroxidase is localized in the stromal thylakoids in which the PS I complex is mainly found, and it can scavenge the hydrogen peroxide photoproduced in PS I (Chapter II). Thus, only a small amount of hydrogen peroxide diffuses to the stroma from its producing site on the thylakoid membranes. When the thylakoid-bound ascorbate peroxidase reduces hydrogen peroxide, the monodehydroascorbate (MDA) radical is produced as the primary oxidation product. Although the concentration of ascorbate in chloroplasts is high, above 10 mM (Foyer et al. 1983, Hossain et al. 1984), it would all be consumed within 80 s, as estimated from the production rate of hydrogen

peroxide, unless the regeneration systems of ascorbate from the MDA radical were operative. Thus, for effective scavenging of the hydrogen peroxide by ascorbate peroxidase, the regeneration of ascorbate from the MDA radical is essential. In addition to being produced by ascorbate peroxidase the MDA radical is also produced as a result of the interaction of ascorbate with superoxide, hydroxyl, and tocopherol chromanoxy radicals, as well as other organic radicals, such as carbon-centered and phenoxy radicals (Bielski 1982). The interaction of ascorbate with the glutathione thiyl radical also generates the MDA radical (Forni et al. 1983). The glutathione radical is produced in reactions with various radicals that include superoxide (Winterbourn 1993).

In the chloroplast stroma, the MDA radical is reduced by the FAD-enzyme MDA reductase with NAD(P)H as the electron donor (Hossain et al. 1984, Hossain and Asada 1985). Moreover, we have shown that the photoreduction of the MDA radical occurrs in thylakoids by a demonstration of the MDA radical-dependent oxygen evolution and the photochemical quenching of Chl fluorescence by the MDA radical. No NAD(P)H-dependent MDA reductase is bound to the thylakoid membranes (Chapter II). Quenching of Chl fluorescence of thylakoids by the MDA radical is inhibited by DCMU and DBMIB, an observation that suggests two possible sites for the photoreduction of the MDA radical in thylakoids, at either Cyt b_6/f or on the reducing side of PS I. In this communication, we describe the identification of the photoreducing site of the MDA radical in the thylakoids as PS I by EPR measurement, and the mediation of the photoreduction of the MDA radical by ferredoxin (Fd). Furthermore, competition for the reduced Fd in PS I between the MDA radical and $NADP^+$ is discussed.

Materials and Methods

Preparation of Fd and FNR from spinach — Fd was purified from spinach leaves according to the method of Buchanan and Arnon (1971). The concentration of Fd was determined from the absorbance at 420 nm and an absorption coefficient of 9.7 mM^{-1} cm^{-1}

(Buchanan and Arnon 1971). FNR was either purchased from Sigma or purified from spinach leaves (Asada and Takahashi 1971). The concentration of FNR was determined from the absorbance at 456 nm and an absorption coefficient of 10.7 mM^{-1} cm^{-1} (Shin 1971).

Isolation of thylakoid membranes from spinach leaves — Spinach leaves from a local market (60 g) were homogenized in a Polytron homogenizer by two 3-s pulses with 250 ml of the homogenizing medium (0.4 M sucrose/2 mM $MgCl_2$/10 mM NaCl/50 mM potassium phosphate, pH 7.0). The homogenate was filtered through 16 layers of cheesecloth and centrifuged at 5,000 x g for 10 min. The pellets were suspended in 250 ml of homogenizing medium without succrose, and washed twice by centrifugation in the same medium under the same conditions. The pelleted thylakoids were suspended in the homogenizing medium at 5 mg Chl ml^{-1} and used as thylakoids. It should be noted here that the present thylakoids were prepared in ascorbate-free medium. Therefore, thylakoid-bound ascorbate peroxidase was inactivated as a result of its lability in the absence of ascorbate (Chapter II), and thus, no production of the MDA radical was to be expected from the reaction of thylakoid-bound ascorbate peroxidase with the hydrogen peroxide that was photogenerated from superoxide. Chl was quantitated by the method of Arnon (1949).

Generation and determination of levels of the MDA radical — The generation and determination of levels of MDA radical were done as described in Chapter II.

Photoreduction of the MDA radical as determined by EPR — The thylakoids were suspended in 700 µl of assay medium that contained 2.5 mM ascorbate and ascorbate oxidase to generate the MDA radical in a flat EPR cell (thickness, 0.4 mm). The photoreduction of the MDA radical was started by illuminating the EPR cell with white light (300 W m^{-2}) or far-red light (≥710 nm, 16 W m^{-2}). Where indicated, a 14-µs single-turnover pulse of light was applied from a xenon discharge lamp with sharp on/off characteristics (XST 103; Walz, Effeltrich Germany). EPR conditions were set as follows: microwave power, 3 mW; receiver gain, 5 x 100; time constant, 0.03 s; modulation amplitude, 0.03 mT; magnetic

field, 338.6 ± 1.0 mT.

Measurement of chlorophyll fluorescence — Modulated Chl fluorescence was measured as described in Chapter II.

Results and Discussion

Photoreduction of the monodehydroascorbate radical is mediated by ferredoxin — When the thylakoids were illuminated while

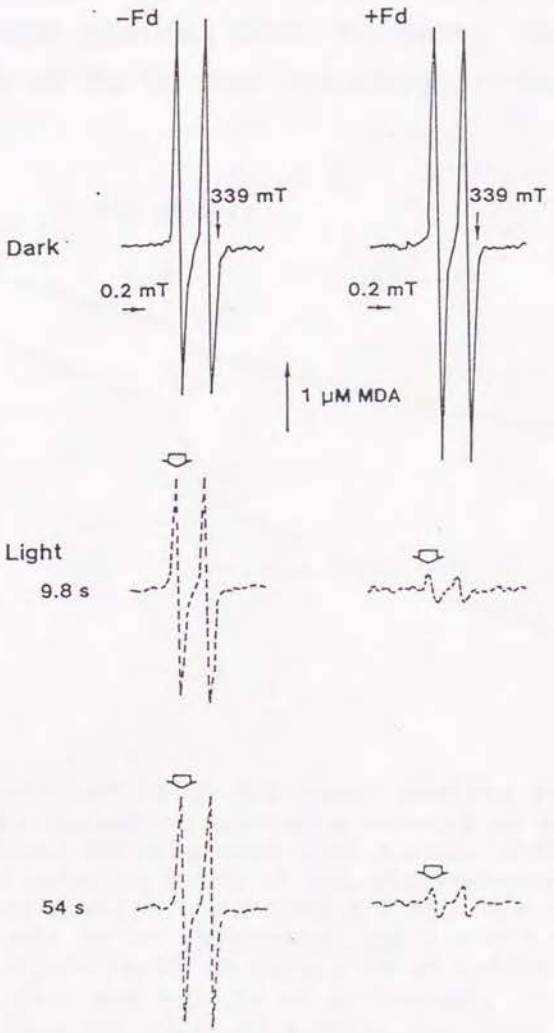


Fig. V-1 *Fd-dependent photoreduction of the MDA radical generated by ascorbate oxidase in spinach thylakoids, as monitored by EPR.* The reaction mixture (70 μl) contained 50 mM HEPES-KOH, pH 7.6/0.4 M sucrose/2 mM $MgCl_2$/10 mM NaCl/0.1 μM nigericin/2 mM ascorbate/sufficient ascorbate oxidase to generate 3.0 μM MDA radical/thylakoid membranes (0.7 μg Chl). Where indicated, 5 μM Fd was added. Conditions for EPR were the same as described in Materials and Methods, with the exception that the sweep time was 43 s for each scan. Indicated times show the time of the recording of the EPR signal at 338.6 mT (open arrow) after the white light had been turned on (300 W m^{-2}).

the MDA radical was being generated by the ascorbate-ascorbate oxidase system, the intensity of the EPR signal from the MDA radical decreased (Fig. V-1, -Fd), consistent with the previous observations (Chapter II). The EPR signal from the the MDA radical decreased more rapidly upon illumination of thylakoids in the presence of Fd (Fig. V-1, +Fd) than in its absence, indicating that Fd transfers electron to the MDA radical.

Photochemical quenching of Chl fluorescence in thylakoids is induced by the MDA radical but, to date, we have failed to observe any effect of Fd on the quenching (Chapter II). When the

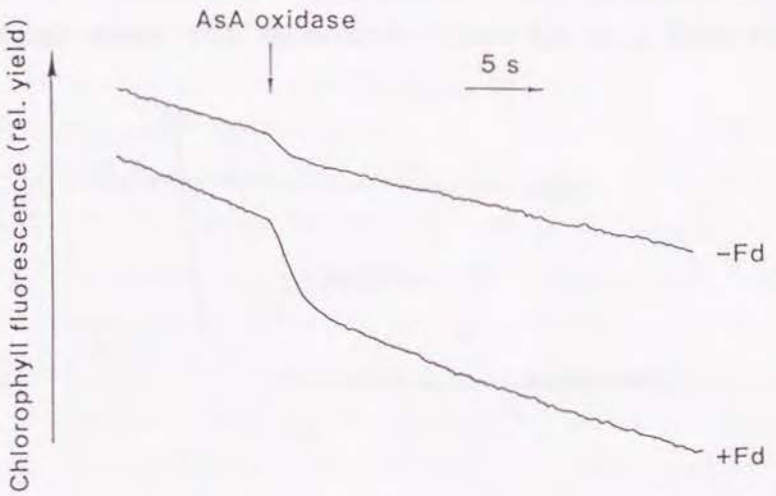


Fig. V-2 *Effects of Fd on the photochemical quenching of Chl fluorescence in thylakoid membranes induced by the MDA radical.* Fluorescence yield was monitored with a weak, modulated measuring light, and the relative yield of Chl fluorescence was induced by saturating actinic white light (300 W m^{-2}). The reaction mixture (1 ml) contained 50 mM HEPES-KOH, pH 7.6/0.4 M sucrose/2 mM $MgCl_2$/0.1 µM nigericin/10 mM NaCl/1 mM ascorbate/thylakoid membranes (20 µg Chl) and 5 µM Fd as indicated. Sufficient ascorbate (AsA) oxidase was added 15 s after the start of illumination by actinic light to give a steady-state concentration of the MDA radical of 2.6 µM in darkness.

intensity of actinic light for the generation of fluorescence is low, as in our previous measurements, namely, when the photogeneration rate of the reductant of the MDA radical is limiting, the

effect of Fd is negligible. However, when the intensity of actinic light is high and the photogeneration rate of the reductant is not limiting, the quenching of Chl fluorescence by Fd is expected, if Fd mediates the reduction of the MDA radical. The results in Fig. V-2 show that Fd mediates the photoreduction of the MDA radical. Thylakoids were illuminated in the presence of an uncoupler by saturating actinic light, the intensity of which was the same as that used for EPR measurements of the MDA radical. Under these conditions, photochemical quenching of Chl fluorescence of thylakoids upon the addition of ascorbate oxidase for the generation of the MDA radical was stimulated by the presence of Fd. Thus, Fd mediates the transfer of electron to the MDA radical when the electron flow is not limiting.

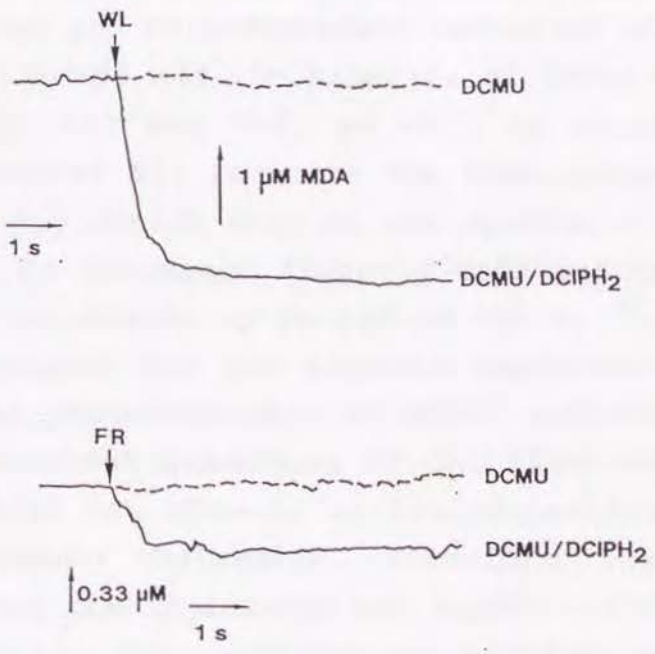


Fig. V-3 *Reduced dichloroindophenol-dependent ($DCIPH_2$-dependent) photoreduction of the MDA radical in spinach thylakoids in the presence of DCMU.* The reaction mixture (70 μl) contained 50 mM HEPES-KOH, pH 7.6/0.4 M sucrose/2 mM $MgCl_2$/0.1 μM nigericin/10 mM NaCl/2 mM ascorbate/sufficient ascorbate oxidase to generate 3.0 μM MDA radical/thylakoid membranes (0.7 μg Chl)/10 μM DCMU/5 μM Fd. Where indicated, 0.1 mM DCIP was added. In the presence of DCIP, the concentration of the MDA radical decreased to 2.4 μM as a result of the reduction of DCIP by ascorbate. Conditions for EPR were the same as described in Materials and Methods. The reaction mixture was illuminated by either white light (WL, 300 W m^{-2}) or far-red light (FR, ≥710 nm; 16 W m^{-2}). After the light had been turned on, the decreases in the intensity of the EPR signal from the MDA radical at 338.6 mT were recorded.

The Fd-mediated reduction site of the MDA radical in thylakoids was determined to be PS I by the following experiments. The Fd-dependent photoreduction of the MDA radical was inhibited by DCMU (Fig. V-3), consistent with the previous results (Chapter II), and also by DBMIB (Chapter II) confirming that PS II is not the site for the reduction of the MDA radical. When thylakoids were illuminated by white light in the presence of DCMU, the MDA radical was decreased as determined by its EPR signal at 338.6 mT only if electron was donated to PS I by the ascorbate-dichloroindophenol system. Furthermore, in the presence of reduced dichloroindophenol, the MDA radical was photoreduced even by far-red light that excited only PS I (Fig. V-3). These results demonstrate clearly that the site of the Fd-dependent photoreduction of the MDA radical is PS I in thylakoids.

Fd-dependent and Fd-independent reduction of the MDA radical in PS I of thylakoids and the kinetics of these reactions — The results in Fig. V-1 and V-2, as well as those from previous experiments (Chapter II) indicate the slow photoreduction of the MDA radical by thylakoids even in the absence of Fd. The rate in the absence of Fd increased linearly upon increases in the concentration of thylakoids up to 160 μg Chl ml^{-1} (Fig. V-4). The thylakoids prepared for the present experiments did not show evidence of the photoreduction of $NADP^+$ and also the $NADP^+$-dependent photochemical quenching of Chl fluorescence in the absence of Fd (data not shown), an indication that little Fd was bound to the present thylakoids. Therefore, the results in Fig. V-4 indicate that the thylakoids are capable of directly reducing the MDA radical via the bound primary electron acceptor in PS I. However, the rate (9.6 μmol MDA mg Chl^{-1} h^{-1}) was only 5% that in the presence of saturating amounts of Fd (192 μmol MDA mg Chl^{-1} h^{-1}), as determined at low concentrations of thylakoids, when the concentration of the MDA radical was 3.1 μM.

In our assay system, the photoreduction of the MDA radical is evaluated as the initial rate of the decrease from a steady-state concentration of the MDA radical in darkness. The concentration of the MDA radical could not be kept at a saturating

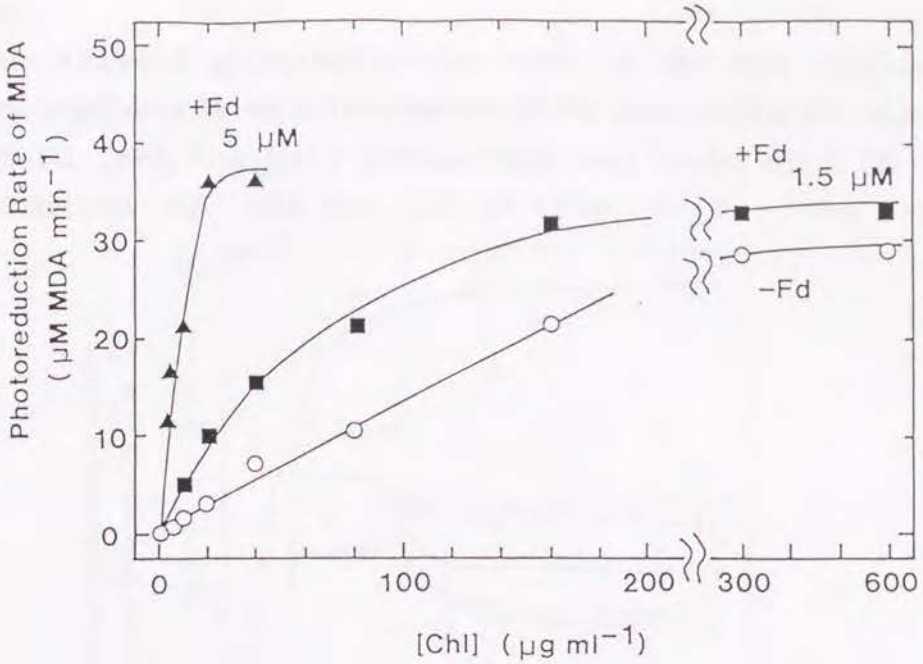


Fig. V-4 *Effects of the concentration of thylakoids on the rate of photoreduction of the MDA radical in the presence of Fd.* The reaction mixture (70 μl) contained 50 mM HEPES-KOH, pH 7.6/0.4 M sucrose/2 mM $MgCl_2$/0.1 μM nigericin/10 mM NaCl/2 mM ascorbate/sufficient ascorbate oxidase to generate 3.1 μM MDA radical. Thylakoid membranes and Fd were added to the reaction mixture at indicated concentrations. After white light (300 W m^{-2}) had been turned on, the rate of photoreduction of the MDA radical was determined from the initial rate of decrease in the intensity of the EPR signal from the MDA radical at 338.6 mT.

value, because of their appreciable rate of spontaneous disproportionation of the radicals (2 X 10^5 M^{-1} s^{-1} at pH 7, Bielski 1982). Under the conditions used for the experiment for which results are shown in Fig, V-4, namely, a steady-state concentration of the MDA radical of 3.1 μM in darkness, the photoreduction rate of the MDA radical was increased by increasing the concentration of Fd, and saturated at reduction rates of around 35 μM min^{-1}. This apparent saturation of the reaction is caused by instrumental limits to the determination of the initial reduction rate of the MDA radical, when the rate is too high in the presence of thylakoids at high concentrations. Therefore, in the following kinetic studies, the Fd-dependent photoreduction of the MDA radical was analyzed at concentrations of thylakoids below 10 μg Chl ml^{-1}, to avoid interference by the Fd-independent photore-

duction.

The initial photoreduction rate of the MDA radical in illuminated thylakoids was increased with increases in the concentration of Fd, and the half saturation was found at 1 μM Fd when the concentration of MDA was 1.1 μM (Fig. V-5). The apparent K_m

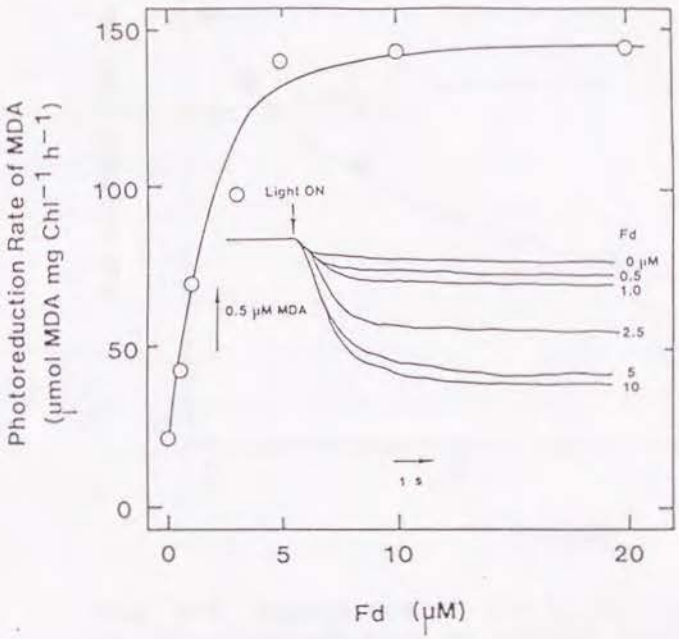


Fig. V-5 *Dependence of the photoreduction of the MDA radical on the concentration of Fd in spinach thylakoids.* The reaction mixture (70 μl) contained 50 mM HEPES-KOH, pH 7.6/0.4 M sucrose/2 mM $MgCl_2$/0.1 μM nigericin/10 mM NaCl/2 mM ascorbate/sufficient ascorbate oxidase to generate 1.1 μM MDA radical/thylakoid membranes (0.7 μg Chl). Fd was added at indicated concentrations. Conditions for EPR are described in Materials and Methods. After white light (300 W m^{-2}) had been turned on, the photoreduction rate of the MDA radical was determined from the initial rate of decrease in the intensity of the EPR signal from the MDA radical at 338.6 mT. Inserts show the time course of the reduction of MDA radicals.

values for the MDA radical in the Fd-dependent photoreduction reaction were determined by measuring the initial photoreduction rate of the MDA radical by EPR at several concentrations of the MDA radical (Fig. V-6). In the absence of Fd, the apparent K_m values for the MDA radical was 8.5 μM, which is very similar to that determined from the MDA radical-dependent oxygen evolution in illuminated thylakoids without the addition of Fd (Chapter II). As the concentration of Fd increased, the affinity of the MDA radical for the reduced Fd in thylakoids increased, as indi-

cated by a decrease in the apparent K_m values for the MDA radical. Thus, the affinity of the MDA radical for a membrane-bound photoreductant is lower than that for the photoreduced Fd in thylakoids.

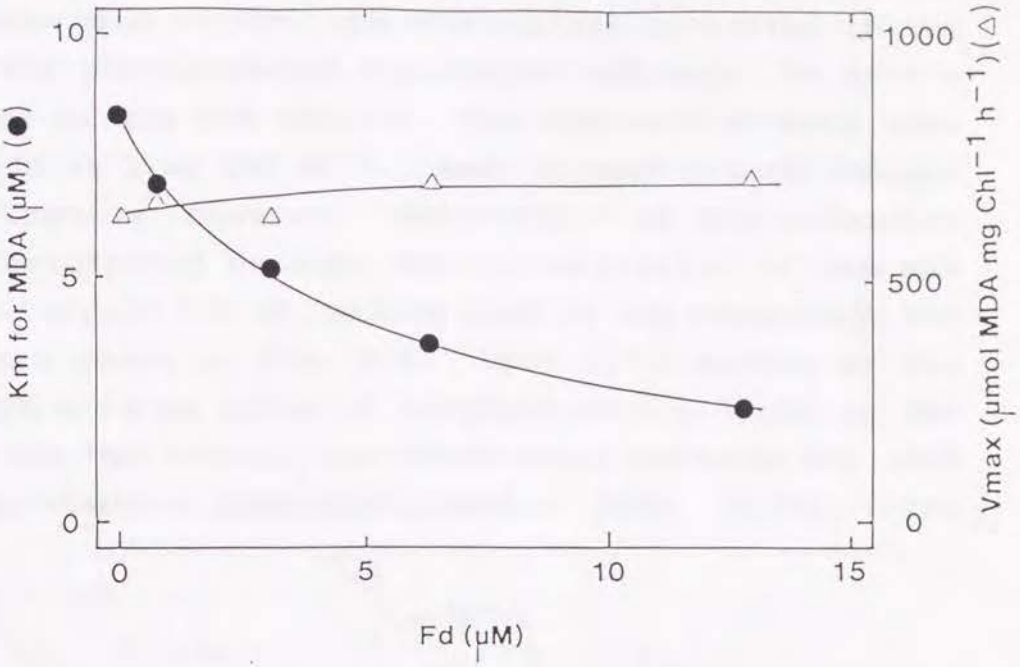


Fig. V-6 *Dependence of the K_m for the MDA radical and the V_{max} of its photoreduction by spinach thylakoids on the concentration of Fd.* The reaction mixture (70 μl) contained 50 mM HEPES-KOH, pH 7.6/0.4 M sucrose/2 mM $MgCl_2$/0.1 μM nigericin/10 mM NaCl/2 mM ascorbate/thylakoid membranes (0.7 μg Chl)/Fd at indicated concentrations. Ascorbate oxidase was added to the reaction mixture to generate the indicated concentrations of the MDA radical up to 3 μM. The initial photoreduction rate of the MDA radical was measured as described in the legend to Fig. V-4. Apparent K_m values for the MDA radical (closed circles) and V_{max} for its photoreduction (open triangles) at several concentrations of Fd were determined from the non-linear regression analysis of the photoreduction rates using Michaelis and Menten's equation (Sakota and Hiromi 1976).

The V_{max} values for the photoreduction of the MDA radical were hardly affected by Fd (Fig. V-6). These values were estimated from the Michaelis-Menten kinetics at saturating concentrations of the MDA radical, which are difficult to maintain, as mentioned above. Therefore, the V_{max} values do not represent the actual, maximum photoreduction rate of the MDA radical. Even in the absence of Fd, the V_{max} value is similar to that in the presence of Fd, however, at physiological concentrations of the MDA radical, Fd accelerates the photoreduction considerably, as discussed below.

Fd-dependent photoreduction of the the MDA radical generated by superoxide radicals in thylakoids — Further evidence for Fd-dependent reduction of the MDA radical can be found in the effect of Fd on the reduction of the the MDA radical generated in the thylakoids by the photoproduced superoxide radicals. To attain detectable level of the MDA radical, the reaction mixture contained thylakoids at 1 mg Chl ml^{-1}. Even at such a high concentration of thylakoids, however, "saturation" of the reduction rate was not anticipated because the concentration of the MDA radical was low, around 0.1 μM, unlike that in the experiment for which results are shown in Fig. V-4. Upon illumination of the thylakoids with a 14-μs pulse of single-turnover light in the absence of Fd, the MDA radical was transiently produced and then decayed via spontaneous disproportionation (Fig. V-7A). The

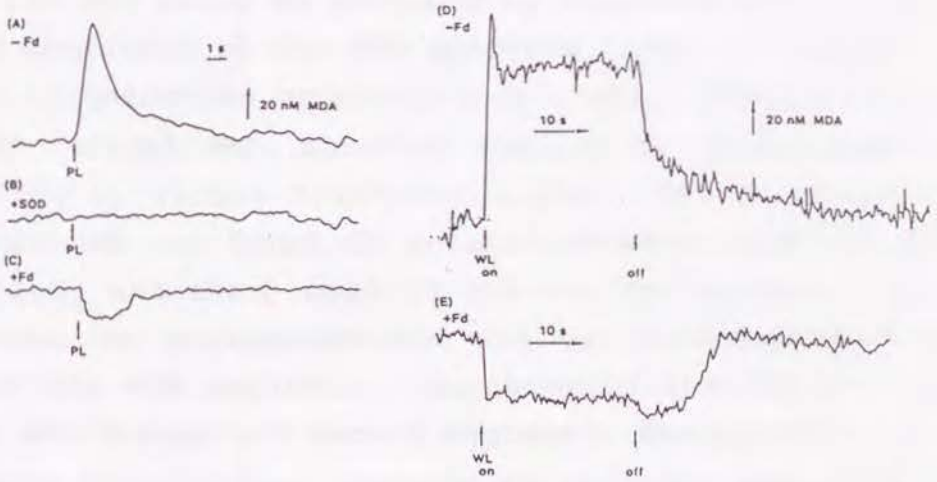


Fig. V-7 *Flash-induced and continuous light-induced production of the MDA radical and its reduction by Fd in spinach thylakoids.* The reaction mixture (70 μl) contained 50 mM HEPES-KOH, pH 7.6/0.4 M sucrose/2 mM $MgCl_2$/10 mM NaCl/0.1 μM nigericin/1 mM ascorbate/thylakoids (70 μg Chl). Under the present conditions, 50 nM MDA radical was produced in darkness by the autooxidation of ascorbate. Where indicated, 30 μM CuZn-superoxide dismutase (SOD) or 5 μM Fd was added to the reaction mixture prior to the illumination by single-turnover pulse light (PL, 1,500 W m^{-2}; 14 μs) or white light (WL, 300 W m^{-2}). The inhibition of EPR signals from the MDA radical at 338.6 mT were monitored. Conditions for EPR were the same as described in Materials and Methods, with the exception that the receiver gain was 1 x 1,000.

transient production of the MDA radical by the single-turnover light was completely inhibited by superoxide dismutase (Fig. V-

7B), an indication that the MDA radical was produced by the univalent oxidation of ascorbate by the superoxide radical generated in PS I (Asada et al. 1974). The reaction rate constant between ascorbate and superoxide has been determined to be 2.7 x 10^5 M^{-1} s^{-1} (Nishikimi 1975). Since the membrane-bound ascorbate peroxidase activity was not retained in the present thylakoids, the production of the MDA radical in the peroxidase-catalyzed reaction by hydrogen peroxide derived from superoxide appeared very unlikely.

In contrast to the absence of Fd, when Fd was added to the thylakoids, no transient production of the MDA radical by the single-turnover light was detected, and rather the EPR signal of the MDA radical was transiently decreased followed by recovery to the dark level (Fig. V-7C). A low level of the MDA radical in the dark (50 nM) would be produced by autooxidation of ascorbate, which was confirmed by its EPR spectrum (data not shown).

Upon illumination by continuous light, production and decay of the MDA radical were observed similar to those observed after illumination by single turn-over light. In the absence of Fd, the MDA radical was found at a level of more than 100 nM during illumination, and this level is assumed to represent a net balance between the photoproduction and the Fd-independent photoreduction of the MDA radical. The decay of the MDA radical after the light was turned off should represent the spontaneous disproportionation (Fig. V-7D). Superoxide radicals should be produced under continuous light in the presence of Fd, since the Fd- and ascorbate-containing reaction mixture gave a similar rate for the photoreduction of dioxygen to that observed in the absence of Fd (data not shown). Therefore, the MDA radical would be photoproduced via a reaction involving superoxide radicals in the presence of Fd. However, the level of the MDA radical did not increase on illumination, and rather was lowered below 10 nM (Fig. V-7E). After turning off of light, the level of the MDA radical remained low for more than 10 s, reflecting the reduction of the MDA radical, generated by the autooxidation of ascorbate, by the reduced Fd that accumulated during illumination.

After all of the reduced Fd has been exhausted, the level of the MDA radical is assumed to be recovered to the dark level. All our observations indicate that the Fd-dependent photoreduction of the MDA radical decreases the radical concentration to almost zero in thylakoids. This is especially the case when the concentrations of the MDA radical is low such as photogenerated by thylakoids themselves, because of a low apparent K_m value for the MDA radical in the presence of Fd (Fig. V-6).

The MDA radical is reduced by reduced ferredoxin that is generated by NADPH and Fd-NADP reductase — To determine whether the photoreduced Fd in PS I directly donates electrons to the MDA radical, the reduction of the MDA radical with the enzymatically reduced Fd was examined. The reduction of the MDA radical was determined by the oxidation of NADPH in the presence of FNR and Fd (Table V-1). In the absence of Fd, the reduction rate of the MDA radical was very low as compared with that in the presence of Fd, indicating the preferential reduction of the MDA radical by reduced Fd, but not by reduced FNR. Previous failure to show the reduction of MDA radicals by the reduced Fd generated in the NADPH-FNR system (Chapter II) would be due to a low concentration of FNR (0.68 μM). We increased the concentration of FNR in the NADPH-FNR system, and found that the MDA radical-dependent oxidation of NADPH was half-saturated by 50 nM FNR. The reduction rate of the MDA radical as determined by the oxidation of NADPH was also dependent on the concentration of Fd, and linearly increased up to 0.5 μM.

Little reduction of the MDA radical by reduced FNR is also sustained by the following observations. The antagonist of $NADP^+$ in the FNR-catalyzed reaction, ATP-ribose, did not show any inhibition of the Fd-mediated photoreduction of the MDA radical in thylakoids at 3.8 mM where the photoreduction of $NADP^+$ was inhibited (data not shown). Thus, it is unlikely that the MDA radical are photoreduced by the reduced form of thylakoid-bound FNR.

Further evidence for the direct reduction of the MDA radical by reduced Fd is the decrease in the steady state concentration

of the MDA radical generated in the ascorbate-ascorbate oxidase system, as determined by EPR, by the reduced Fd produced in the NADPH-FNR system. The decrease in the steady state concentration of the MDA radical was negligible in the absence of Fd, but was enhanced by Fd. The concentration of the MDA radical was further lowered by an increase in the concentration of NADPH which accelerated the reduction rate of Fd (Table V-1).

Table V-1 *The reduction of the MDA radical by the reduced Fd generated with the NADPH-Fd-NADP reductase.* The reaction mixture (1 ml) contained 50 mM HEPES-KOH, pH 7.6/0.4 M sucrose/2 mM $MgCl_2$/10 mM NaCl/0.5 mM ascorbate/320 nM FNR/10 μM Fd where indicated and NADPH as indicated. The reduction of the MDA radical was measured as a decrease for 7.5 s in the absorbance at 340 nm assuming an absorption coefficient of 6.2 mM^{-1} cm^{-1}, after the addition of ascorbate oxidase, which generated the MDA radical at a steady state concentration of 1.2 μM in the absence of FNR. The oxidation of NADPH due to diaphorase activity of FNR and to autooxiation of Fd as observed before the addition of ascorbate oxidase was corrected. The reduction of the MDA radical was also measured using the same reaction mixture, as a decrease in the steady state concentration of the MDA radical determined by EPR 2 min after the addition of ascorbate oxidase, which generated the MDA radical at a steady state concentration of 1.3 μM in the absence of FNR. Where indicated, NADPH had been added to the reaction mixture, before ascorbate oxidase was added.

System (concentration of NADPH)	Reduction rate of the MDA radical (μmol reduced min^{-1} μmol FNR^{-1})	Steady-state concentration of MDA radical (μM)
NADPH → FNR → MDA		
0 mM	0	1.3
0.1 mM	19	1.3
1.0 mM	nd	1.2
NADPH → FNR → Fd → MDA		
0 mM	0	1.3
0.1 mM	282	0.93
1.0 mM	nd	0.34

nd, not determined.

Under the conditions of Table 1, the steady state concentration of reduced Fd was determined to be below 0.1 μM from an decrease in absorbance at 420 nm, when the concentration of NADPH was 0.1 mM (data not shown), and that of MDA radical 0.93 μM by EPR (Table V-1). At the steady state, the production rate (v_p, 0.32 μM s^{-1}) of the MDA radical by the ascorbate-ascorbate oxidase system is equal to the sum of the reduction rate (k_{Fd}[MDA][redFd]) of the MDA radical by reduced Fd and the disproportionation rate (k_{sp}[MDA]^2) of the MDA radicals. Thus,

$$v_p = k_{Fd}[MDA][redFd] + k_{sp}[MDA]^2,$$

where k_{Fd} is the second-order rate constant of the reduction of MDA radical by reduced Fd, [MDA] and [redFd] are the steady state concentrations of the MDA radical and reduced form of Fd, respectively, and k_{sp} is the rate constant of the disproportionation of MDA radicals and given as 10^5 M^{-1} s^{-1} at pH 7.6 (Bielski 1982). The k_{Fd} is estimated to be higher than 2.7 x 10^6 M^{-1} s^{-1} using the above equation. The similar estimation also is done for the Fd-dependent photoreduction of MDA radicals in thylakoids under the conditions of Fig. V-5 at 10 μM Fd. The steady state concentrations of MDA radicals and reduced Fd were 0.1 μM and below 0.2 μM, respectively, and v_p was 0.32 μM s^{-1}. The k_{Fd} in the thylakoids system is estimated to be 5-fold higher than that in the NADPH-FNR system; 1.6 x 10^7 M^{-1} s^{-1}. Contribution of the spontaneous disproportionation of MDA radicals is 34% in NADPH-FNR system, and 0.3% in thylakoid-system that of the reduction by Fd.

Competition between $NADP^+$ and the MDA radical for photoreduced Fd in thylakoids — The present results indicate that, in thylakoiods, most of the MDA radical is reduced by the photoreduced Fd in PS I. This conclusion raises the question of whether $NADP^+$ and the MDA radical compete for photoreduced Fd in the thylakoids. The results in Figure V-8 indicate competition between $NADP^+$ and the MDA radical for the reductant. The MDA radical at less than micromolar levels suppressed the photoreduction of $NADP^+$ at milimolar levels by reduced Fd. Conversely, $NADP^+$ affected the photoreduction of the MDA radical. The photoreduction rate of the MDA radical was decreased as the concen-

tration of $NADP^+$ increased, again indicating the competition between the MDA radical and $NADP^+$ for photoreduced Fd (Fig. V-9A). The photoreduction rate of the MDA radical at 3 μM was decreased by 50% by $NADP^+$ at 200 μM, which is the saturating concentration for the photoreduction of $NADP^+$ in thylakoids (Furbank and Badger 1983).

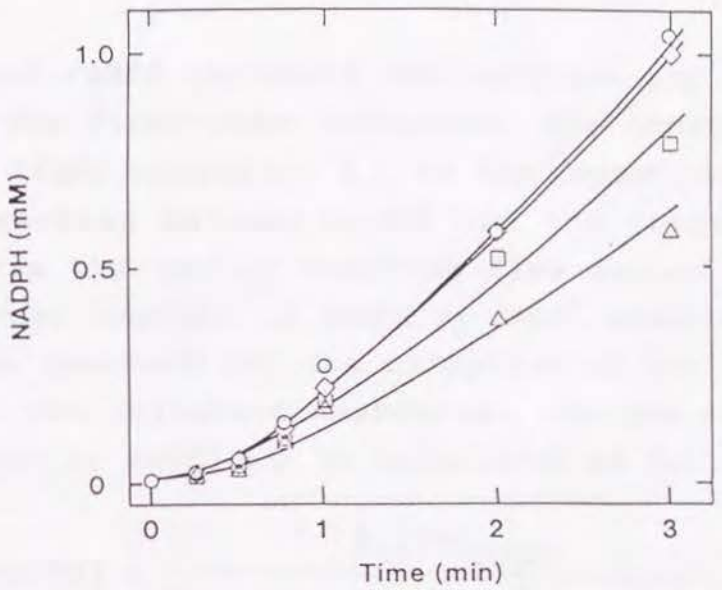
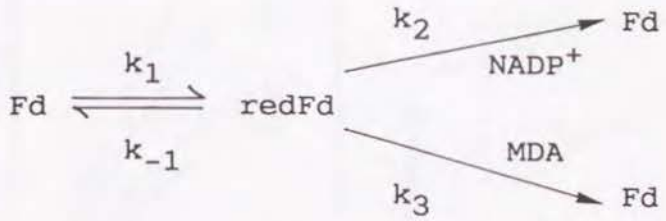


Fig. V-8 *Inhibition of the photoreduction of $NADP^+$ by the MDA radical in spinach thylakoids.* The reaction mixture (1 ml) contained 50 mM HEPES-KOH, pH 7.6/0.4 M sucrose/2 mM $MgCl_2$/10 mM NaCl/2.5 mM ascorbate/5 μM Fd/0.1 M nigericin/thylakoid membranes (60 μg Chl)/2 mM $NADP^+$. After incubation in darkness for 5 min, the reaction mixture in a test tube was illuminated by a 300-watt iodine projector lamp (320 W m^{-2}) at 25°C. At indicated times, an aliquot of the reaction mixture was sampled, and the concentration of photoreduced NADPH was determined from the increase in absorbance at 340 nm and an absorption coefficient of 6.2 mM^{-1} cm^{-1}. Ascorbate oxidase was added 30 s before the illumination to generate the MDA radical at the following steady-state concentrations in darkness: circles, 0 μM; diamonds, 0.7 μM; squares, 1.6 μM; triangles, 3.3 μM. Since the concentration of the MDA radical was immediately decreased upon illumination (see Fig. V-5), the actual concentrations of the MDA radical during measurements of the photoreduction of $NADP^+$ were lower by least one order of magnitude than those in darkness.

The apparent K_m value for the MDA radical in its photoreduc-

tion was proportional to the concentration of $NADP^+$ (Fig. V-9B). This relationship can be explained by the following reaction scheme:



where Fd and redFd represent the oxidized and reduced forms of Fd; k_1 is the first-order reduction rate constant of Fd, which depends on light intensity; k_{-1} is the second-order rate constant for the reaction between redFd and the intersystem electron carriers via the cyclic electron flow around PS I; k_2 is the oxidation rate constant of redFd by $NADP^+$ mediated by FNR; and k_3 is the rate constant for the oxidation of the redFd by the MDA radical on the thylakoid membranes. In the steady state, the concentration of redFd can be calculated as follows:

$$[\text{redFd}] = \frac{k_1[\text{Fd}]_{\text{total}}}{k_1 + k_{-1} + k_2[\text{NADP}^+] + k_3[\text{MDA}]},$$

where $[\text{Fd}]_{\text{total}} = [\text{Fd}] + [\text{redFd}]$. The photoreduction rate of the MDA radical (v) is given by the following equation:

$$v = k_3[\text{redFd}][\text{MDA}] = \frac{V_{max}[\text{MDA}]}{K_m + [\text{MDA}]},$$

where V_{max} is the maximum photoreduction rate of the MDA radical and equals $k_1[\text{Fd}]_{\text{total}}$, and K_m is $(k_1 + k_{-1})/k_3 + k_2[\text{NADP}^+]/k_3$. This equation is analogous to that of Michaelis and Menten, and the "K_m" is the value for the the MDA radical in its photoreduction at a fixed concentration of $NADP^+$. When K_m values for the MDA radical are plotted against the concentration of $NADP^+$ (Fig. V-9B), the slope of the straight line gives the ratio of k_3 to k_2. The ratio was estimated to be 34, indicating the greatly preferable photoreduction of the MDA radical than that of $NADP^+$

in PS I.

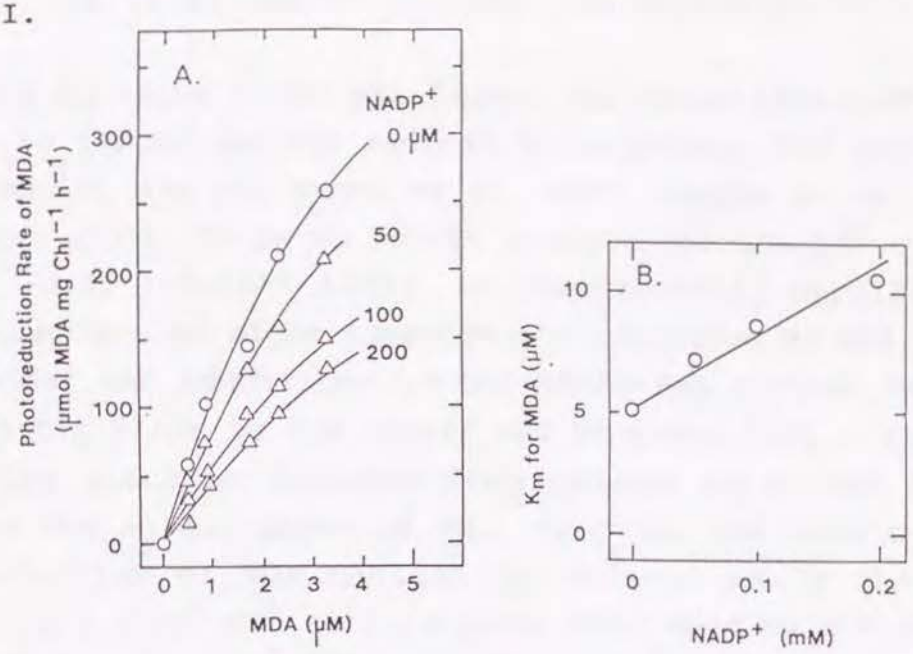


Fig. V-9 *Inhibition of the photoreduction of the MDA radical by NADP$^+$ in spinach thylakoids.* (A) The reaction mixture (70 μl) contained 50 mM HEPES-KOH, pH 7.6/0.4 M sucrose/2 mM $MgCl_2$/10 mM NaCl/2.5 mM ascorbate/5 μM Fd/0.1 μM nigericin/thylakoid membranes (0.7 μg Chl). NADP$^+$ was added at the indicated concentrations. The concentrations of the MDA radical in the reaction mixture were adjusted, as indicated, by changing the concentration of ascorbate oxidase added to the reaction mixture before illumination. Initial rates of photoreduction of the MDA radical were measured as described in the legend to Fig. V-4. (B) Apparent K_m values for the MDA radical during its photoreduction at the indicated concentrations of NADP$^+$ were determined from the non-linear regression analysis using Michaelis and Menten's equation (Sakota and Hiromi 1976) and they were plotted against the concentration of NADP$^+$.

Concluding Remarks — The photoreduction site of the MDA radical in thylakoids has been identified as the reducing side of PS I, since the MDA radical was photoreduced by PS I that has been excited by far-red light and white light in the presence of DCMU if electrons are donated to PS I (Fig. V-3). Participation of Fd in the photoreduction of the MDA radical in thylakoids was confirmed by the decrease in levels of the MDA radical, as determined by the intensity of the EPR signal (Fig. V-1, V-4 and V-5) and also from quenching of Chl fluorescence by the MDA radical (Fig. V-2). The requirement for Fd is additional evidence for the participation of PS I in the photoreduction. Further, from the reduction of the MDA radical by the Fd reduced by the NADPH-FNR system (Table V-1), it is clear that in thylakoids the photoreduced Fd directly reduces the MDA radical to ascorbate.

This is the first report showing the reduction of a radical by Fd.

The E_m value (-420 mV; Tagawa and Arnon 1962) of Fd is low enough to reduce the MDA radical to ascorbate (MDA radical/ascorbate couple, 320 mV; Nanni et al. 1980, Sapper et al. 1982). On the other hand, Fd is an acidic protein and the p*Ka* of MDA radical is -0.45 (Bielski 1982), an electrostatic repulsion between the two molecules might suppress the reduction of MDA radicals by Fd. Under the conditions of the NADPH-FNR system, however, the Fd probably binds to FNR (Knaff and Hirasawa 1991), which facilitates the electron transfer from reduced Fd to MDA radicals by masking the acidic patch of Fd. Even so, the rate constant for the reduction of MDA radical by reduced Fd in the NADPH-FNR system (2.7×10^6 M^{-1} s^{-1}) is lower than that by the photoreduced Fd in PS I (1.6×10^7 M^{-1} s^{-1}). This difference suggests that the thylakoid membranes where Fd binds would provide a circumstance, which would further cancel out the electrostatic repulsion for facilitation of the reduction of MDA anion radicals by Fd.

Even in the absence of Fd, the MDA radical could be photoreduced, but its rate is only 5% that in the presence of Fd (Fig. V-4 and V-5). Further, the K_m value for the MDA radical is higher than that in the presence of Fd (Fig. V-6). These results show that in the absence of Fd the MDA radical is photoreduced by a membrane-bound electron carrier, different from the peripheral Fd. The F_A/F_B cluster in *psa* C of PS I (Golbeck 1992) has the E_m values of -470 and -560 mV (Oh-oka et al. 1991) which are low enough to reduce the MDA radical to ascorbate, and is a likely site for the photoreduction of the MDA radical in the absence of Fd. The Fd-independent photoreduction could not lower the the MDA radical below 100 nM when the MDA radical is generated at physiological, low levels, while the Fd-dependent photoreduction maintained the levels of the MDA radical at almost zero (Fig. V-7), indicating that at physiological concentration of the MDA radical the Fd-dependent reaction has a higher affinity for the MDA radical than the Fd-independent one does and could function for the regeneration of ascorbate under physiological conditions.

Dioxygen is univalently reduced to the superoxide anion radical in PS I (Asada et al. 1974) and, subsequently, hydrogen

peroxide is produced via the disproportionation of the superoxide anion radicals in a reaction catalyzed by superoxide dismutase. Thylakoid-bound ascorbate peroxidase catalyzes the reduction of the hydrogen peroxide by ascorbate, producing the MDA radical on the reducing side of PS I (Chapter II). Studies of competition for the photoreduced Fd between $NADP^+$ and the MDA radical in PS I (Fig. V-8 and V-9) indicate the reduction of the MDA radical at more than 30-fold greater rate than that of $NADP^+$. The present results confirm that the actual electron acceptor produced upon the addition of hydrogen peroxide to chloroplasts or thylakoids is the MDA radical, and they infer the following two observations. First, when hydrogen peroxide is added to intact chloroplasts under illumination, the fixation of carbon dioxide ceases while the hydrogen peroxide is being scavenged. After all the hydrogen peroxide added to chloroplasts has been scavenged, the fixation of carbon dioxide starts again (Nakano and Asada 1980). This observation can be accounted for by the preferential photoreduction of the MDA radical generated by hydrogen peroxide. Second, the photochemical quenching of Chl fluorescence is induced upon the addition of hydrogen peroxide to either chloroplasts (Neubauer et al. 1990) or thylakoids (Chapter II). This phenomenon can be explained by the photoreduction of the MDA radical generated in the reaction of thylakoid-bound ascorbate peroxidase with the hydrogen peroxide. Such quenching has actually been observed when the MDA radical is generated by the ascorbate-ascorbate oxidase system (Chapter II). When actinic light for the measurement of fluorescence does not limit the photoreduction of the MDA radical, namely, at high intensities of actinic light, the participation of Fd in the photoreduction of the radical can be demonstrated (Fig. V-2).

It has been assumed that the MDA radical produced by the reaction catalyzed by ascorbate peroxidase is reduced to ascorbate by MDA reductase, which is localized in the stroma with NADPH or NADH as the electron donor (Hossain et al. 1984, Hossain and Asada 1985). As indicated by this study, the photoreduced Fd in PS I is preferentially used to reduce the MDA radical and, therefore, the production of NADPH at PS I is suppressed while the thylakoid-bound ascorbate peroxidase generates the MDA radi-

cal, and little NADPH is available to MDA reductase. Thus, in the vicinity of the thylakoid membranes, the contribution of MDA reductase to the regeneration of ascorbate seems to be very low or negligible

We propose a new scheme for the scavenging of superoxide and hydrogen peroxide which includes the regeneration of ascorbate in chloroplast thylakoids (Fig. V-10). The superoxide anion radi-

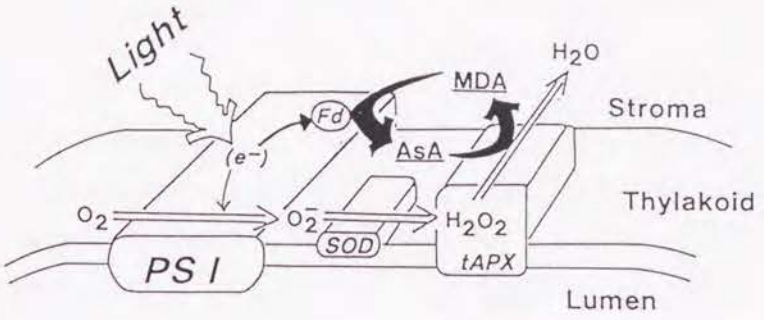


Fig. V-10 *The proposed thylakoid scavenging system for superoxide radicals and hydrogen peroxide in chloroplasts.* For details, see the text.

cals photoproduced in PS I within the thylakoid membranes diffuse to the stroma and disproportionate to hydrogen peroxide and dioxygen in reaction catalyzed by thylakoid-bound or peripheral superoxide dismutase (SOD; Hayakawa et al. 1984, 1985, Kanematsu and Asada 1990). The hydrogen peroxide is reduced to water by ascorbate (AsA) in a reaction catalyzed by the thylakoid-bound ascorbate peroxidase (tAPX), with production of the MDA radical (Chapter II, III). The MDA radical is then reduced by the photoreduced Fd, which regenerates ascorbate for the electron donor of the thylakoid-bound ascorbate peroxidase.

When the superoxide radicals and hydrogen peroxide are failed to interact with the thylakoid scavenging system, they are scavenged by the stromal scavenging system, which is the same as the system proposed previously (Asada and Takahashi 1987). The stromal scavenging system involves a) stromal superoxide dismutase (Kanematsu and Asada 1990), b) reduction of hydrogen peroxide in a reaction catalyzed by the stromal ascorbate peroxidase (Nakano and Asada 1981,1987, Chen and Asada 1989), c) reduction of the MDA radical by NAD(P)H in a reaction catalyzed by MDA reductase (Hossain et al. 1984, Hossain and Asada 1985), d)

spontaneous disproportionation of the MDA radicals to dehydroascorbate (DHA) and AsA, e) reduction of DHA to AsA by GSH in a reaction catalyzed by DHA reductase (Hossain and Asada 1984b), f) reduction of $NADP^+$ by reduced Fd in a reaction catalyzed by Fd-NADP reductase, and g) reduction of GSSG by NADPH in a reaction catalyzed by glutathione reductase (Halliwell and Foyer 1978, Connel and Mullet 1986).

Chapter VI

Acquisition of Hydrogen Peroxide-Scavenging System during the Evolution of Cyanobacteria

Photoproduction of various species of active oxygen is inevitable in photosynthetic organisms. The two major primary species of active oxygen are the superoxide anion radical, produced by the autooxidation of the primary electron acceptor of PSI and of reduced ferredoxin, and the singlet dioxygen which is produced by pigment-photosensitized reactions. Because of the high reactivity of active oxygen with cellular components, its immediate scavenging is indispensable to the organisms, and the cells suffer photooxidative damage or photoinhibition unless scavenging systems are operative (Asada and Takahashi 1987).

Cyanobacteria were the first photosynthetic organisms to acquire a system for the oxidation of water for the electron donation to the reaction center, with the resultant evolution of dioxygen. Hence, the cyanobacteria themselves should have systems for scavenging active oxygen to protect their cells from photooxidative damage. The photoreduction of dioxygen to produce active oxygen in cyanobacteria and eukaryotic algae has been demonstrated in intact cells (Patterson and Myers 1973, Radmer and Kok 1976, Radmer and Ollinger 1980, Peltier and Thibault 1985) and in isolated thylakoids (Honeycutt and Krogmann 1972, Hammans et al. 1977).

It has been shown that cyanobacteria contain carotenoids and tocopherols in the thylakoid membranes for the scavenging of singlet dioxygen and of lipid radicals. Cyanobacteria also contain superoxide dismutase (Abeliovich et al. 1974, Asada et al. 1977) to scavenge the superoxide radicals, and it has been

identified as an Fe-enzyme in the cytosol (Asada et al. 1975, Misra and Keele 1975, Lumsden et al. 1976, Csèke et al. 1979) and an Mn-enzyme in the thylakoids (Okada et al. 1979). The presecnce of superoxide dismutase in cyanobacteria is not surprising since even anaerobic photosynthetic bacteria, such as *Chromatium* and *Chlorobium*, contain Fe-superoxide dismutase (Kanematsu and Asada 1978a,b). These observations suggest that the very low concentration of dioxygen in the atmosphere prior to the appearance of cyanobacteria (Berkner and Marshall 1965) was a problem for these anaerobic bacteria and necessitated the effective scavenging of superoxide radicals. The superoxide radicals are disproportionated in a reaction catalyzed by superoxide dismutase to produce hydrogen peroxide and dioxygen. The hydrogen peroxide is scavenged by an ascorbate-specific peroxidase in angiosperm chloroplasts. The oxidized ascorbate, monodehydroascorbate (MDA) radical, is reduced to AsA dependent on ferredoxin which is photoreduced in photosystem I of thylakoids (Chapter V) and MDA reductase using NADPH localized in the stroma (Hossain et al. 1984). Thus, the hydrogen peroxide photoproduced in thylakoids is scavenged by the scavenging system composed of ascorbate peroxidase and the regeneration of ascorbate using photoreductants.

In this chapter, the mechanism of hydrogen peroxide scavenging in the prokaryotic algae cyanobacteria and eukaryotic algae is described. I have found that although ascorbate peroxidase does not exist in any cyanobacteria tested, the scavenging system of hydrogen peroxide was composed of the peroxidase which uses photoreductant as the electron donor in some cyanobacteria, similar to angiosperm chloroplasts and composed only of catalase in other cyanobacteria. Further it was elucidated that in eukaryotic algae the hydrogen peroxide photoproduced was scavenged by a peroxidase reaction using ascorbate as the electron donor like angiosperm chloroplasts. On the basis of above results, evolution of scavenging system of hydrogen peroxide is discussed.

Materials and Methods

Culture of cyanobacteria — *Anabaena variabilis, Anabaena cylindrica, Anacystis nidulans, Nostoc muscorum, Aphanothece halophytica, Plectonema boryanum, Fremyella diplosiphon, Tolypothrix tenuis* were grown in the Kratz-Myers medium (Kratz and Myers 1955) at 25°C (3 klx and 1% CO_2). *Synechocystis* 6803 was cultured in the BG-11 medium (Allen 1968) at 30°C (4 klx and 3% CO_2). *Synechococcus* 7002 was cultured in medium A of Stevens et al. (1973) at 30°C (4 klx and 3% CO_2). *Euglena gracilis* and *Chlamydomonas reinhardtii* were grown in the media described by Cramer and Myers (1952) and Orth et al. (1966), respectively. Cells after the mid-log phase of growth were harvested by centrifugation at 3,000 x g or 10,000 x g (*S.* 6803 and *S.* 7002) for 10 min and washed with 50 mM potassium phosphate, pH 7.0.

Assays of enzymes and Chl — The washed cells were sonicated (30 watts, 10 min at 1-min interval) in 50 mM potassium phosphate, pH 7.0, which contained 0.1 mM EDTA, 330 mM sorbitol and 0.5 mM ascorbate. The supernatant was used for the enzymatic assays, after centrifugation (10,000 x g for 10 min) of the sonicated cell lysate. Ascorbate peroxidase activity was determined as described by Nakano and Asada (1981), in a reaction mixture (1 ml) that contained 50 mM potassium phosphate, pH 7.0, 0.1 mM hydrogen peroxide and 0.5 mM ascorbate. The hydrogen peroxide-dependent oxidation of ascorbate was determined by a decrease in the absorbance at 290 nm, using an absorption coefficient of 2.8 mM^{-1} cm^{-1}.

Chl in *Euglena* and *Chlamydomonas* was determined by the method of Arnon (1949). Chl *a* in cyanobacteria was determined by the method of Marsac and Houmard (1988).

Synthesis of $H_2{}^{18}O_2$ — $^{18}O_2$-labeled H_2O_2 was prepared by the glucose-glucose oxidase system in $^{18}O_2$ (Asada and Badger 1984). The reaction mixture (2 ml; 10 mM potassium phosphate, pH 6.0, 10 mM glucose, 0.5 mM EDTA and 0.5 mM KCN) in the vessel of an oxygen-electrode was bubbled to remove $^{16}O_2$. Cyanide was added to inhibit contaminating catalase in the preparation of glucose

oxidase. The vessel was stoppered, and bubbles of $^{18}O_2$ introduced by a gas syringe were allowed to dissolve in the medium. The reaction was allowed to proceed for 5 h at 25°C in the presence of glucose oxidase (1 mg in 50 µl; Sigma, Sr Louis, MO, USA), with additional supplies of $^{18}O_2$-bubbles whenever they disappeared. The reaction was terminated by addition 40 µl of 1 M HCl, after which unreacted $^{18}O_2$ was depleted by bubbling with argon. The reaction mixture was neutralized to pH 7.0 with KOH and passed through a column of Dowex-1 (Cl^-)(0.5 x 2.5 cm) to remove cyanide. The concentration of $H_2{}^{18}O_2$ prepared in this way was about 3 mM, as measured with the assay using catalase, and its atom% of ^{18}O was about 80.

Evolution of $^{18}O_2$ and $^{16}O_2$ in cells — A gas-permeable membrane-mass spectrometer system was used to determine the evolution of dioxygen isotopes from water and $H_2{}^{18}O_2$. A teflon membrane (100 µm thick) was placed at the bottom of the cylindrical reaction vessel (10 mm in diameter) to separate the medium from the high-vacuum inlet to a quadrupole mass analyzer (MSQ 150A, ULVAC) (Mano et al. 1987). The washed cells suspended in 2 ml of 10 mM potassium phosphate, pH 7.0, which contained 2 mM EDTA, were placed in the reaction vessel and illuminated at 25°C by an iodine lamp of a projector at 400 W m^{-2} to exhaust the inorganic carbon, until the evolution of $^{16}O_2$ was ceased. Then, the suspensions of cells were bubbled with argon to remove dioxygen, and then incubated for 3 min in the dark. Then $H_2{}^{18}O_2$ was added to the cells in the dark or light, and the evolution of $^{16}O_2$ and $^{18}O_2$ was determined simultaneously at 5-s intervals. The amounts of unreacted $H_2{}^{18}O_2$ were determined from the evolution of $^{18}O_2$ after the addition of catalase after the reaction.

Effect of hydrogen peroxide on chlorophyll fluorescence — The fluorescence of chlorophyll a in the cells of cyanobacteria was monitored with a pulse-amplified modulation fluorometer as described in chapter II.

Results and Discussion

Ascorbate peroxidase in eukaryotic algae and cyanobacteria — Ascorbate peroxidase was detected in the eukaryotic algae *Euglena gracilis* and *Chlamydomonas reinhardtii* (Table VI-1) in accordance with the previous reports (Shigeoka et al. 1980a,b, Yokota et al. 1988). However, the activities were lower than that in spinach chloroplasts on the basis of chlorophyll content. Further, AsA peroxidase has been found in the eukaryotic alga *Zooxanthella* (*Symbiodium* sp.) (Lesser and Shick 1989).

One of the characteristic properties of AsA peroxidase from angiosperms is its inactivation in AsA-depleted medium (Hossain et al. 1984a, Nakano and Asada 1987, Chen and Asada 1989, Chapter II, IV in this study). I found that this inactivation also occurred with AsA peroxidase in extracts and in cells of *Euglena*. When a cell extract was diluted with AsA-depleted medium to lower the concentration of AsA to below 0.1 μM, the activity of AsA peroxidase was lost with a half time of about 5 min. No inacti-

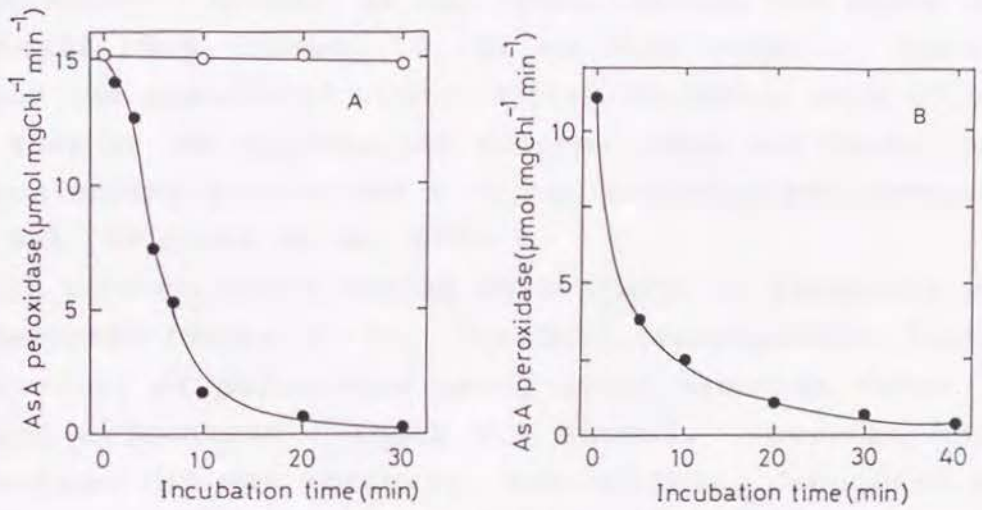


Fig. VI-1 *The inactivation of AsA peroxidase by depletion of AsA in an extract (A) and cells (B) of Euglena.* (A) The harvested cells were sonicated at 1 mg Chl ml^{-1} in the suspension medium (50 mM HEPES-KOH, pH 7.0, 330 mM sorbitol, 0.1 mM EDTA and 20 μM AsA) and centrifuged at 15,000 x g for 1 min. The supernatant was diluted 200-fold with AsA-depleted suspension medium and incubated at 25°C (closed circles). The undiluted supernatant (open circles) also was incubated under the same conditions, and the respective activities were assayed. At the indicated times, each extract was assayed in an AsA-containing reaction mixture, and the activity of AsA peroxidase was determined. (B) The cells (36 μg Chl ml^{-1}) suspended in 10 mM $NaHCO_3$ were incubated at 25°C with 2.6 mM H_2O_2 in the dark for the indicated times. The cells were sonicated in the suspension medium and the activity of AsA peroxidase was assayed after centrifugation.

vation was found when the extract was diluted with a medium that contained 20 μM AsA (Fig. VI-1A). Because of the low activity of catalase in *Euglena* (Asada et al. 1977), the treatment of the cells with hydrogen peroxide in the dark was expected to oxidize AsA via the peroxidase-catalyzed reaction and to inactivate the AsA peroxidase as a result of the depletion of AsA. The cells were treated with 2.6 mM hydrogen peroxide and the activity of AsA peroxidase in the cell extract was determined. The results in Fig. VI-1B indicate that AsA peroxidase was inactivated in the cells with the half-time similar to that observed in the case of the dilution of the extracts. As judged from the half-time of inactivation of AsA peroxidase in the extract and in the cells under AsA-depleted conditions, AsA peroxidase in *Euglena* appears to resemble the cytosolic isozyme rather than the chloroplast isozyme of plants. The chloroplast isozyme of AsA peroxidase in plants is inactivated with a half-time of 10 - 30 s in AsA-depleted medium (Hossain et al. 1984a, Nakano and Asada 1987, Chen and Asada 1989, Chaper II, IV in this study). The cytosolic isozyme was associated with a higher oxidation rate of pyrogallol than that of the chloroplast isozyme (Chen and Asada 1989). The *Euglena* enzyme also shows a higher activity for pyrogallol than with AsA (Shigeoka et al. 1980a).

In cyanobacteria tested no activity of ascorbate peroxidase was detected (Table VI-1). Further, cyanobacteria tested showed no activity of peroxidase using other electron donor, NAD(P)H, GSH and cytochrome *c* (data not shown). However, heat-stable peroxidase (HS-PX) activity, not activity dependent on enzyme protein, using ascorbate as the electron donor was detected in following species; *Anabaena cylindrica, Anabaena variabilis, Synechocystis* 6803, *Nostoc muscorum, Anacystis nidulans, Plectonema boryanum, Synechococcus* 7002. The activities of HS-PXs using ascorbate as the electron donor ranged from 0.10 to 0.90 μmol ascorbate oxidized mg Chl^{-1} min^{-1}, which was about ten times lower than the activity of ascorbate peroxidase in *Euglena* and spinach chloroplasts (data not shown). Tel-Or, *in the IXth International Congress on Photosynthesis*, JAPAN (1992), showed

that compound which catalyze HS-PX reaction and found it to be oligopeptide which chelates iron in its active center.

Table VI-1. *Activities of ascorbate peroxidase in extracts from cyanobacteria and eukaryotic algae and photoreductant peroxidase in cyanobacteria and eukaryotic algae: a comparison with the activities of ascorbate peroxidase and photoreductant peroxidase in spinach chloroplasts* . Activities of ascorbate peroxidase were determined as described in Materials and Methods and expressed as the oxidation rate of ascorbate (µmol ascorbate oxidized mg Chl^{-1} min^{-1}). Activities of photoreductant-peroxidase were calculated from maximal evolution rate of $^{16}O_2$ upon the addition of $H_2{}^{18}O_2$ to the cells of algae and spinach chloroplasts (80 µM H_2O_2 to 58.4 µg Chl a ml^{-1} *Plectonema boryanum*, 25 µg Chl a ml^{-1} *Anacystis nidulans*, 20 µg Chl a ml^{-1} *Synechococcus* 7002, 25 µg Chl a ml^{-1} *Nostoc muscorum*, 26 µg Chl a ml^{-1} *Anabaena cylindrica*, 20 µg Chl a ml^{-1} *Anabaena variabilis* and 29 µg Chl a ml^{-1} *Synechosystis* 6803, 69 µM H_2O_2 to 47 µg Chl ml^{-1} *Euglena*, 80 µM H_2O_2 to 30 µg Chl ml^{-1} *Chlamydomonas*, 40 µM H_2O_2 to 11 µg Chl ml^{-1} spinach chloroplasts[a]) in the light and expressed as the scavenging rate of hydrogen peroxide (µmol H_2O_2 reduced mg Chl^{-1} min^{-1}). n.d. not determined.

Species	AsA peroxidase	Photoreductant peroxidase
Cyanobacteria		
Plectonema boryanum	0.00	0.0
Anacystis nidulans	0.00	0.0
Nostoc muscorum	0.00	0.0
Synechococcus PCC 7002	0.00	0.0
Fremyella diplosiphon	0.00	n.d
Aphanothece halophytica	0.00	n.d
Tolypothrix tenuis	0.00	n.d
Anabaena cylindrica	0.00	3.0
Anabaena variabilis	0.00	2.2
Synechocystis PCC 6803	0.00	0.4
Eukaryotic algae		
Euglena gracilis	4.80	0.8
Chlamydomonas reinhardtii	0.51	0.6
Angiosperm		
Spinach chloroplasts[a]	2.5-23	2.0

a) Data from Hossain et al. (1984) and Asada and Badger (1984).

Scavenging of hydrogen peroxide in eukaryotic algae — *Euglena* cells contain AsA peroxidase (Table VI-1). In contrast to the enzyme in spinach chloroplasts, AsA peroxidase in *Euglena* is localized not in the chloroplasts, but in the cytosol (Shigeoka et al. 1980b). To determine whether a photoreductant produced in the thylakoids is linked to reduction of the oxidation product of AsA by the peroxidase reaction, the evolution of $^{16}O_2$ and $^{18}O_2$ from *Euglena* cells was monitored, after addition of $H_2{}^{18}O_2$, in the light and dark. In the present monitoring system, if one molecule of $H_2{}^{18}O_2$ is disproportionated by catalase, a half molecule of $^{18}O_2$ is evolved in either the light or the dark. When $H_2{}^{18}O_2$ is reduced to water by a peroxidase-catalyzed reaction, using a photoreductant generated in the thylakoids, $^{16}O_2$ is evolved from $H_2{}^{16}O$ in PS II only in the light. Generation of a half molecule of $^{16}O_2$ for each molecule of $H_2{}^{18}O_2$ added is expected from the stoichiometry of the reaction, since four reducing equivalents can be donated to the peroxidase for each molecular of dioxygen evolved, and two reducing equivalents are consumed for the reduction of one molecule of hydrogen peroxide to water. Thus,

$$2\ H_2{}^{16}O \longrightarrow {}^{16}O_2 + 4\ H^+ + 4\ [e^-]$$
$$2\ H_2{}^{18}O_2 + 4\ H^+ + 4\ [e^-] \longrightarrow 4\ H_2{}^{18}O$$
$$\overline{\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad}$$
$$2\ H_2{}^{16}O + 2\ H_2{}^{18}O_2 \longrightarrow 4\ H_2{}^{18}O + {}^{16}O_2$$

Even when $H_2{}^{18}O_2$ is reduced by a peroxidase, no $^{16}O_2$ is evolved if the electron donor is provided by endogeneous reductants or by respiration. In addition, non-enzymatic reduction of hydrogen peroxide by cellular components would not result in the evolutin of either $^{16}O_2$ or $^{18}O_2$. In intact spinach chloroplasts, one molecule of $^{16}O_2$ is evolved with the disappearance of two molecules of $H_2{}^{18}O_2$ in the light, indicating the scavenging of hydrogen peroxide by a peroxidase with a photoreductant as the electron donor (Asada and Badger 1984).

No $^{18}O_2$ was evolved from $H_2{}^{18}O_2$ in *Euglena* cells in either the light or the dark conditions by the addition of $H_2{}^{18}O_2$.

This result may be due to a low or absent activity of catalase in *Euglena* (Asada et al. 1977). In the light, $^{16}O_2$ was evolved, but none was detected in the dark (Fig. VI-2), indicating that

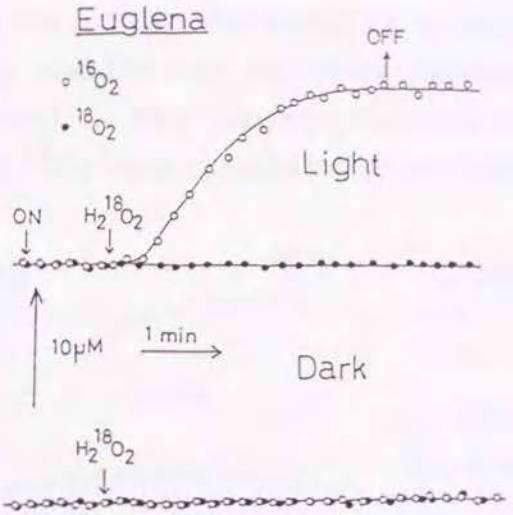


Fig. VI-2 *Light-dependent decomposition of* $H_2{}^{18}O_2$ *and evolution of* $^{16}O_2$ *and* $^{18}O_2$ *in the cells of Euglena gracilis.* The reaction mixture (0.5 ml) contained the cells (23.7 μg Chl) in 50 mM HEPES-KOH, pH 7.0. $H_2{}^{18}O_2$ (69 μM) was added 1 min after the light was turned on (400 W m^{-2}) (ON), and no $H_2{}^{18}O_2$ was found after the reaction. The dark control was kept in the dark during the entire process, and 13 μM $H_2{}^{18}O_2$ was decomposed during the dark reaction.

the peroxidase system operates to scavenge hydrogen peroxide with a photoreductant as the electron donor. With the disappearance of 69 μM $H_2{}^{18}O_2$, 21 μM $^{16}O_2$ were evolved in the light, thus, about 60% of the decomposition of hydrogen peroxide can be accounted for by the peroxidase-catalyzed reaction linked to photosynthetic electron transport. The remaining hydrogen peroxide would be reduced by a peroxidase using endogeneous reductants. Similar results were obtained when *Chlamydomonas* cells were tested under the same conditions, but $^{18}O_2$ was evolved at a low rate (about 40% of that of $^{16}O_2$) in both the light and the dark, probably because of the presence of catalase in the cells (data not shown). Thus, the cells of the two eukaryotic algae scavenge

hydrogen peroxide through the peroxidase reaction as also occurs in angiosperm chloroplasts.

Scavenging of hydrogen peroxide in cyanobacteria -- *A. cylindrica* and *S.* 6803 generated $^{16}O_2$ on addition of $H_2{}^{18}O_2$ to illuminated cells but not in the dark (Fig. VI-3A and B). The illuminated cells of *A. variabilis* also showed the same $H_2{}^{18}O_2$-dependent $^{16}O_2$ evolution as that observed with *A. cylindrica* (data not shown). The light-dependent evolution of $^{16}O_2$ on addition of $H_2{}^{18}O_2$ was completely inhibited by 10 μM DCMU (Fig.

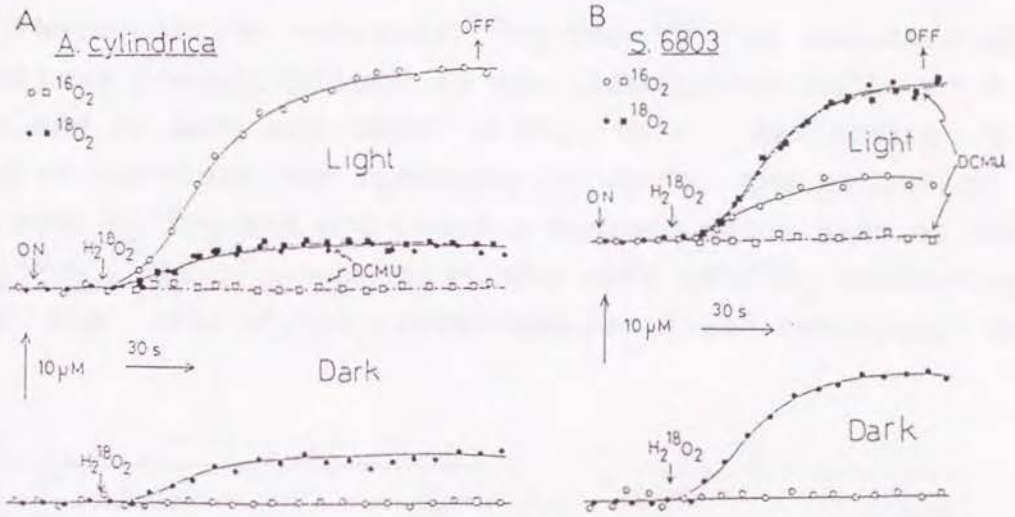


Fig. VI-3 *Light-dependent decomposition of* $H_2{}^{18}O_2$ *and evolution of* $^{16}O_2$ *and* $^{18}O_2$ *in the photoreductant-peroxidase containing (A) A. cylindrica and (B) S. 6803.* The reaction mixture (1.0 ml) contained the cells (*A. cylindrica*, 26 μg Chl a; *S.* 6803, 29.0 μg Chl a), in 50 mM potassium phosphate, pH 7.0. $H_2{}^{18}O_2$ (80 μM) was added 30 s after the light was turned on (400 W m^{-2}) (ON). The dark controls were kept in the dark during the entire process. Where indicated, 10 μM DCMU (dotted lines, square and closed square) was added before the addition of $H_2{}^{18}O_2$. With the disappearance of 80 μM $H_2{}^{18}O_2$, 33 μM $^{16}O_2$ (*A. cylindrica*) and 9.5 μM $^{16}O_2$ (*S.* 6803) were generatd in the light. Thus, 83% (*A. cylindrica*) and 24% (*S.* 6803) of the decomposition of hydrogen peroxide was accounted for by the peroxidase-catalyzed reaction linked to the photosynthetic electron transport. The amount of $^{18}O_2$ evolved was 7 μM (*A. cylindrica*) and 22 μM (*S.* 6803) in both the light and dark. Thus, 9% (*A. cylindrica*) and 55% (*S.* 6803) of the hydrogen peroxide was decomposed by the catalase-catalyzed reaction. The remaining hydrogen peroxide would be reduced by a peroxidase using endogeneous reductants.

VI-3). The inhibition by DCMU confirmed that the electron donor for the peroxidase is supplied through the electron transport

system in the thylakoids.

The three species of cyanobacteria generated $^{18}O_2$ from $H_2{}^{18}O_2$, in a reaction catalyzed by catalase, at the same rate in the light and the dark (Fig. VI-3). The evolution of $^{18}O_2$ was not affected by DCMU under either light or dark conditions, but it was completely suppressed by KCN (1 mM), an inhibitor of catalase (data not shown). The ratio of the rate of decomposition of 80 μM $H_2{}^{18}O_2$ by catalase to that by the peroxidase was high in *S.* 6803 (55%) as compared with that in *A. cylindrica* (9%) and *A. variabilis* (11%).

The evolution rates of $^{16}O_2$ and $^{18}O_2$ on addition of $H_2{}^{18}O_2$ at various concentrations to the illuminated cells of *A. cylindrica* and *S. 6803* are shown in Fig. VI-4. Reflecting a high K_m value of catalase for hydrogen peroxide, the evolution rate of $^{18}O_2$ from $H_2{}^{18}O_2$ did not reach a maximum value even at 200 to 300 μM $H_2{}^{18}O_2$. If I assume that the rate of $^{16}O_2$ evolution represents the rate of the peroxidase-catalyzed reaction, that is,

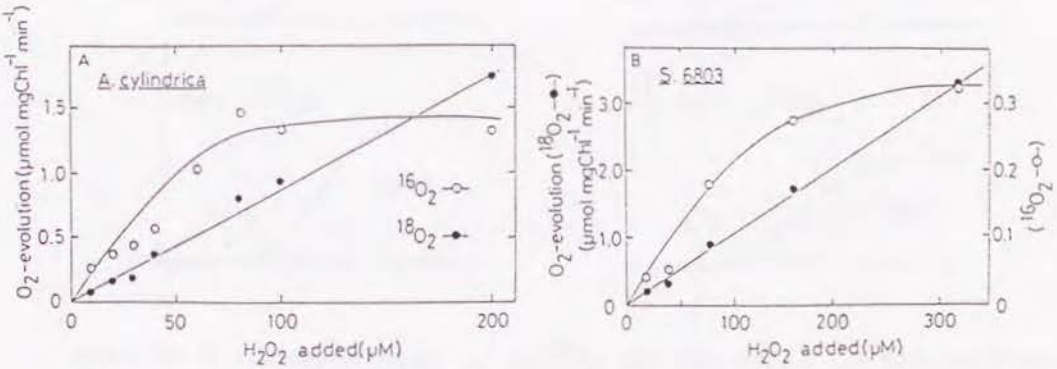


Fig. VI-4 *Effect of $H_2{}^{18}O_2$ concentration on $^{16}O_2$ and $^{18}O_2$ evolution in illuminated cells of (A) A. cylindrica and (B) S. 6803.* The reaction mixture (1.0 ml) contained the cells (25 μg Chl a) in 50 mM potassium phosphate, pH 7.0, and $H_2{}^{18}O_2$ was added at the indicated concentrations. The other conditions were the same as in the legend to Fig. VI-3, and the maximum evolution rates of $^{16}O_2$ and $^{18}O_2$ were determined.

substrate of peroxidase presents at a saturating concentration for the peroxidase in illuminated cells, the concentration of

hydrogen peroxide for the half-maximum rate of $^{16}O_2$ evolution represents the K_m value of the peroxidase for hydrogen peroxide. The concentrations of hydrogen peroxide for the half-maximum rate of reaction were about 40 μM (*A. cylindrica*) and 80 μM (*S.* 6803), similar to the K_m value of AsA peroxidase from plants (Nakano and Asada 1987, Chen and Asada 1989), suggesting that the concentration of hydrogen peroxide can be reduced by a low concentration of the peroxidase. At the low concentrations of hydrogen peroxide that are assumed to be present in cells, hydrogen peroxide would be mainly scavenged by the peroxidase-catalyzed reaction using a photoreductant as the electron donor.

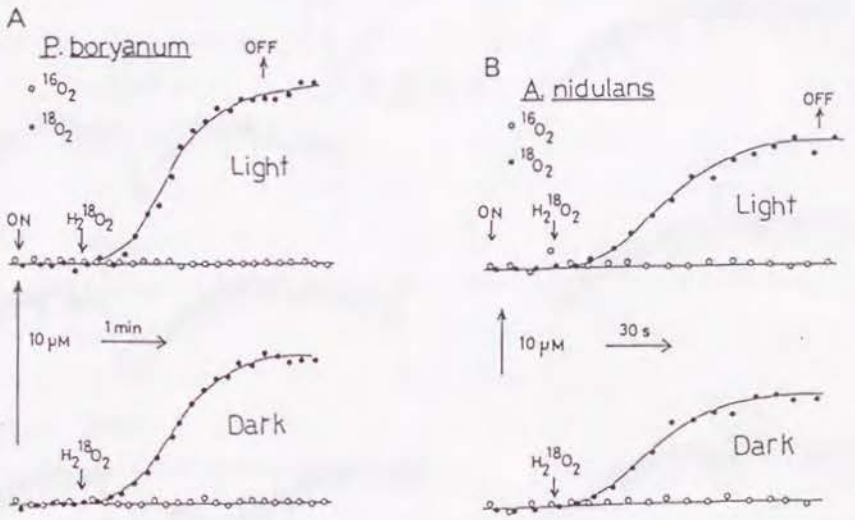


Fig. VI-5 *Decomposition of $H_2{}^{18}O_2$ in the cells of the photoreductant peroxidase-lacking (A) P. boryanum and (B) A. nidulans.* To the reaction mixture (*P. boryanum*, 29.2 μg Chl a in 0.5 ml of 50 mM HEPES-KOH, pH 7.0; *A. nidulans*, 25 μg Chl a in 1 ml of 50 mM potassium phosphate, pH 7.0), $H_2{}^{18}O_2$ (*P. boryanum*, 36 μM; *A. nidulans*, 80 μM) was added at indicated times after the light was turned on (400 W m^{-2}) (ON). During the light incubation, 22 μM $H_2{}^{18}O_2$ (*P. boryanum*) and 32 μM $H_2{}^{18}O_2$ (*A. nidulans*) were decomposed. Dark control was kept in the dark during the entire process. During the dark incubation, 18 μM $H_2{}^{18}O_2$ (*P. boryanum*) and 34 μM $H_2{}^{18}O_2$ (*A. nidulans*) were decomposed.

On the other hand, cyanobacteria, *P. boryanum* and *A. nidulans*, showed no evidence of evolution of $^{16}O_2$ on addition of

$H_2{}^{18}O_2$ to the illuminated cells. Figure VI-5 shows the results for *P. boryanum* and *A. nidulans* (*Synechococcus* 6301). Upon the addition of $H_2{}^{18}O_2$ only $^{18}O_2$ was evolved, and it was evolved at similar rates in the light and dark, indicating that hydrogen peroxide is disproportionated by catalase and that the peroxidase system using photoreductant as electron donor does not operate.

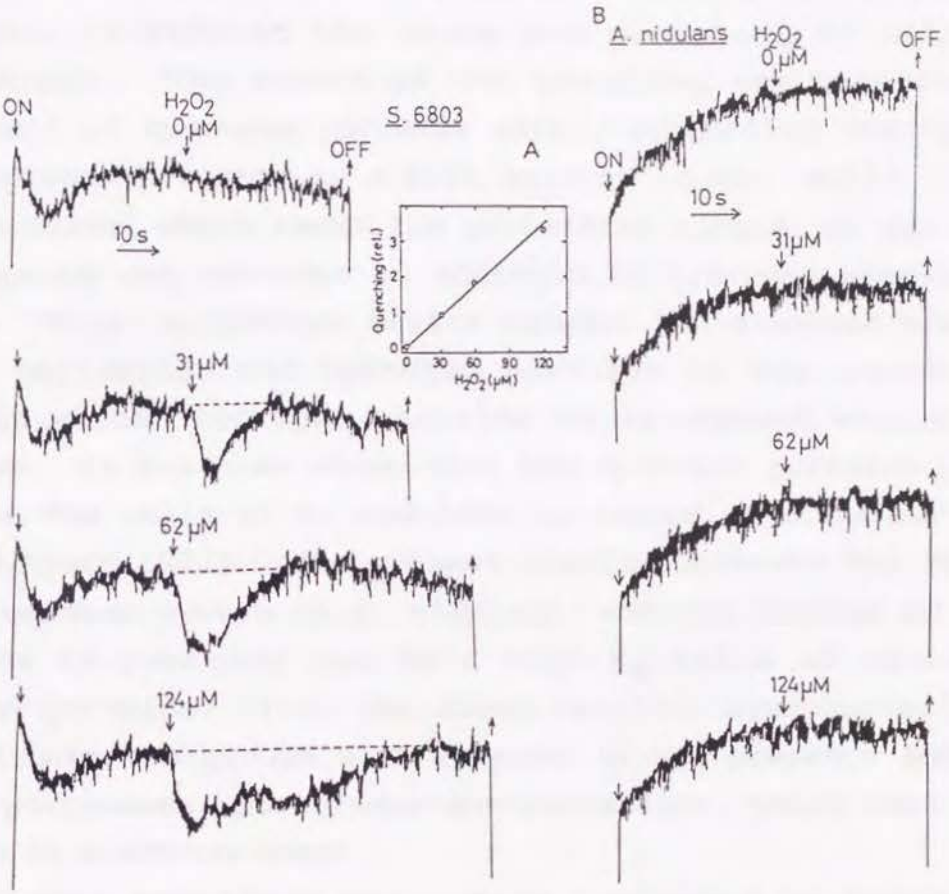


Fig. VI-6 *The effect of hydrogen peroxide concentration on chlorophyll a fluorescences in the photoreductant peroxidase-containing S. 6803 (A) and the photoreductant peroxidase-lacking A. nidulans (B).* The reaction mixture (0.8 ml) contained the cells (20 μg Chl a) in the BG-11 medium. Hydrogen peroxide was added after the fluorescence reached F_m at the concentrations indicated. The relative quenching was determined from the area below the dotted lines after addition of hydrogen peroxide.

Further evidence for the operation of the peroxidase system using photoreductant as electron donor in the cells of *S.* 6803

comes from the quenching of fluorescence by hydrogen peroxide (Fig. VI-6A). If hydrogen peroxide is reduced to water by the peroxidase-catalyzed reaction using the photoreductant produced in the thylakoids, then hydrogen peroxide works as an effective electron acceptor in the cells. It has been shown that this is indeed the case in illuminated intact chloroplasts of spinach (Neubauer and Schreiber 1989). When hydrogen peroxide was added to illuminated cells after the maximum level of fluorescence was attained, it induced the transient quenching of chlorophyll fluorescence. The extent of the quenching was proportional to the amount of hydrogen peroxide added, supporting the hypothesis that hydrogen peroxide is a Hill oxidant in the cells. However, in *A. nidulans* which lacks the peroxidase system, no quenching of fluorescence was observed on addition of hydrogen peroxide (Fig. VI-6B). Thus, no linkage exists between the electron transport of the thylakoids and hydrogen peroxide in the cyanobacterial cells in which hydrogen peroxide is decomposed exclusively by catalase. It has been shown that the hydrogen peroxide photoproduced in the cells of *A. nidulans* is leaked to a medium (Patterson and Myers 1973). The present results indicate the absence of the peroxidase system in *A. nidulans*, and the leakage of hydrogen peroxide is probably due to a high K_m value of catalase for hydrogen peroxide. From the above results, cyanobacteria can be divided into two groups with respect to the presence and absence of the peroxidase, photoreductant-peroxidase, which uses photoreductant as electron donor.

Whether ascorbate peroxidase functions in scavenging of hydrogen peroxide in cyanobacteria like chloroplasts of higher plants and eukaryotic algae was studied as described above. However, no activity of ascorbate peroxidase was detected in cyanobacteria, regardless of the presence and absence of the photoreductant-peroxidase activity (Table VI-1). Cyanobacteria tested showed no activity of peroxidase using other electron donor, NAD(P)H, GSH and cytochrome *c* (data not shown). Further, the existence of HS-PX in cyanobacteria was not related to that of the photoreductant peroxidase in cyanobacteria. Although

Plectonema boryanum and *Anacystis nidulans* show the activities of HS-PXs, these cyanobacteria did not show any activity of photoreductant peroxidase. What electron donor functions in scavenging of hydrogen peroxide in cyanobacteria which shows the activity of the photoreductant-peroxidase is now on study.

Concluding remarks — When cyanobacteria appeared on the earth, three thousand to two thousand million years ago, the atmospheric concentration of dioxygen was perhaps ten thousand times lower than the present atmospheric level (0.002%, Berkner and Marshall 1965). The atmospheric concentration of dioxygen has gradually increased as a result of the evolution of dioxygen by cyanobacteria themselves. At an early phase of the evolution of cyanobacteria, the scavenging of hydrogen peroxide by catalase would have been sufficient to protect the cells from oxidative damage. The photoproduction of superoxide in PSI occurs within the thylakoid membranes (Takahashi and Asada 1988). The apparent K_m for dioxygen of the production of superoxide in the thylakoids is 2 - 3 μM (Asada and Nakano 1977) and the K_m increases with increases in light intensity (Takahashi and Asada 1982). The concentration of dioxygen in water equilibrated with the atmosphere when cyanobacteria appeared on the earth is estimated to be 0.025 μM. The actual concentration of dioxygen in the cells of cyanobacteria would have been only a little higher than this value in the light, since it has been shown that the gradient of the oxygen concentration between illuminated spinach chloroplasts and their surroundings is small as a results of diffusion (Steigner et al. 1977). Thus, the cellular concentration of dioxygen in cyanobacteria was lower by at least two orders of magnitude than the apparent K_m for dioxygen of the photoproduction of superoxide when appeared on the earth.

Accompanying by an increase in the atmospheric concentration of dioxygen, the photoproduction rate of superoxide should have increased and the production rate of its disproportionation product, hydrogen peroxide, should also have increased. Thus, an effective peroxidase system was required, such that the cellular concentration of hydrogen peroxide could be lowered to protect

the cells from oxidative damage. In this respect, the presence or absence of Photoreductant-peroxidase should be one of the criteria for the assessment of the evolutionary stage of cyanobacteria. Our results suggest that photosynthetic organisms acquired the system for the scavenging of hydrogen peroxide by photoreductant-peroxidase during the evolution of cyanobacteria, which, at first, acquired the ability of oxygenic photosynthesis on the earth and supplied oxygen to the atmosphere of earth. This photoreductant peroxidase for the scavenging of hydrogen peroxide, acquired by cyanobacteria, has been conserved in eukaryotic algae and the chloroplasts of angiosperms, and ascorbate peroxidase was acquired during the evolution of eukaryotic algae. Note that superoxide dismutase has been found in cyanobacteria (*Plectonema boryanum, Nostoc verrucosum, Anabaena cylindrica, Anabaena variabilis, Spirulina platensis* (Asada et al. 1977), and in *Anacystis nidulans* (Csėke et al. 1979)). Furthermore, I detected superoxide dismutase activity in *Synechosystis* 6803 and *Synechococcus* 7002 (data not shown). Thus, superoxide dismutase is present in cyanobacteria, irrespective of the presence and absence of photoreductant-peroxidase, suggesting the importance of the scavenging of superoxide for the suppression of oxidative damage.

Chapter VII

Summary

Oxygen metabolism in chloroplasts is composed of two major reactions. The first one is an oxygen evolution resulting from a photooxidation of water in photosystem II (PS II). The oxygen evolved from PS II is the electron acceptor producing bioenergy in all aerobic organisms to keep their life. The second one, which I am interesting in, is the Mehler reaction and univalent reduction of dioxygen in photosystem I (PS I) by light, resulting in the formation of superoxide anion radical (O_2^-). The O_2^- is disproportionated by superoxide dismutase to water and hydrogen peroxide. The second reaction is the reaction which produces the active species of oxygen, toxic to all organisms, but its production is inevitable even under the favorable conditions to plants. Thus, if the scavenging system of the active species of oxygen does not effectively operate, plants suffer from an oxidative damage by the active species of oxygen. My first project in this study was to elucidate how hydrogen peroxide photoproduced in chloroplast thylakoid membranes is scavenged to protect the Calvin cycle enzymes localized in chloroplast stroma from oxidative damages.

Thylakoid-bound ascorbate peroxidase

First, a peroxidase activity in thylakoid membranes where hydrogen peroxide is photoproduced was assayed using several electron donors. Then, a peroxidase using ascorbate as electron donor (thylakoid-bound ascorbate peroxidase, tAPX) similar to that localized in stroma was found in thylakoid membranes (Chapter II Table II-1, II-3). Further, tAPX was localized in stroma thylakoids at higher concentrations than that of tAPX in grana thylakoids (Chapter II Table II-2). PS I is localized in the stroma thylakoids where dioxygen is univalently photoreduced. For its immediate scavenging of hydrogen peroxide as soon as it

is produced it is very reasonable that APX is localized in the stroma thylakoids. For example, thylakoids which showed an activity of APX in fact could scavenge hydrogen peroxide photoproduced in PS I, but thylakoids without APX activity could not (Chapter II Fig. II-3). This is the first evidence that tAPX in fact scavenges the hydrogen peroxide produced via the Mehler reaction in chloroplast thylakoids.

The production rate of O_2^- in chloroplasts is 240 μM s^{-1} and the production rate of hydrogen peroxide is estimated to be 120 μM s^{-1} (Asada and Takahashi 1987). Thus, although a concentration of ascorbate in a chloroplast is high, over 10 mM, ascorbate would be completely consumed by the reaction of APX within one minute, and APX could not operate unless a regeneration system of ascorbate exists. Next, whether the enzymes for regenerating ascorbate, monodehydroascorbate (MDA) radical reductase (MDA reductase) and dehydroascorbate (DHA) reductase do operate in thylakoids similar to stroma or not was studied. No activities of these enzymes, however, were detected (Chapter II Table II-1). It was found that the amount of ascorbate consumed during the Mehler reaction in APX-bound thylakoids was lower than the value estimated from stoichiometry of the Mehler reaction (Chapter II Fig. II-4). This result indicates that ascorbate is regenerated during the Mehler reaction and the regeneration system of ascorbate is localized in thylakoids. Also, it was shown that hydrogen peroxide quenched a chlorophyll fluorescence of APX-bound thylakoids (Chapter II Fig. II-5). These results suggest that an oxidation product of APX reaction is photoreduced, and this suggestion is supported by the following results. The one-electron oxidized product of ascorbate by APX, MDA radical, quenched the chlorophyll fluorescence of thylakoids (Chapter II Fig. II-6). MDA radical-dependent oxygen evolution was observed (Chapter II Fig. II-9). Further, EPR signal of MDA radical disappeared under the light (Chapter II Fig. II-8). Thus, it was elucidated that the primary oxidation product of ascorbate, MDA radical, produced by tAPX reaction during the Mehler reaction is photoreduced to ascorbate, and the regeneration system of ascorbate

operates in thylakoids using the photoreductant. The photoreduction of MDA radicals by thylakoids is the first evidence that a radical is to be the Hill oxidant.

Molecular properties of thylakoid-bound ascorbate peroxidase

So far, contradictory results about the existence of ascorbate peroxidase in thylakoids have been reported. Groden and Beck (1979) reported that APX is bound to thylakoids, but Nakano and Asada (1981) reported that is not true. In this study, I gave one united answer against this problem. That is to say, similar to stromal APX, tAPX is rapidly inactivated with a half time of 15 s by the depletion treatment of ascorbate from tAPX (Chapter II Fig. II-2). Thus, if we want to detect the activity of tAPX, ascorbate is necessary during the preparation of thylakoids. This is a reason why APX has not been found in thylakoids for a long time.

Next, I isolated tAPX from spinach thylakoids and studied the molecular properties of the isolated tAPX. As tAPX was not removed from thylakoids by chelate reagents, salts or chaotropic reagents , but solubilized by detergents, it was thought that tAPX is tightly bound to the thylakoid membranes (Chapter II see text). Octylglucoside-solubilized tAPX was purified to a single protein using chromatographic techniques (Chapter III Table III-1). This is the first report that a peroxidase was purified from thylakoid membranes. Molecular properties of tAPX obtained from the purified tAPX are as follows: (1) the molecular size of tAPX from spinach is 10 kDa larger than that of stromal APX from spinach, and an antibody against tea stromal APX binds to tAPX from spinach, suggesting that tAPX has common epitopes to the stromal APX (Chapter III Fig. III-1, III-2). (2) From the result that a homology of NH_2-terminal amino acid sequence of tAPX with that of tea stromal APX is high (Chapter III Fig. III-3), it is suggested that domain of tAPX for the binding to thylakoids is not the NH_2-terminal of tAPX. On the other hand, the NH_2-terminal amino acid sequence of tAPX does not show any homology with those of spinach, pea and *Arabidopsis* cytosol APXs (Chapter III

Fig. III-3). This supports an idea that the ancestor of ascorbate peroxidase first diverged to give cytosolic and chloroplastic isozymes, and the ancestor of chloroplastic ascorbate peroxidase diverged further to give the stromal and thylakoid-bound ascorbate peroxidase. (3) Spinach tAPX gives an absorption spectrum characteristic of ferric high-spin state, and has one molecule of protoheme IX in its reaction center (Chapter III Fig. III-4). (4) Spinach tAPX shows a high affinity for ascorbate as an electron donor. (5) The oxidation mechanism of ascorbate by tAPX obeys the peroxidase ping-pong kinetics, similar to classical plant peroxidase (Chapter III Fig. III-5). In this study the reaction mechanism of APX was first elucidated, and the rate constants, k_1 and k_3, were estimated to be 1.2×10^7 M^{-1} s^{-1} and 2.1×10^6 M^{-1} s^{-1}, respectively (Chapter III Fig III-5). (6) In the reaction of APX the ratio of hydrogen peroxide to ascorbate is very important. Because the ratio is higher, the activity of APX is more rapidly decreased (Chapter III Fig. III-5). This result suggests that APX is damaged by hydrogen peroxide and gave the breakthrough for me to solve the inactivation mechanism of APX in the absence of ascorbate.

The above enzymatic properties of the purified tAPX were consistent with those of the enzyme in intact thylakoids and in detergent-solubilized thylakoids. Thus, the purified tAPX seems to have the same properties as the enzyme in its membrane-bound state. These results suggest that the site of reduction of hydrogen peroxide by tAPX is exposed to the stromal side of the thylakoid membranes where the ferredoxin-dependent regeneration of ascorbate occurs (Chapter V).

Inactivation mechanism of ascorbate peroxidase in the absence of ascorbate

The unique characteristic of ascorbate peroxidase different from other peroxidase, for example, horseradish peroxidase, cytochrome *c* peroxidase, glutathione peroxidase, NAD(P)H peroxidase and so on, is its rapid inactivation in the absence of electron donor (Nakano and Asada 1987, Chen and Asada 1989). Thylakoid-

bound APX and APX in *Euglena* are also the case (Chapter II Fig. II-2, Chapter VI Fig. VI-1). In the study shown in Chapter III Fig. III-5, it was found that APX is damaged by hydrogen peroxide which is a substrate for APX. From the result, I propose a hypothesis on the inactivation mechanism of APX by the depletion treatment of ascorbate; APX is damaged by hydrogen peroxide produced from the autooxidation of a small amount of ascorbate present in the system catalyzed with contaminated metal ions, because APX cannot scavenge hydrogen peroxide in the absence of ascorbate. Next, I tried to prove this hypothesis using the purified thylakoid-bound ascorbate peroxidase.

Although by the depletion treatment of ascorbate from tAPX under aerobic conditions its activity was rapidly decreased, under anaerobic conditions the activity was maintained (Chapter IV Fig. IV-2). This result supports that an autooxidation product during the treatment gives an damage to tAPX. Further, superoxide dismutase did not suppress the inactivation of tAPX in the absence of ascorbate, but catalase did inhibit the inactivation (Chapter IV Fig. IV-2), indicating that the autooxidation product which inactivates tAPX is hydrogen peroxide. Thus, APX is very lablie in hydrogen peroxide, in spite of being peroxidase.

How the damage of APX by hydrogen peroxide proceeds was studied. The reaction mechanism of APX is the peroxidase ping-pong (Chapter III Fig. III-5), and hydrogen peroxide binds to native APX to form Compound-I, two-electron oxidized APX. If ascorbate is absent, the compound-I of APX is accumulated. I thought that the accumulation of the compound-I is the cause for APX inactivation in the absence of ascorbate. In fact, when the formation of Compound-I of tAPX by hydrogen peroxide was inhibited by ligating CN^- to a heme of tAPX before the depletion treatment of ascorbate, the inactivation of tAPX is suppressed (Chapter IV Fig. IV-3). Further, it was confirmed that the Compound-I of tAPX was rapidly degradated in the absence of ascorbate (Chapter IV Fig. IV-5). The degradation rate constant, k_i, of the Compound-I was given as first order (Chapter IV Fig. IV-4, IV-6), supporting the result that in the absence of ascorbate

tAPX is inactivated with first order kinetics. From these results the inactivation mechanism of APX in the absence of ascorbate was elucidated. In the absence of ascorbate hydrogen peroxide produced via autooxidation binds to APX, and the Compound-I is accumulated, leading to its degradation.

Photoreduction of the MDA radical by ferredoxin

The inactivation mechanism of ascorbate peroxidase gave me an concept; under physiological conditions, ascorbate must be regenerated. If it is not so, APX cannot only scavenge hydrogen peroxide, what is worse, but is also inactivated by the hydrogen peroxide. In the previous study (Chapter II), it was elucidated that during the operation of APX to scavenge the hydrogen peroxide photoproduced in thylakoids ascorbate is regenerated by the photoreduction of MDA radicals in thylakoids. Next, I studied a relationshinp between the site where APX functions and the site where MDA radicals are photoreduced, from the point of their functions in the scavenging of hydrogen peroxide on thylakoids. The photoreduction site of MDA radicals was identified in thylakoids.

The photoreduction of MDA radicals in thylakoids was inhibited by DCMU which inhibits the linear electron flow between Q_A and Q_B, and also by DBMIB which inhibits the electron flow between plastoquinone-pool and Cyt *b/f* (Chapter II Fig. II-6, II-8). It is suggested that PS II is excluded from the candidate for the photoreduction site of MDA radicals. On the other hand, the light-dependent disappearence of EPR signal of MDA radicals (Chapter V Fig. V-1, V-5, V-7) and MDA radical-dependent quenching of chlorophyll fluorescence (Chapter V Fig. V-2) were stimulated by ferredoxin (Fd), which is the physiological electron acceptor in PS I. This suggests that Fd mediates the electron transfer from PS I to MDA radicals. The facts which confirm that MDA radicals are photoreduced by PS I via Fd are as follows. Even in the presence of DCMU, the EPR signal of MDA radicals disappeared dependent on light, by the addition of $DCIPH_2$ to donate electrons directly to PS I (Chapter V Fig. V-3). Further,

by illumination of far-red light which excites only PS I the EPR signal of MDA radicals also disappeared (Chapter V Fig. V-3). Thus, the photoreduction of MDA radicals functions at the same site where APX scavenges hydrogen peroxide in thylakoids, and it is very effective for APX to get the electron donor.

Monodehydroascorbate radical is reduced directly by the Fd photoreduced in PS I, because the Fd reduced by NADPH via ferredoxin-NADP oxidoreductase (FNR) reduced MDA radical to ascorbate (Chapter V Table V-1) and ATP ribose which inhibits the binding of $NADP^+$ to FNR did not show any effect on the Fd-dependent photoreduction of MDA radicals (Chapter V). As E_m value (-420 mV;Tagawa and Arnon 1962) of Fd from spinach chloroplasts is low enough to reduce MDA radical to ascorbate (MDA radical/ascorbate, 320 mV;Nanni et al. 1980, Sapper et al. 1982), it is likely that the reduced form of Fd donates electrons to MDA radicals.

The reactivity of MDA radical gives the possibility that MDA radicals are reduced by several kinds of Fd in organisms. Ferredoxins which have reaction center of 2Fe-2S are also found in green algae and cyanobacteria (Knaff and Hirasawa 1991), similar to higher plants. This suggests that in these photosynthetic organisms also ascorbate might be regenerated by the photoreduction of MDA radicals in PS I. Further, MDA radicals might be reduced to ascorbate by adrenodoxin or putidaredoxin. Adrenodoxin in adrenal cortex mitochondria which reaction center is also 2Fe-2S shows a low potential (E_m = -274 mV) and reduces cytochrome P-450 via NADPH-adrenodoxin reductase. Putidaredoxin (E_m = -240 mV) in *Pseudomonas putida* transfers electrons to cytochrome P-450 for the degradation of campher via NADPH-putidaredoxin reductase. If it is so, the reduction of MDA radicals would be a new physiological function of these ferredoxins.

The photoreduction of MDA radicals by the Fd photoreduced in PS I gave me a question whether MDA radicals and $NADP^+$ which is also a physiological electron acceptor in PS I compete each other for the reduced Fd. In fact, the photoreduction of $NADP^+$ by thylakoids was suppressed in the presence of MDA radicals and the extent of the suppression was increased as the concentration of

MDA radicals was increased (Chapter V Fig. V-8). Adversely, $NADP^+$ inhibited the photoreduction of MDA radicals (Chapter V Fig. V-9). These results show that there is a competition between MDA radical and $NADP^+$ for their photoreductant in PS I. From the concentration dependency of $NADP^+$ on the inhibition of MDA radical photoreduction, the apparent rate constant for the photoreduction of MDA radicals by the reduced Fd was estimated to be 30 fold higher than that for $NADP^+$ photoreduction (Chapter V Fig. V-9). Thus, it is suggested that the reduced Fd in PSI preferentially donates electrons to MDA radicals to regenerate ascorbate and to scavenge hydrogen peroxide by APX than to $NADP^+$ even though a fixation of CO_2 is suppressed (see text in Chapter V).

From these studies on the localization of APX in thylakoids (Chapter II), the characterization of thylakoid-bound ascorbate peroxidase (Chapter III, IV) and the photoreduction site of MDA radicals in thylakoids (Chapter V), I could answer the first question in this thesis. The primary scavenging system of hydrogen peroxide is on thylakoids and scavenges hydrogen peroxide photoproduced in PS I before its diffusion to stroma (Chapter V Fig. V-10). Due to this system, Calvin cycle enzymes can apparently turnover for the fixation of CO_2 without photooxidative damage.

Now what I think about the scavenging system of O_2^- and hydrogen peroxide in chloroplasts is as follows (Fig. VII-1). This system is divided to the two system; thylakoid-scavenging system (A)(in this study) and stroma-scavenging system (B). It is supposed that the stroma-scavenging system is localized in the vicinity of the thylakoid-system and supports the system. The whole system is composed of a)diffusion to the stroma of O_2^- photoproduced within the PS I-localized stroma thylakoid membranes, b) disproportionation of O_2^- catalyzed by thylakoid-bound or peripheral SOD, c) reduction of hydrogen peroxide by ascorbate (AsA) catalyzed with tAPX, d) reduction of MDA radicals (MDA) by reduced Fd, e) diffusion of O_2^- and hydrogen peroxide to the

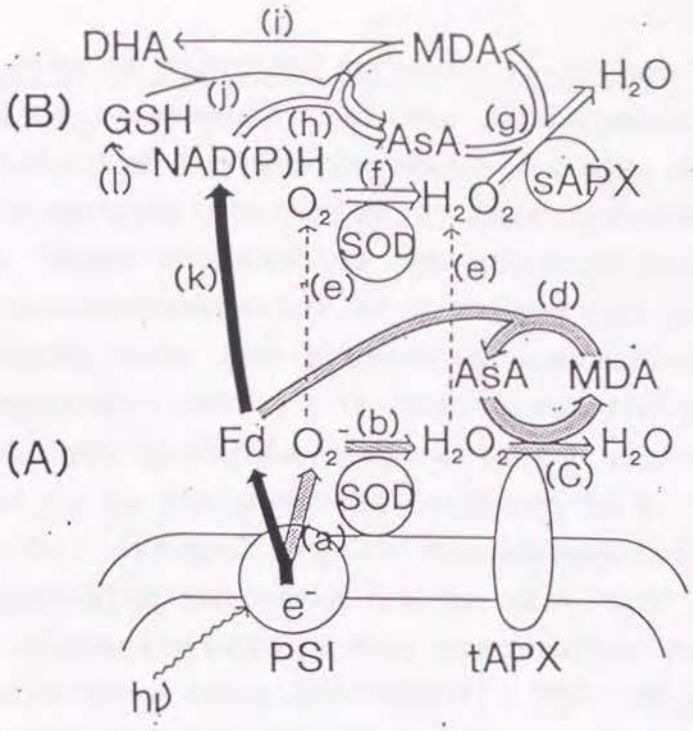


Fig. VII-1 *Thylakoid- and Stroma-scavenging systems of superoxide and hydrogen peroxide.* The whole system is composed of thylakoid-scavenging system (A) and stroma-scavenging system (B); a) diffusion to the stroma of superoxide photoproduced within the PS I-localized stromal thylakoid membranes, b) disproportionation of superoxide catalyzed by thylakoid-bound or peripheral superoxide dismutase (SOD), c) reduction of hydrogen peroxide by ascorbate (AsA) catalyzed with thylakoid-bound ascorbate peroxidase (tAPX), d) reduction of monodehydroascorbate (MDA) radical by reduced ferredoxin (Fd), e) diffusion of superoxide and hydrogen peroxide to the stroma, f) disproportionation of superoxide catalyzed by stromal SOD, g) reduction of hydrogen peroxide catalyzed by stromal APX (sAPX), h) reduction of MDA by NAD(P)H catalyzed with MDA reductase, i) spontaneous disproportionation of MDA to dehydroascorbate (DHA) and AsA, j) reduction of DHA to AsA by GSH catalyzed with DHA reductase, k) reduction of $NADP^+$ by reduced Fd catalyzed with Fd-NADP reductase, and l) reduction of GSSG by NADPH catalyzed with glutathione reductase.

stroma, f) disproportionation of O_2^- catalyzed by stromal SOD, g) reduction of hydrogen peroxide catalyzed by stromal APX (sAPX), h) reduction of MDA by NAD(P)H catalyzed with MDA reductase, i) spontaneous disproportionation of MDA to dehydroascorbate (DHA) and AsA, j) reduction of DHA to AsA by GSH catalyzed with DHA reductase, k) reduction of $NADP^+$ by reduced Fd catalyzed with Fd-NADP reductase, and l) reduction of GSSG by NADPH catalyzed with glutathione reductase.

Scavenging system of hydrogen peroxide in algae

It would be reasonable that the prokaryotic algae cyanobacteria which have the two photosystems are the origin for oxygen evolving photosynthetic organisms. When cyanobacteria appeared on the earth, three thousand to two thousand million years ago, the atmospheric concentration of dioxygen was perhaps ten thousand times lower than the present atmospheric level (0.002%, Berkner and Marshall 1965). At that time, the production rates of O_2^- and hydrogen peroxide derived of O_2^- would be negligible, because K_m for O_2 in its photoreduction in PS I is 2-3 μM (Asada and Nakano 1977). After that, as the atmospheric oxygen concentration was gradually increased as the result of the evolution of dioxygen by cyanobacteria, the production rate of hydrogen peroxide should have been increased. This should require the acquisition of the defence system against hydrogen peroxide to cyanobacteria for their living. After the acquisition of the system in cyanobacteria, eukaryotic algae would appear with the scavenging system of O_2^- and hydrogen peroxide, and accerelates the increase of oxygen concentration in the atmosphere. After that, as the ozone layer was formed and the UV light was cut, the land plant would be extensively evolved. In the previous study (Chapter II-V), the scavenging system of hydrogen peroxide in higher plant chloroplasts was elucidated. My next question was when the scavenging system of hydrogen peroxide was acquired during the evolution of the photosynthetic organisms.

The activities of peroxidase in the eukaryotic algae *Euglena* and *Chlamydomonas* were assayed using several electron donors, and the peroxidases which use ascorbate as electron donor similar to that in higher plant chloroplasts were found (Chapter VI Table VI-1). However, APXs in these eukaryotic algae were slowly inactivated in the absence of ascorbate with a half time of 5-10 min (Chapter VI Fig. VI-1), which value is 40 folds higher than that of APX in higher plant chloroplasts (Chapter IV). This results suggested that the compound-I of APX in eukaryotic algae tolerates hydrogen peroxide more than do that of APX in higher plant chloroplasts.

Whether in eukaryotic algae APX functions to scavenge hydrogen peroxide using photoreductants, similar to APX in higher plant chloroplasts (Chapter II-V), or not was studied. On addition of $H_2{}^{18}O_2$ to *Euglena* and *Chlamydomonas*, $^{16}O_2$ derived from water was evolved in the light and not in the dark (Chapter VI Fig. VI-2). From the molar ratio of $H_2{}^{18}O_2$ consumed to $^{16}O_2$ evolved in the light, it was found that in these cells also the peroxidase reaction functions to scavenge hydrogen peroxide using ascorbate as the photoreductant produced in thylakoids.

Further, in cyanobacteria the scavenging mechanism of hydrogen peroxide was studied. On addition of $H_2{}^{18}O_2$ to *Anabaena variabilis* and *Synechocystis* 6803, $^{18}O_2$ due to the catalase reaction and $^{16}O_2$ were evolved in the light, but only $^{18}O_2$ was evolved in the dark (Chapter VI Fig. VI-3). The coupling of the evolution of $^{16}O_2$ in the light with the photochemical reactions in thylakoids was shown by the facts that the evolution in the light was inhibited by DCMU and the chlorophyll *a* fluorescence was quenched by the addition of hydrogen peroxide to these cells (Chapter VI Fig. VI-3, VI-6). These results show that in these cyanobacteria the peroxidase using photoreductants (photoreductant peroxidase) functions to scavenge hydrogen peroxide. On the other hand, in *Anacystis nidulans* and *Plectonema boryanum* the photoreductant peroxidase activity was not detected and hydrogen peroxide was decomposed only by catalase (Chapter VI Fig. VI-5, VI-6). Thus, cyanobacteria can be divided into two groups, the first group scavenges hydrogen peroxide with the photoreductant peroxidase, and the second one only scavenges hydrogen peroxide with catalase.

The acquisition of the photoreductant peroxidase would give the tolerance against the oxidative damage by hydrogen peroxide to cyanobacteria. The Michaelis constant K_m for hydrogen peroxide of catalase is high. Although 1 M hydrogen peroxide is decomposed at a very rapid rate of 10^7 s^{-1}, the scavenging rate at μmolar levels of hydrogen peroxide which inhibits Calvin-cycle enzymes is very low (10 s^{-1}). Thus, to scavenge the low concentration of hydrogen peroxide a high concentration of catalase is

required as that in peroxisome of higher plants. On the other hand, K_m for hydrogen peroxide (40-80 μM) of the photoreductant peroxidase is lower than that of catalase (Chapter VI Fig. VI-4) and the low concentration of hydrogen peroxide could be rapidly scavenged by lower concentrations of the peroxidase.

Different from eukaryotic algae and higher plant chloroplasts, in the extracts of cyanobacteria peroxidase activities using various electron donors were not detected, but only catalase activity was detected (Chapter VI Table VI-1). What peroxidase functions to scavenge hydrogen peroxide in cyanobacteria which show the activity of the photoreductant peroxidase is now on study.

From above results, it is concluded that the photoreductant peroxidase was acquired during the evolution of cyanobacteria, and after that ascorbate peroxidase was acquired during the evolution of eukaryotic algae and functions as the photoreductant peroxidase, and this scavenging system is conserved in chloroplasts of angiosperm.

Thylakoid- and stromal-scavenging systems are the molecular basis of the O_2-dependent electron flow

Is the photoreduction of dioxygen in PS I of thylakoids to produce O_2^- really the source of oxygen toxicity in plants? If it is so, as described by me, the two contradictory reactions in oxygen metabolism of chloroplasts coexist in the point of advantage to plants. Is the idea that the Mehler reaction is the positive use of dioxygen allowable? The reports which support this idea have been recently published. For example, in the absence of dioxygen chloroplasts suffer from photodamage (Wu et al. 1991). In the absence of dioxygen chloroplasts cannot start the fixation of carbon dioxide (Egneus et al. 1975). In algae, dioxygen plays a role in the light-dependent concentrating system of inorganic carbon (Sültemeyer et al. 1993). These results suggest that an electron flow from PS I to O_2 could relax the photoinhibition and contribute to the formation of ATP for photosynthesis.

A group of Schreiber has proposed the mechanism where O_2 -dependent electron flow plays the essential role in the protection against photoinhibition (Schreiber and Neubauer 1990, Schreiber et al. 1991, Neubauer and Yamamoto 1992, Reising and Schreiber 1992, Hormann et al. 1993). The turnover of the thylakoid- and stroma-scavenging systems in chloroplasts accompanied with the Mehler reaction means that O_2 and MDA radical function as Hill oxidants. This electron flow can form pH gradient across the thylakoids and membrane-energization in thylakoids, resulting in the down-sizing of the PS II by which photoenergy exceeding the capacity of CO_2-assimilatory electron flow is leaked as heat (Weiss and Berry 1987, Genty et al. 1989, Krieger et al. 1992). Thus, it is suggested that the photoreduction of O_2 and MDA radical suppresses the photoinhibition. The thylakoid-scavenging system of hydrogen peroxide (Chapter V) gives the molecular basis for the regulation of PS II by the O_2-dependent electron flow (Schreiber and Neubauer 1990), and this system would be the system acquired during the evolution of plants to use dioxygen.

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