

**Studies of Adenosine 5'-phosphosulfate Reducing
Enzymes in Higher Plants**

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Abbreviations

AMP	adenosine 5'-monophosphate
APS	adenosine 5'-phosphosulfate
ATP	adenosine 5'-triphosphate
CBB	coomassie brilliant blue
DEAE	diethylaminoethyl
DTE	dithioerythritol
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
FW	fresh weight
GSH	glutathione
Hepes	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPLC	high-performance liquid chromatography
ICPS	inductivity coupled plasma atomic emission spectrometer
NADPH	nicotineamide adenine dinucleotide phosphate
OAS	<i>O</i> -acetyl-L-serine
PAGE	polyacrylamide gel electrophoresis
PAPS	3'-phosphoadenosine 5'-phosphosulfate
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid

CONTENTS

Introduction		1
Chapter 1.	Non-radioactive Adenosine 5'-phosphosulfate Sulfotransferase Assay by Coupling with Sulfite Reductase and <i>O</i> -Acetylserine-(thiol)lyase.	5
Chapter 2.	Purification and Characterization of Adenosine 5'-phosphosulfate Sulfotransferase from Radish.	20
Chapter 3.	Molecular Evolution of Adenosine 5'-phosphosulfate Reduction Pathway.	29
Summary		38
References		39
Publications		48
Acknowledgments		49
Appendix		50

Introduction

Inorganic sulfate taken up by microorganisms and higher plants is converted to organic sulfur compounds. A series of reactions leading to cysteine from sulfate is called sulfate assimilation. Sulfate assimilation pathway is shown in Fig. 1. Most of the steps in the pathway are similar both in microorganisms and higher plants. Generally, animals do not have sulfate assimilation pathway. At first, sulfate anion is taken up into the cell by sulfate transporter or sulfate permease. cDNAs encoding sulfate transporter have been cloned in several organisms. In higher plants, cDNAs were cloned in *Stylosanthes hamata* (Smith et al, 1995), *Hordeum vulgare* (Hawkesford and Smith 1997) and *Arabidopsis thaliana* (Takahashi et al, 1996, Takahashi et al, 1997). There are two classes of sulfate transporters in higher plants; one is constitutively expressed and another is induced by sulfur deficient condition (Hawkesford and Smith 1997). The first reaction in sulfate assimilation is an activation of sulfate to adenosine 5'-phosphosulfate (APS) catalyzed by ATP sulfurylase (EC 2.7.7.4). In *E. coli* and yeast (Thomas, et al, 1997), APS is converted to another active form of sulfate, 3'-phospho adenosine 5'-phosphosulfate (PAPS) by APS kinase (EC 2.7.1.24), then PAPS is reduced to sulfite by PAPS reductase (EC 1.8.99.4). PAPS reductase in these organisms required thioredoxin as an electron-donor. PAPS is also considered to be a sulfate donor in transfer reactions of several sulfotransferase. In sulfur-reducing bacteria, APS is directly converted to sulfite by APS reductase. APS reductase is contained FAD and Fe-S cluster in the enzyme (Lampreia et al, 1990). In algae and higher plants, APS is thought to be converted to sulfite by APS sulfotransferase, which catalyzes transfer of the sulfo-group of APS to a carrier compound with a thiol group. APS kinase and PAPS reductase were also reported in spinach (Burnell and Anderson, 1973, Schwenn, 1989). More detailed evidence is required to prove the involvement of PAPS in sulfate assimilation pathway of higher plants. PAPS is also used as a sulfate donor of sulfotransferase in higher plants. Sulfite is converted sulfide by sulfite reductase (EC 1.8.1.2) coupled with ferredoxin in higher plants or NADPH in *E. coli* and yeast as an electron-donor. In the final step of the assimilation, sulfide is converted to cysteine by *O*-acetylserine(thiol)lyase (cysteine synthase; EC 4.2.99.8) with *O*-acetylserine (OAS), which is supplied from serine by serineacetyltransferase (EC 2.3.1.30). In yeast, when homoserine is served instead of serine, homocysteine is produced. Cysteine is a key sulfur

containing compound which leads to other important sulfur containing compounds such as methionine, glutathione (GSH), and proteins. Among the enzymes involved in sulfate assimilation pathway, only APS sulfotransferase had not been purified until 1993 when this study was started.

The enzymes in the sulfate assimilation passway are located mainly in chloroplast and cytosol in higher plants. One isoform of *O*-acetylserine(thiol)lyase is also located in mitochondria. Isolated genes encoding the enzymes involved in the assimilation pathway in higher plants are listed in Table 1.

APS sulfotransferase is thought to be an enzyme which catalyzes the transfer of sulfo-group of APS to a carrier compound with a thiol group in the assimilatory pathway in higher plants. In *in vitro* reaction, dithiothreitol (DTT) or GSH is required for the reaction and thought to be a carrier. But *in vivo* a carrier has not been determined. It has been supposed that GSH (Tsang and Schiff 1978) or a somewhat larger molecular weight compound (Schmidt and Schwenn 1971) is functioned as a carrier. APS sulfotransferase activity was susceptible to regulatory factors like reduced sulfur sources (SO_2 , H_2S and cysteine, Brunold 1976, Wyss and Brunold 1979, Wyss and Brunold 1980), heavy metal treatment (Nussabaum et al. 1988) and sulfur and nitrogen nutrition (Brunold and Suter 1984, Brunold et al, 1987, Haller et al, 1986). Although there were many physiological studies on APS sulfotransferase, the enzyme had been only partially purified from *Chlorella* (Hodson and Schiff 1971) and spinach (Schmidt 1976). In algae, APS sulfotransferase was purified from *Euglena gracilis* (Li and Schiff 1991), and marine macroalgae, *Porphyra yezoensis* (Kanno et al. 1996). Recently APS reductase genes were isolated from *Arabidopsis thaliana* (Gutierrez-Marcos et al.1996, Setya et al. 1996). These authors reported that APS reductase catalyzed the direct conversion of APS to sulfite. Recombinant proteins of plant APS reductase had activity converting APS to sulfite, when thiols compounds existed at the high concentration. Recombinant APS reductase also had a low PAPS reductase activity. However, the components of the reaction mixture are the same between APS sulfotransferase and recombinant APS reductase, so that it is impossible to distinguish APS reductase from APS sulfotransferase.

Thus, the step converting APS to sulfite is still not defined in higher plants. Characterization of APS sulfotransferase (or APS reductase) and APS kinase will provide valuable information to understand mechanism and control of the sulfate assimilation

pathway. In this study, I try to understand the step converting APS to sulfite in higher plants. The non-radioactive assay method for APS sulfotransferase is described in Chapter 1. Purification of APS sulfotransferase from radish seedlings and its characterization are described in Chapter 2. Finally, I discuss molecular evolution of the step converting APS to sulfite in Chapter 3.

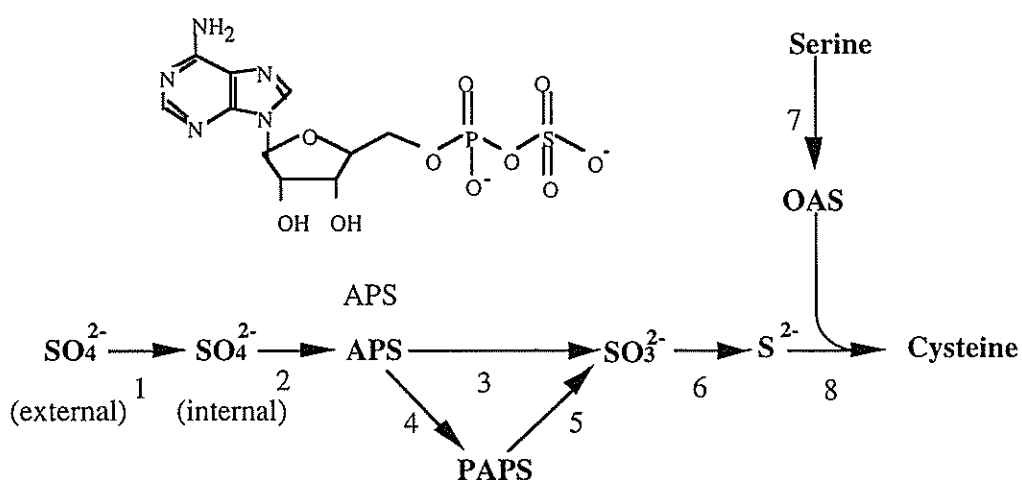


Fig. 1 Sulfate assimilation pathway

SO_4^{2-} , sulfate; APS, adenosine 5'-phosphosulfate; PAPS, 3'-phospho-adenosine 5'-phosphosulfate; OAS, *O*-acetylserine

[1] sulfate transporter; [2] ATP sulfurylase; [3] APS sulfotransferase or APS reductase; [4] APS kinase; [5] PAPS reductase; [6] sulfite reductase; [7] serineacetyltransferase; [8] *O*-acetylserine(thiol)lyase;

Table 1. Isolated genes encoding enzymes involved in sulfate assimilation pathway in higher plants

Gene	Species	Reference
Sulfate transporter	<i>Arabidopsis thaliana</i>	Takahashi et al, 1996
		Takahashi et al, 1997
	<i>Stylosanthes hamata</i>	Smith et al, 1995
	<i>Hordeum vulgare</i>	Hawkesford and Smith 1997
ATP sulfurylase	<i>Arabidopsis thaliana</i>	Leustek et al, 1994
		Klonus et al, 1995
		Murillo and Leustek 1995
	<i>Solanum tuberosum</i>	Klonus et al, 1994
APS kinase	<i>Arabidopsis thaliana</i>	Arz et al, 1994
APS reductase	<i>Arabidopsis thaliana</i>	Gutierrez-Marcos et al, 1996
		Setya et al, 1996
Sulfite reductase	<i>Arabidopsis thaliana</i>	Brühl et al, 1996
	<i>Zea mays</i>	Ideguchi et al, 1995
O-Acetylserine(thiol)lyase	<i>Arabidopsis thaliana</i>	Hell et al, 1994
	<i>Capsicum annuum</i>	Römer et al, 1992
	<i>Spinacia oleacea</i>	Hell et al, 1993
		Rolland et al, 1993
		Saito et al, 1992
		Saito et al, 1993
		Saito et al, 1994
	<i>Triticum aestivum</i>	Youssefian et al, 1993
Serineacetyltransferase	<i>Arabidopsis thaliana</i>	Ruffet, et al, 1995
		Roberts and Wray 1996
		Bogdanova and Hell 1997
	<i>Citrullus vulgaris</i>	Saito et al, 1995

Chapter 1. Non-radioactive Adenosine 5'-phosphosulfate Sulfotransferase Assay by Coupling with Sulfite Reductase and *O*-Acetylserine(thiol)lyase.

Introduction

Currently, APS sulfotransferase activity is assayed based on release of [^{35}S] SO_2 from [^{35}S]APS in the presence of dithioerythritol (DTE), DTT or GSH (Schiff and Levinthal, 1968). [^{35}S]APS used as a substrate have to be synthesized in the laboratory by the complexed procedures. This method is stime-consuming, although ensitive. The difficulty in the enzyme assay is one of the reason why APS sulfotransferase has not been purified from higher plants. Therefore, we have attempted to develop an alternative and nonradioactive assay method for APS sulfotransferase.

Relationship of APS sulfotransferase activity with sulfur nutrition was reported in *Lemna minor* (Brunold et al, 1987), tropical legume *Macroptilium atropurpureum* (Bell et al, 1995), and suspension cell culture of *Nicotiana sylvestris* (Jenni et al, 1980) and *Rosa* sp. (Haller et al, 1986). They reported a long term response of the enzyme activity in the plants grown with or without sulfate. Since sulfur is one of the essential macroelements in plants, contents of sulfur-containing compounds in the plants must be changed. In *Lemna* (Datko et al, 1978), *Macroptilium* (Clarkson et al, 1983) and spinach (Dietz 1989) grown under different sulfur nutrition conditions, changes in the contents of sulfur-containing compounds were reported.

This chapter describes a rapid and sensitive assay method for APS sulfotransferase. The method we developed is based on a coupled reaction with sulfite reductase and *O*-acetylserine(thiol)lyase to yield cysteine from APS (Fig. 2). Changes in APS sulfotransferase activity and contents of sulfur-containing compounds in the plants grown under the different sulfur nutrition conditions are also described.

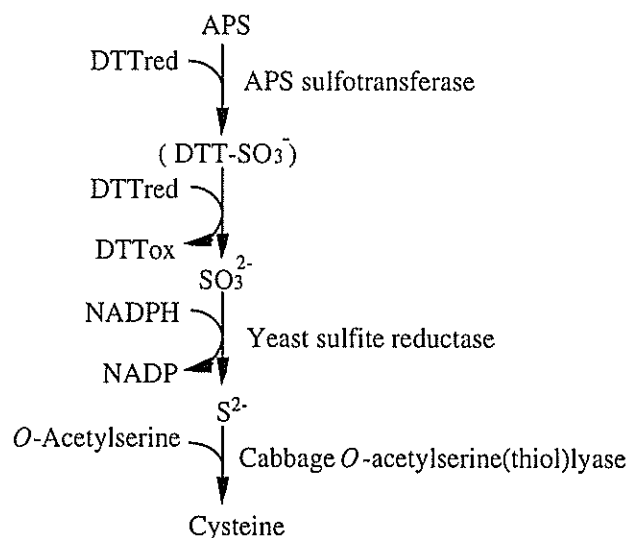


Fig. 2. Principle of APS sulfotransferase assay.

APS sulfotransferase catalyzes the transfer of sulfo-group of APS to DTT. In the presence of excess DTT, sulfite is spontaneously eliminated from the DTT-SO₃⁻ formed. Then, the sulfite formed is converted to cysteine by coupling with sulfite reductase and *O*-acetylserine-(thiol)lyase.

Materials and Methods

Materials

Authentic APS was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Trimethylamine-*N*-sulfonic acid was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). All other reagents were of analytical grade.

Plant materials

Radish (*Raphanus sativus* L.) seeds after soaking in tap water for 1 day were placed in plastic containers with tap water and germinated in the dark for 1 day. Germinated seeds were then placed in the greenhouse and grown for another 4 to 5 days with a nutrient solution. Harvested shoots were stored at -80°C until use. Nutrient solution contained 16.4 mM N (1.4 mM NH₄-N and 15 mM NO₃-N), 0.65 mM P, 4 mM K, 3.9 mM Ca, 2.0 mM Mg, 2.1 mM S, 21 μM Mn, 0.6 μM Zn, 48 μM Fe, 0.3 μM Cu, 14 μM B, and 0.2 μM Mo in tap

water. Cl was supplied from tap water.

Soybean (*Glycine max* Merrill cv. Shirojishi) seeds were germinated in a plastic container with vermiculite in a greenhouse. When primary leaves were fully expanded, seedlings were transplanted into 1/5000 Wagner pots (2 seedlings per pot) containing 3-l nutrient solution with aeration. The solution was a quarter-strength of Hoagland solution. The nutrient solutions were replaced with the fresh one every 5 days. When the first trifoliate leaves were fully expanded, the pots were drained, washed, flushed with distilled H₂O and refilled with 1/2 strength modified Hoagland solution, containing 1 mM (S-1) or 0 mM sulfate (S-0) (all the sulfate salts were replaced with chloride ones). The nutrient solutions were replaced with the fresh one every 3 days. Seedlings were harvested 0 and 10 days after the treatment, weighed, and stored at -80°C.

Other plant materials were obtained at local markets.

Synthesis of APS

APS was synthesized by the method described previously (Cherniak and Davidson, 1964) with some modifications to obtain a large amount of the purified one. Trimethylamine-*N*-sulfonic acid was used instead of triethylamine-*N*-sulfonic acid in the process of sulfonation. The APS synthesized was further purified. Hereafter, all steps were done at 4°C. The mixture containing APS was put on a DEAE-Toyopearl 650M column (2 x 20 cm) and eluted with 200 mM potassium phosphate buffer, pH 8.0. APS was eluted after AMP. Fractions containing APS were combined, concentrated to about 2 mM, and stored at -20°C until use. Purification of APS on DEAE-Toyopearl 650M resulted in 99% purity on a HPLC chart. The APS synthesized was identified by comparison of the retention time with commercially available APS on HPLC mounting Wakobeads I-DEAE column (6 x 100 mm, Wako Pure Chemicals Ind. Ltd, Osaka, Japan). The mobile phase was 150 mM potassium phosphate buffer, pH 6.9. Detection was done by absorbance at 260 nm. A molecular absorption coefficient of 15,200 was used for APS (Baddiley and Letters, 1957).

Preparation of O-acetylserine(thiol)lyase from cabbage leaves

O-acetylserine(thiol)lyase activity was assayed essentially by the method described previously (Lunn et al, 1990). The reaction mixture was composed of 20 μmoles of

potassium phosphate buffer (pH 7.5), 1 μ mole of 2-mercaptoethanol, 2 μ moles of OAS, 2.5 μ moles of Na_2S , and the enzyme solution in a final volume of 1 ml. After incubation at 30°C for 10 min, cysteine formed was measured colorimetrically (Gaitonde, M. K., 1967). One unit (U) was defined as the amount of the enzyme catalyzing formation of 1 μ mole of cysteine per min under these conditions. *O*-acetylserine(thiol)lyase was partially purified by the method described previously (Masada et al, 1975) with some modifications. Fresh cabbage leaves (1 kg) were homogenized in a Waring blender for 5 min with 200 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA and 0.1% mercaptoethanol (200 mM PEM buffer, pH 8.0). The homogenate was filtered through 4 layers of gauze and the filtrate was centrifuged at 10,000 $\times$ g for 10 min. The supernatant was heated at 70°C for 3 min, cooled quickly, and then centrifuged at 10,000 $\times$ g for 10 min. The supernatant was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (30-80%). The resulting precipitate was dissolved in 30 mM PEM buffer, pH 8.0, containing 10% glycerol (30 mM PEMG buffer, pH 8.0) and dialyzed overnight against the same buffer. The dialyzed enzyme solution was purified by DEAE cellulose column chromatography and gel filtration with Sephadex G-100. Thus, the enzyme was purified 100-fold with a specific activity of 366 U/mg protein. Active fractions were pooled, concentrated, and stored at -80°C until use. Under these conditions, the enzyme was stored without a significant loss of activity more than one year.

Preparation of NADPH-dependent sulfite reductase from baker's yeast

NADPH-dependent sulfite reductase activity was measured by coupling with *O*-acetylserine(thiol)lyase (Von Arb and Brunold, 1983). The reaction mixture was composed of 25 μ moles of Hepes/NaOH buffer (pH 8.0), 2 μ moles of OAS, 10 μ moles of DTT, 1 μ mole of NADPH, 2 μ moles of Na_2SO_3 , 0.3 U of *O*-acetylserine(thiol)lyase, and the enzyme solution for sulfite reductase in a final volume of 1 ml. After the incubation for 20 min at 30°C, cysteine formed was measured colorimetrically. One unit (U) was defined as the amount of the enzyme catalyzing formation of 1 μ mole of cysteine per min under these conditions. Sulfite reductase was partially purified by the method described previously with some modifications (Yoshimoto and Sato, 1968). Baker's yeast cells (300 g) were suspended in 3 l of chilled 300 mM PEM buffer, pH 7.0, and disrupted in a Dyno-mill (Willy A. Bachofen, Switzerland). The homogenate was centrifuged at 10,000 $\times$ g for 10 min. The supernatant was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (30-50%). The precipitate obtained was

dissolved in 50 mM PEMG buffer, pH 7.0, and dialyzed overnight against the same buffer. The dialyzed enzyme solution was further purified by DEAE-Toyopearl 650M column chromatography with a linear gradient of 100 to 150 mM KCl in the 50 mM PEMG buffer, pH 7.0. Thus, the enzyme was purified 75-fold with a specific activity of 0.063 U/mg protein. Active fractions were pooled, concentrated, and stored at -80°C until use. Under the condition, the enzyme could be stored more than one year without any significant loss of activity.

APS sulfotransferase assay

The standard reaction mixture for APS sulfotransferase assay contained 25 μ moles of potassium phosphate buffer (pH 8.0), 500 μ moles of Na_2SO_4 , 100 μ moles of Na_2CO_3 , 4 μ moles of OAS, 30 μ moles of DTT, 1 μ mole of NADPH, 200 nmoles of APS, 0.01 U of sulfite reductase, 0.3 U of *O*-acetylserine(thiol)lyase, and an aliquot of the enzyme solution in a final volume of 1 ml. The final pH of the reaction mixture was 9.0. After the incubation for a definite period at 30°C, the reaction was stopped by addition of 0.2 ml of 1.5 M trichloroacetic acid. After the mixture was centrifuged at 10,000 $\times g$ for 2 min, the acid ninhydrin reagent was added to the supernatant to measure cysteine formed colorimetrically (Baddiley et al. 1957). One unit (U) was defined as the amount of the enzyme catalyzing formation of 1 μ mole of cysteine per min under the condition.

Preparation of enzyme solution

All steps were done at 4°C unless otherwise noted. Fresh leaves were homogenized in a Waring blender for 5 min with 2 volumes of 300 mM PEM buffer, pH 8.0. The homogenate was filtered through 4 layers of gauze, and centrifuged at 10,000 $\times g$ for 10 min. The supernatant was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (40-60%). The resulting precipitate was dissolved in a small volume of 50 mM PEMG buffer, pH 8.0, and dialyzed overnight against the same buffer. The dialyzed enzyme solution was referred to as "enzyme preparation A". The enzyme preparation A was put on a DEAE cellulose column (2 $\times$ 20 cm). After the column was washed with 50 mM PEMG buffer, pH 8.0, proteins were eluted with the same buffer containing 500 mM KCl. The active fractions were pooled, precipitated by 60% saturation of $(\text{NH}_4)_2\text{SO}_4$. The precipitate dissolved in 50 mM PEMG buffer, pH 8.0, was dialyzed against the same buffer. The dialyzed enzyme thus obtained was referred

to as "enzyme preparation B", and stored at -80°C until use. Summary of a typical purification from spinach leaves is shown in Table 2.

Table 2 Summary of purification of APS sulfotransferase from spinach leaves

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)
(NH ₄) ₂ SO ₄ fractionation ^a	5830	2.00	0.000343
DEAE cellulose	864	2.80	0.00324

^a Started from 1.8 kg of spinach leaves.

Protein measurement

Protein was measured by the method of Bradford (1976) with bovine serum albumin as the standard protein.

Measurement of sulfur-containing compounds

For protein-S, the freeze-dried samples were homogenized with 10% TCA and centrifuged at 7000 *g* for 5 min. The precipitate was washed twice with ethanol and once with acetone. The final precipitation was dried, digested by nitric acid, and subjected to ICPS analysis (ICP-AES SPS1500VR Seiko Instruments Inc.).

For sulfate, 150 mg fresh material was freeze-dried and was homogenized in a mortar with a pestle in 3 ml of H₂O. The homogenate was transferred to a test tube with a screw cap. The homogenate was autoclaved for 10 min and centrifuged at 3000 *g* for 10 min. The supernatant (20 µl) was applied to anion chromatography on a Shimadzu HPLC system (conductivity detector, CDD-6A; column, IC-A1). Mobile phase was 2.5 mM phthalic acid, and 2.4 mM Trishydroxymethylaminomethane.

Results and Discussion

Sulfite measurement

I had attempted to measure sulfite by coupling with sulfite reductase and *O*-acetylserine(thiol)lyase. NADPH-dependent sulfite reductase and *O*-acetylserine(thiol)lyase used here were purified partially from Baker's yeast and cabbage, respectively. The reaction mixture for sulfite measurement was the same as that for the APS sulfotransferase assay, but APS and APS sulfotransferase were omitted. To this reaction mixture, a definite amount of sulfite was added as Na_2SO_3 . When the reaction was done with 100 nmol of sulfite, cysteine formation increased linearly with respect to incubation time (up to 30 min) and the amount of sulfite reductase (up to 0.95 mg protein) (data not shown). When either sulfite reductase or *O*-acetylserine(thiol)lyase was omitted from the reaction mixture containing sulfite (100 nmol), no cysteine formation was observed. This suggests that the sulfite reductase used here did not contain *O*-acetylserine(thiol)lyase activity or *vice versa*. Heat-denaturing of sulfite reductase or *O*-acetylserine(thiol)lyase prevented cysteine formation. Addition of Na_2SO_4 (500 μmol) and Na_2CO_3 (100 μmol), which were required for maximizing APS sulfotransferase activity as mentioned below, did not reduce cysteine formation. When the reaction mixture was incubated with different concentrations of sulfite (1 to 300 nmol), cysteine formed increased linearly with the increase in sulfite (Fig. 3). The rate of conversion from sulfite to cysteine was constantly about 60% under the conditions used. Thus the amount of sulfite in the reaction mixture could be assessed by the method used here.

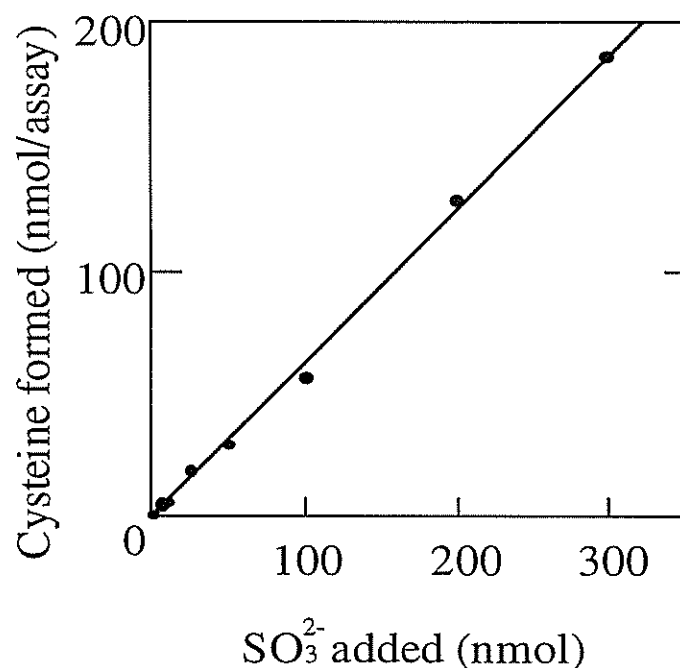


Fig.3. Sulfite measurement by coupling with sulfite reductase and *O*-acetylserine(thiol)lyase.

Definite amounts of sulfite were added to the mixture for APS sulfotransferase assay instead of APS and APS sulfotransferase. After the incubation at 30°C for 30 min, the cysteine formed was measured.

APS sulfotransferase assay

When APS and the crude enzyme solution of APS sulfotransferase (preparation A) were added to the reaction mixture for sulfite measurement described above and incubated at 30°C, cysteine was formed. Therefore, we further examined conditions for optimizing the enzyme reaction of APS sulfotransferase. Cysteine formation was dependent on APS (Table 3). The amount of cysteine formed increased linearly with increase in APS up to 100 nmol and became constant at the concentration more than 100 nmol (Fig. 4). Therefore, 200 nmol of APS was used as the substrate. Omitting APS sulfotransferase solution from the reaction mixture or addition of heat-denatured APS sulfotransferase instead of the native one resulted in no cysteine formation (Table 3). Cysteine formation was also dependent on sulfite reductase and *O*-acetylserine(thiol)lyase as expected. Amounts of

sulfite reductase and *O*-acetylserine(thiol)lyase were sufficient under the conditions used, since increasing amounts of these enzymes did not increase the cysteine formed. Since APS sulfotransferase activity is reported to increase by higher concentrations of some salts (Schmidt, 1975b, Suter et al., 1992), Na₂SO₄ and Na₂CO₃ were added to the reaction mixture. APS sulfotransferase activity was increased two-fold by addition of 500 mM Na₂SO₄ and 100 mM Na₂CO₃. High ionic strength would stabilize the enzyme activity. Without OAS and NADPH, cysteine formation was not observed. Increasing amounts of OAS and NADPH did not increase the cysteine formed under the conditions used. Thus, APS sulfotransferase activity could be assessed by this method.

To assess the requirement of DTT for the APS sulfotransferase, 2-mercaptoethanol-free APS sulfotransferase was prepared by dialysis of the preparation A against the buffer without 2-mercaptoethanol. The activity of APS sulfotransferase thus obtained was reduced to about 50% of the activity of the enzyme with 2-mercaptoethanol. Omitting DTT from the enzyme reaction mixture resulted in less than 10% cysteine formation compared to cysteine formation with DTT, suggesting that DTT is required for APS sulfotransferase reaction to form sulfite. Addition of MgCl₂ have no effect on spinach APS sulfotransferase, although it was required for *Euglena* APS sulfotransferase.

Cysteine formation increased with increase in incubation time (Fig. 5) and the amount of the enzyme added (Fig. 6). APS in the reaction mixture was completely converted to other compounds under the conditions used here, since APS was not detected by HPLC analysis after 30 min of incubation (data not shown). A part of APS was converted to sulfite. The fate of other part of APS is not known, but it may hydrolyzed by phosphatase, which are abundant in plant tissues. As a conclusion, we established a new, rapid assay method for APS sulfotransferase.

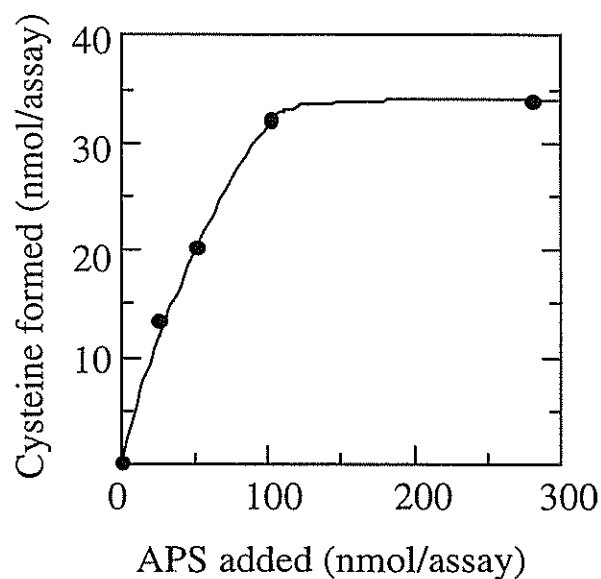


Fig. 4. APS sulfotransferase activity as a function of APS concentration. The standard reaction mixture for APS sulfotransferase was used with exception of addition of various amounts of APS. Enzyme preparation B (0.50 mg protein) was used as APS sulfotransferase. After the incubation at 30°C for 30 min, the cysteine formed was measured.

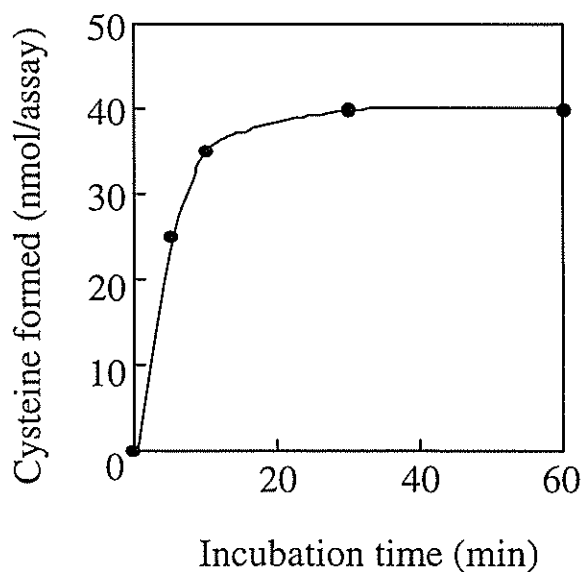


Fig. 5. APS sulfotransferase activity as a function of incubation time. The standard reaction mixture for APS sulfotransferase was used. Enzyme preparation B (0.50 mg protein) was used as APS sulfotransferase. After the incubation at 30°C for definite periods, the cysteine formed was measured.

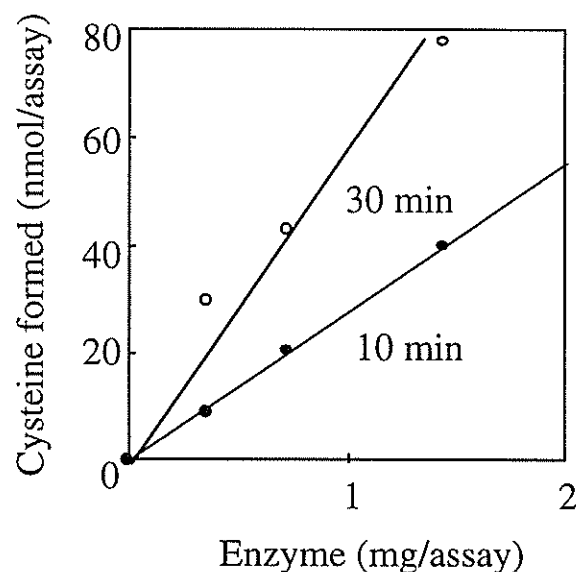


Fig. 6. APS sulfotransferase activity as a function of enzyme concentration. The standard reaction mixture for APS sulfotransferase was used with the exception of addition of various amounts of APS sulfotransferase (enzyme preparation B). After the incubation at 30°C for 30 min, the cysteine formed was measured.

Distribution of APS sulfotransferase activity in higher plants

We used the new enzyme assay method to estimate APS sulfotransferase activity in some higher plants. Crude enzyme extracts were prepared by the method described above for enzyme preparation A. APS sulfotransferase activity was detected from leaves of all of the higher plants tested (Table 4). Chinese leek showed the highest activity among the plants tested. The activities obtained here were comparable to those reported so far (Schmidt, 1975a).

Table 3 Requirements for APS sulfotransferase assay in extract from spinach leaves

Reaction mixture	APS sulfotransferase activity with enzyme preparation (% to the complete mixture)	
	A	B
Complete	100 ^a	100 ^b
-APS	24	6
-Na ₂ SO ₄	35	53
-Na ₂ CO ₃	47	47
-DTT	12 ^c	- ^d
-OAS	6	-
-NADPH	6	-
-APS sulfotransferase	3	-
Heat-denatured sulfite reductase	9	-
Heat-denatured <i>O</i> -Acetylserine(thiol)lyase	8	-
Heat-denatured APS sulfotransferase	7	-

^a With the complete reaction mixture containing enzyme preparation A (6.48 mg protein /assay), 85 nmoles of cysteine were formed.

^b With the complete reaction mixture containing enzyme preparation B (0.40 mg protein /assay), 32 nmoles of cysteine were formed.

^c Enzyme solution free from 2-mercaptoethanol was used as described in the text. A value indicates the activity relative to that with the complete reaction mixture containing 2-mercaptoethanol-free enzyme solution.

^d Not determined

Table 4 Distribution of APS sulfotransferase activity in leaves of various plants^a

Plant	Total protein (mg/g FW)	Total activity (mU/g FW)	Specific activity (mU/mg protein)
<i>Allium odorum</i>	2.40	0.43	0.18
<i>Brassica chinenisis</i> var. Komatuna	8.94	1.33	0.15
<i>Brassica oleracea</i> var. capitata	1.04	0.50	0.48
<i>Glycine max</i>	5.81	1.92	0.33
<i>Petroselinum sativum</i>	5.63	1.58	0.30
<i>Raphanus sativus</i> L.	6.30	1.00	0.16
<i>Spinacea oleracea</i>	5.50	1.86	0.34

^aAPS Sulfotransferase activity was measured in the standard reaction mixture with "enzyme preparation A" as described in Materials and Methods. Incubation time was 30 min.

Increase in radish APS sulfotransferase

One-week old radish seedlings were treated with various concentration of (NH₄)₂SO₄ for 2 h at 25°C. The enzyme solution after (NH₄)₂SO₄ fractionation was used as the enzyme source. The enzyme activity was activated by 10 mM of (NH₄)₂SO₄. The most effectively; about two-fold higher in terms of specific activity (Fig. 7). In *Lemna*, or suspension cell culture of *Nicotiana sylvestris*, it was reported that addition of reduced sulfur like cysteine to the culture medium was effective to decrease APS sulfotransferase activity in a few hours (Brunold et al, 1987, Jenni et al, 1980). *In vitro* condition, APS sulfotransferase was activated by some salts like Na₂SO₄ or NaCl (Suter et al, 1992). The finding here was first report that addition of sulfate increase APS sulfotransferase activity *in vivo*.

Effects of sulfur-deficient treatments on APS sulfotransferase activity

Soybean seedlings grown under the sulfur-deficient condition were used for measurement of sulfate and protein-S, and for assay of APS sulfotransferase (Fig. 8). During 10 days of sulfur-deficient treatment, the plants (S-0) looked almost similar to the control (S-1) plants. Fresh weight of S-0 plant had no significant difference to the control one (S-1). However, APS sulfotransferase activity in leaves of S-0 plant was that of the only 30% of control one, and protein-S was about 70% of the control one, and sulfate content in the leaves of S-0 plant was about 10% of the control one. The sulfate content of plant is, therefore, a more sensitive indicator of sulfur deficiency than the total sulfur content, as reported by Freney et al (1978). Sulfur-deficient plant seem to reduce sulfate to cysteine quickly and incorporated it into protein-S. Earlier works showed that APS sulfotransferase activity increased two or three fold in a few days on treatment with sulfate deficient nutrient solution (*Lemna*, *Macrotidium*, *Rosa* sp.) (Brunold et al, 1987, Bell et al, 1995, Haller et al, 1986). They also showed that APS sulfotransferase activity was the highest 2 or 3 days after the sulfur-deficient treatment. Our result showed that APS sulfotransferase activity decreased 10 days after sulfate deficient treatment, more detailed research will be required to clear regulation of APS sulfotransferase activity.

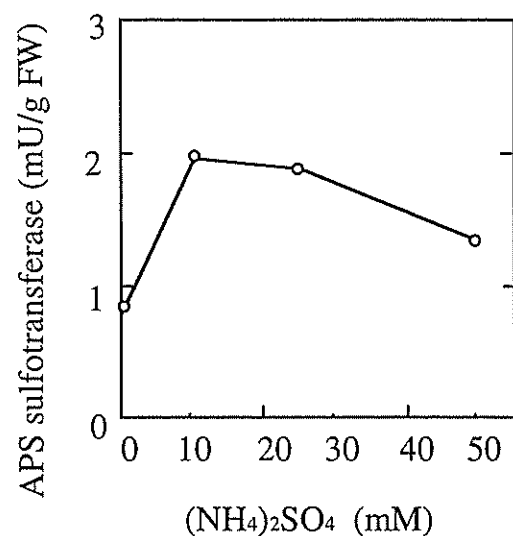


Fig. 7. Changes in radish APS sulfotransferase activity. One-week old radish seedlings were treated with (NH₄)₂SO₄ for 2 h at 25°C. The solution after the (NH₄)₂SO₄ fractionation was used as the enzyme source.

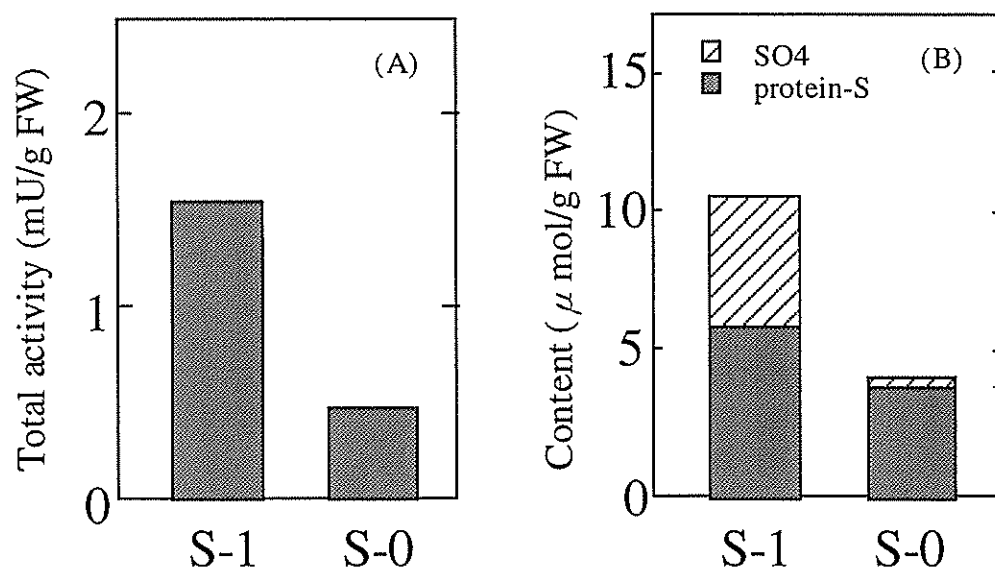


Fig. 8. Effects of sulfur-deficiency on APS sulfotransferase activity and content of sulfate and protein.

(A) APS sulfotransferase activity was measured after (NH₄)₂SO₄ treatment. (B) Content of sulfate and protein-S in soybean leaves.

Chapter 2. Purification and Characterization of Adenosine 5'-phosphosulfate Sulfotransferase from Radish.

Introduction

An enzyme responsible for formation of sulfite from APS has not been purified from higher plants, although it is one step in the sulfate assimilation pathway. So far, APS sulfotransferase has been purified from *Euglena gracilis* (Li and Schiff 1991), and marine macroalgae, *Porphyra yezoensis* (Kanno et al. 1996). *Euglena* enzyme were purified 10,000 fold and *P. yezoensis* one 3,000 fold. *Euglena* enzyme is homo-tetramer and *P. yezoensis* one homo-octamer. However, in higher plants, it was only partially purified from spinach (Schmidt 1976), probably due to instability of APS sulfotransferase like *P. yezoensis* one. To obtain purified APS sulfotransferase is crucial to understand sulfite formation from APS. This chapter describes purification of APS sulfotransferase from radish seedlings and its properties.

Material and methods

Materials

Authentic APS was obtained from Sigma Chemical Co. (St. Louis, MO, USA). AMP Sepharose and Phenyl Sepharose HP column was obtained from Pharmacia Biotech Inc. (Uppsala, Sweden), Cosmosil 5Diol-300 was obtained from Nacalai tesque (Kyoto, Japan), Marker protein kits for SDS-PAGE and gel filtration were obtained from Bio-Rad (Hercules, CA, USA) and Sigma Chemical Co.. All other reagents were of analytical grade.

Purification of APS sulfotransferase

All purification procedures were performed at 4°C unless otherwise noted. One-week old radish (*Raphanus sativus* L.) seedlings (kaiwardaikon) obtained at a local market were treated with 10 mM (NH₄)₂SO₄ for two h at 25°C and frozen at -80°C until use.

Frozen radish seedlings (600 g) were homogenized in a Waring blender for 5 min with 50 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA, 1 mM ascorbic acid and 30 mM sucrose. The homogenate was filtered through 2 layers of gauze and the filtrate was centrifuged at 10,000 g for 15 min. To the supernatant $(\text{NH}_4)_2\text{SO}_4$ was added to obtain proteins precipitating between 40% and 80% saturation. The resulting precipitate was dissolved in 50 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA, 10% glycerol (PEG buffer) and dialyzed overnight against the same buffer. The dialyzed enzyme solution was applied on a DEAE cellulose column (1 x 10 cm). After the column was washed with PEG buffer, proteins were eluted with the same buffer containing 150 mM KCl. The active fractions were pooled, concentrated, and desalted. It was further applied on a AMP Sepharose column (1 x 15 cm). Proteins were eluted with the PEG buffer. The active fractions were pooled and concentrated. To this solution, $(\text{NH}_4)_2\text{SO}_4$ (243 mg/ml) was added and the solution was applied on a Phenyl Sepharose HP column (1.6 x 10 cm). Proteins were eluted with a linear gradient of 1.8 to 0 M $(\text{NH}_4)_2\text{SO}_4$. The active fraction was pooled, concentrated, and stored at -80°C until use. Under this condition, the enzyme was stored without a significant loss of the activity more than one year.

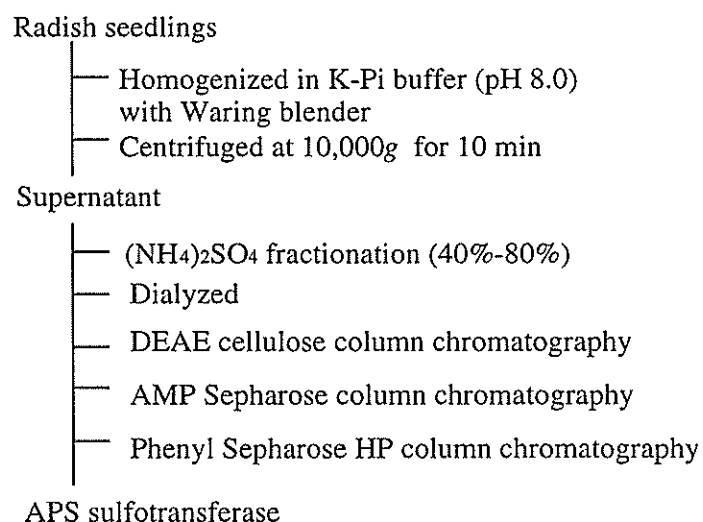


Fig. 9 Purification of radish APS sulfotransferase

Assay of APS sulfotransferase

APS sulfotransferase was assayed as described in chapter 1 (Ara and Sekiya 1997a). One unit (U) was defined as the amount of the enzyme catalyzing formation of 1 μ mol of cysteine per min under the condition.

Molecular weight

Mr of native enzyme was measured by gel filtration with Cosmosil 5Diol-300 column. Mobile phase was 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. Flow rate was 0.5 ml/min. The standard proteins (Sigma) were ferritin (Mr 443,000), β -amylase (Mr 200,000), alcohol dehydrogenase (Mr 150,000), carbonic anhydratase (Mr 29,000).

Mr of the subunit was determined by SDS-PAGE (Laemmli, 1970) with standard marker proteins (myosin, 200,000; β -galactosidase, 166,000; bovine serum albumin, 82,000; ovalbumin, 45,000).

Protein measurement

Protein was measured by the method of Bradford (1976) with bovine serum albumin as the standard protein.

Results and Discussion

Purification of APS sulfotransferase

One-week old radish seedlings were treated with 10 mM $(\text{NH}_4)_2\text{SO}_4$ were used for purification. APS sulfotransferase was purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation, and column chromatography with DEAE cellulose, AMP Sepharose and Phenyl Sepharose. Among there, AMP Sepharose was effective, since most proteins were eluted after the APS sulfotransferase (Fig. 10). APS sulfotransferase activity was found in two fraction^s eluted at 1.7-1.5 M $(\text{NH}_4)_2\text{SO}_4$ (peak 1) and 0.7-0.5 M $(\text{NH}_4)_2\text{SO}_4$ (peak 2) (Fig. 11). Peak 1 and 2 were designated as APS sulfotransferase 1 and 2, respectively. Native PAGE of the elutants from the Phenyl Sepharose column shown that the peak 1 was homogeneous while peak 2 contained a few contaminated proteins. SDS-PAGE also showed that the peak 1

was a single protein band (Fig. 12). Thus, one of the isoforms (APS sulfotransferase 1) was purified to homogeneity with an activity yield of 22% and a specific activity of 25.8 U/mg protein. Summary of the purification was shown in Table 5. The concentrated purified enzyme could be stored at -80°C without any significant loss of the activity. APS sulfotransferase precipitated by 80% saturated $(\text{NH}_4)_2\text{SO}_4$ was also stable during storage at -20°C, although the activity of the enzyme in the buffer alone was almost lost at 4 °C.

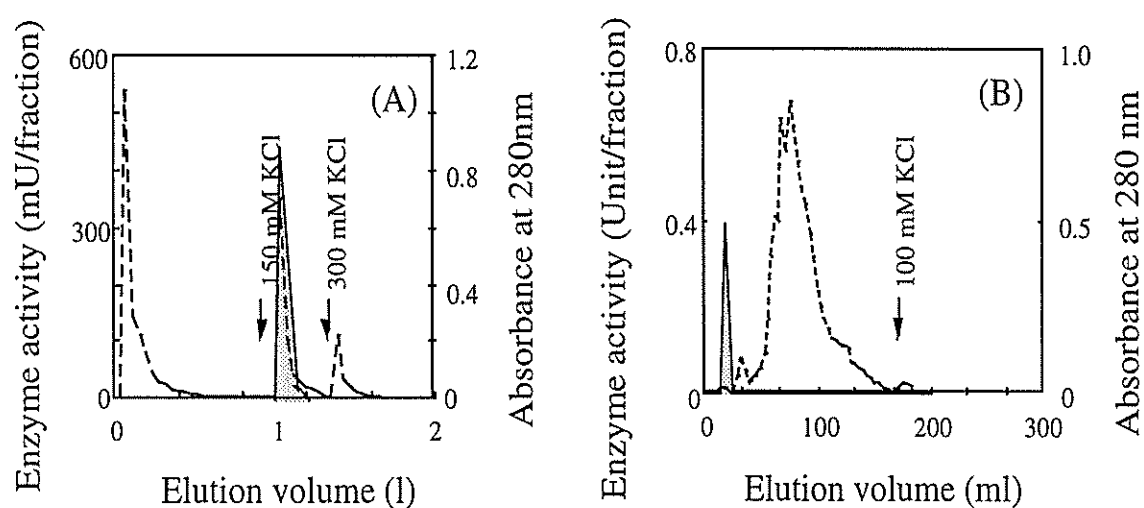


Fig. 10. Elution profiles of DEAE cellulose (A) and AMP Sepharose (B) column chromatography.

The active fraction after the $(\text{NH}_4)_2\text{SO}_4$ fractionation was applied on DEAE cellulose column. After washing with PEG buffer, proteins were eluted stepwise with PEG buffer containing KCl. The active fraction was applied on AMP Sepharose column. Proteins were eluted with PEG buffer. Dashed line (---) indicate absorbance at 280 nm and shaded area APS sulfotransferase activity.

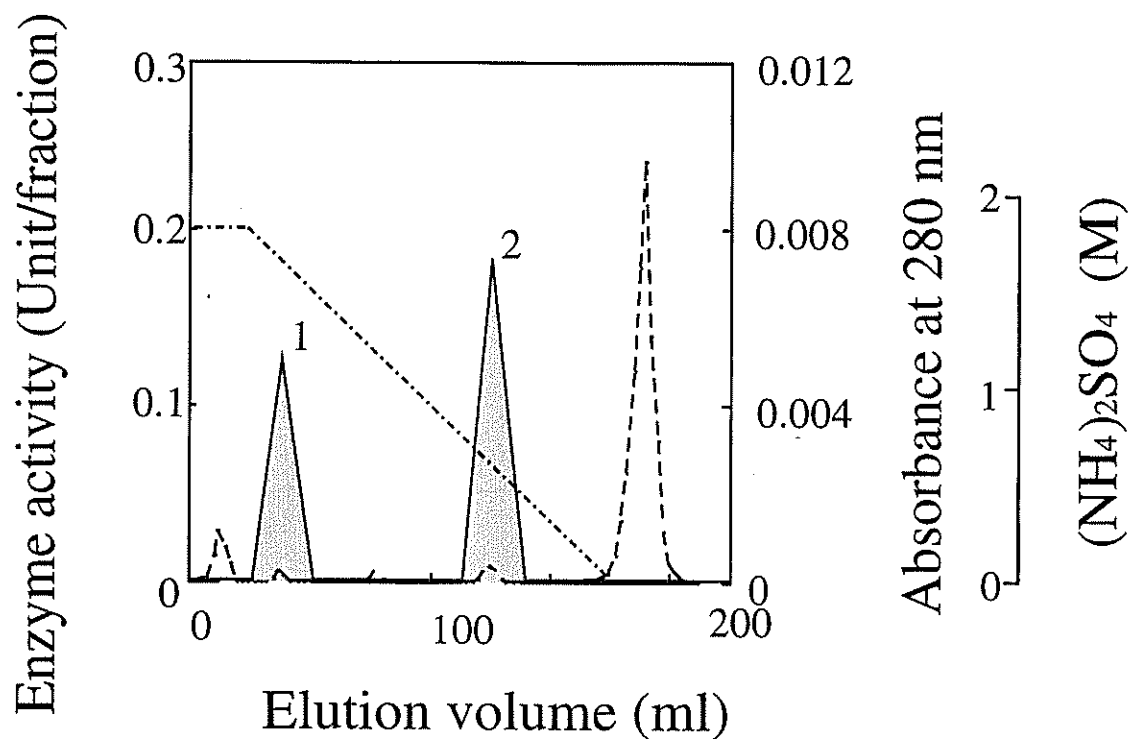


Fig. 11. Elution profile of Phenyl Sepharose HP column chromatography. The active fraction after AMP Sepharose column chromatography was applied on Phenyl Sepharose HP column. Proteins were eluted stepwise with a gradient of 1.8 to 0 M $(\text{NH}_4)_2\text{SO}_4$ in PEG buffer. Dashed line indicates absorbance at 280 nm and shaded area APS sulfotransferase activity. Dashed-pointed line indicates the concentration of $(\text{NH}_4)_2\text{SO}_4$.

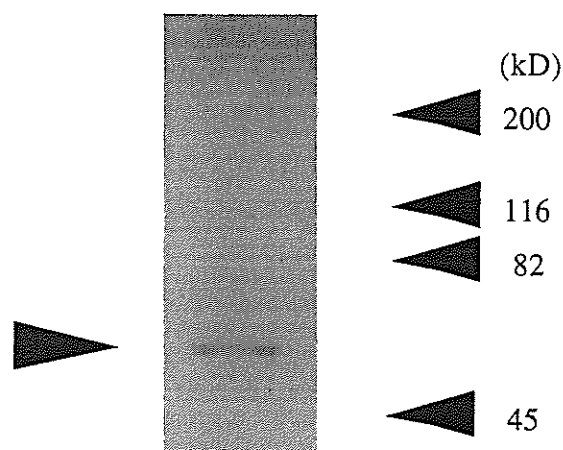


Fig. 12. SDS-PAGE of purified APS sulfotransferase from radish. SDS-PAGE was performed on a 12.5% gel and stained with CBB.

Table 5 Purification of APS sulfotransferase from radish seedlings.

Procedure	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg protein)	Fold
(NH ₄) ₂ SO ₄	225	600	2.7	1
DEAE cellulose	45	395	8.8	3
AMP Sepharose	0.3	320	1070	395
Phenyl Sepharose HP 1	0.005	129	25800	9560
	2	0.02	190	9500
				3520

Molecular weight.

Mr of radish APS sulfotransferase was estimated to be 225,000 by gel filtration. SDS-PAGE showed a single protein band and its Mr was 58,000 (Fig. 12, 13). Therefore it was concluded that radish APS sulfotransferase is a homotetrameric protein.

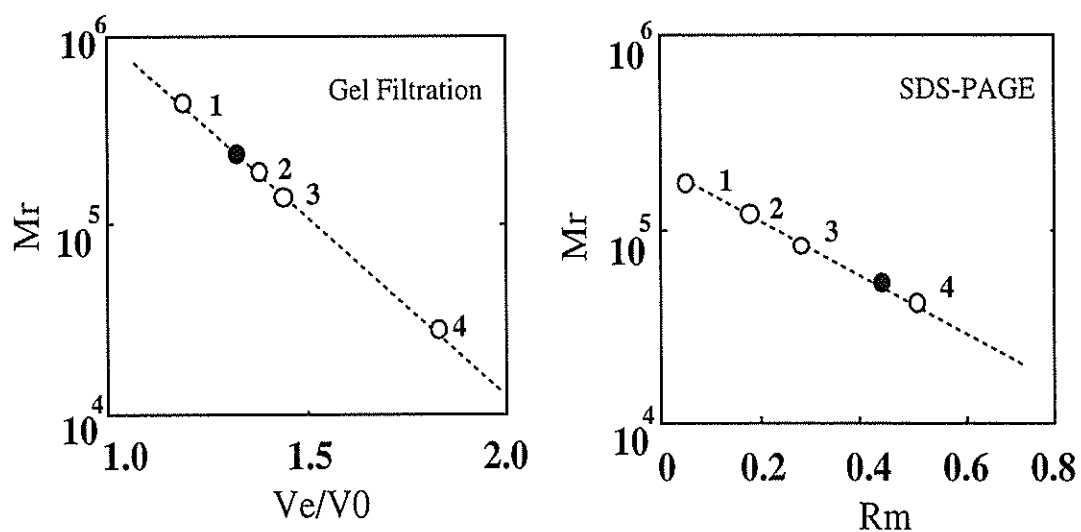


Fig. 13 Molecular weight of radish APS sulfotransferase 1

APS acted as a substrate for APS sulfotransferase in the presence of DTT (10 mM) to yield sulfite, but not in the absence of DTT. DTT (10 mM) was replaced by 20 mM 2-mercaptoethanol: but the amount of sulfite formed reduced to 30%. Without DTT, no sulfite was released from APS. 3'-Phosphoadenosine 5'-phosphosulfate (PAPS, 0.4 mM) and adenosine 5'-monosulfate (0.4 mM) did not act as a substrate in the presence and absence of DTT (10 mM) (Fig. 14). Thus, radish APS sulfotransferase is highly specific to APS. In Fig. 14, the structure of each compound simulated by SYBYL 6.3 using ATP X-ray structural data from database of Cambridge CSD as a starting structure (Kennard et al, 1971) is shown.

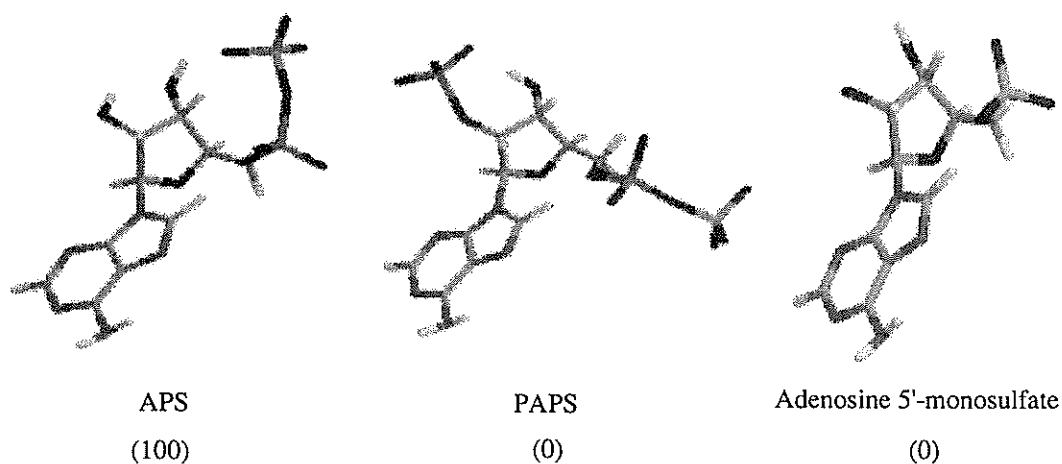


Fig. 14. Structure of APS, PAPS and Adenosine 5'-monosulfate and substrate specificity of radish APS sulfotransferase
Figures under the name of the compounds represent APS sulfotransferase activity relative to that with APS in percent.

Properties of radish APS sulfotransferase

Several APS sulfotransferase properties are listed in table. 6. The subunit composition of radish APS sulfotransferase is different from those of *Euglena* (Li and Schiff 1991) and *P. yezoensis* (Kanno et al, 1996). *Euglena* one is a tetramer of 25kd subunit and *P. yezoensis* one is an octamer of 43kd subunit (Li and Schiff 1991, Kanno et al, 1996). Native Mr of the radish enzyme is smaller than those of *P. yezoensis* (Kanno et al, 1996) and *Chlorella* ones (Hodson and Schiff 1971), and larger than spinach (Schmidt 1976) and Norway spruce ones (Suter et al, 1992). Apparent Km value for APS was 41 μ M with radish APS sulfotransferase. This value was similar to 13 μ M with spinach APS sulfotransferase (Schmidt 1976) and 29 μ M with Norway spruce one (Suter et al. 1992), but different from 0.1 μ M with *Euglena* one and 2.1 μ M with *P. yezoensis* one. These suggest that algae and higher plant could have a different type of APS sulfotransferase. AMP (2 mM) inhibited APS sulfotransferase activity completely. This result is consistent with previous works with several plants (Schmidt 1976, Suter et al., 1992). N terminal amino acid analysis were done using 10 μ g of purified enzyme, but significant amino acid signals were not detected. N-terminal amino acid of radish APS sulfotransferase may be blocked. Recently, it was reported that APS kinase have a weak activity of APS sulfotransferase (Schiffmann and Schwenn, 1994, Arz et al. 1994). However, the Mr of the enzyme was different and antibody raised against recombinant *Arabidopsis thaliana* APS kinase (Schiffmann and Schwenn, 1994) did not react with purified radish APS sulfotransferase. Therefore, APS sulfotransferase activity found in APS kinase may not function *in vivo*.

Table 6 Properties of APS sulfotransferase.

Species	Purification	Molecular weight		Km (μ M) (APS)
		Native	Subunit	
<i>Euglena gracilis</i>	H	120,000	25,000	0.1
<i>Porphyra yezoensis</i>	H	350,000	43,000	2.1
<i>Chlorella</i>	P	330,000	-	-
Spinach	P	110,000	-	13.0
Norway Spruce	P	115,000	-	29.0
Radish	H	255,000	58,000	41.0

H, purified to homogeneity. P, purified partially.

Chapter 3. Molecular Evolution of Adenosine 5'-phosphosulfate Reduction Pathway

Introduction

An step converting APS to sulfite has not been clarified so far in higher plants. Recent review showed that three sulfur assimilatory pathways are proposed for higher plants (Hell, 1997). First one of them is bound-sulfite pathway, in which APS sulfotransferase is responsible. This enzyme was purified as described in Chapter 2. Second one is a direct reduction pathway, in which APS reductase is responsible. cDNA encoding APS reductase was cloned from *Arabidopsis thaliana* (Gutierrez-Marcos et al, 1996, Setya et al, 1996). Recombinant APS reductase converted APS to sulfite with DTT *in vitro*, although the reaction mechanism of APS reductase is still unknown. However, the reaction components in the assay of APS reductase and APS sulfotransferase are almost the same. The difference in the proposed reaction mechanisms for both enzyme is whether the sulfo-group of APS is transferred first to an exogenous carrier with a thiol-group for APS sulfotransferase or to an endogenous thiol-group in the enzyme itself for APS reductase reaction. Therefore, both enzymes are probably the same. Third one is composed of APS kinase and PAPS reductase like *E. coli* and yeast. cDNAs encoding these enzymes were isolated from *A. thaliana*. In *Arabidopsis*, Mr of APS kinase is 30,000 and APS reductase is 46,000 to 51,000. PAPS reductase is only reported from spinach (Schwenn 1989). However, *Arabidopsis* APS kinase had a weak activity of APS sulfotransferase and *Arabidopsis* APS reductase had a weak activity of PAPS reductase. In bacteria and yeast, the pathway composed of APS kinase and PAPS reductase, in archae APS reductase pathway is well known. So I have attempted to compare DNA sequences data and discuss the molecular evolution of APS reduction step. In the discussion, DNA sequences for APS reductase was used for higher plants, since genes encoding APS sulfotransferase had not been isolated from higher plants.

Materials and Methods

Data

All sequences were retrieved from the GenBank and the EMBL sequence repositories, and several complete genome sequence database (Fleischmann et al, 1995, Fraser et al, 1995, Himmelreich et al, 1996, Kaneko et al, 1996, Bult et al, 1996, Smith, et al, 1997, Kunst et al, 1997, Klenk et al, 1997, Kawarabayasi et al. 1998a,b). Homologous gene sequences were surveyed through both BLAST submissions and nomenclature searches of the database, and the DNA sequence were translated into the predicted amino acid sequence.

Software

BLAST was used for homology search. GENEWORKS 2.51 (IntelliGenetics, Inc.) and MOTIFVIEW were used for domain and motif analyses of each gene sequence. MOTIFVIEW were made by perl (see in Appendix) and run on form of cgi on WEB server.

Results and Discussions

Domain analysis

I obtained data for about 20 functionally assigned genes of the enzymes involved in the APS reducing pathway, from GenBank etc. Amino acid sequences were aligned using the default settings of CLASTALW on GENEWORKS under PAM250 matrix. Domain structures in the alignment of these genes are shown in Fig. 15. APS reductase (plants), PAPS reductase (bacteria), and APS kinase (plants and bacteria) had highly homologous areas as shown by the oblique lined area. These areas may be a nucleotide binding domain, to which APS, PAPS and ATP bind. DNA sequence of *Arabidopsis thaliana* APS reductase had a thioredoxin domain conserved and had two redox-active motifs (CXXC). This motif existed in redoxin superfamily such as thioredoxin, glutaredoxin, rubredoxin, and protein disulfide isomerase or disulfide oxidoreductase. Dipeptide pattern inside this motif of plant APS reductase was almost identical to that of glutaredoxin. Glutaredoxin catalyzes the transfer of an electron from GSH to disulfide bond in protein so that APS reductase in

plants can use GSH as an electron donor and not require thioredoxin (Setya et al, 1996), although *E. coli* PAPS reductase required thioredoxin for *in vitro* reaction. This suggest that plant APS reductase gene may be a chimeric gene of PAPS reductase and thioredoxin.

Definition of APS and PAPS binding motifs

I have attempted to find possible APS and PAPS binding motif in oblique lined areas in Fig. 15. APS kinase may bind APS, PAPS, ATP, APS reductase APS, and PAPS reductase PAPS. Our hypothesis was that APS or PAPS binding motif has a similar structure of the ATP binding motif. ATP binding motif is DXXG...GXXXXGK (Prosite database, Bairoch, 1992). I paid an attention to three amino acids, D, G, and K. MOTIFVIEW was written by perl and was operated on WEB server. It converts amino acid sequence to an easily-looking form of defined amino acid pattern. Submotif were defined from the ATP binding motif (Table. 7). G(X)₂₋₆G and D(X)₁₋₃G was named as L2 to L6, and S1 to S3, respectively. MOTIFVIEW can select these submotifs easily and delete many unrelated patterns made by D, K, G (for example, "...KKK...", and ".....GGK....."). As a result, APS kinase had L4K...S1...S2...L4-6, PAPS reductase S1...L4-6, and APS reductase S2...L4-6. It is predicted from these findings that APS binding motif is DXXG...G(X)₄₋₆G and PAPS binding motif is DXG...G(X)₄₋₆G.

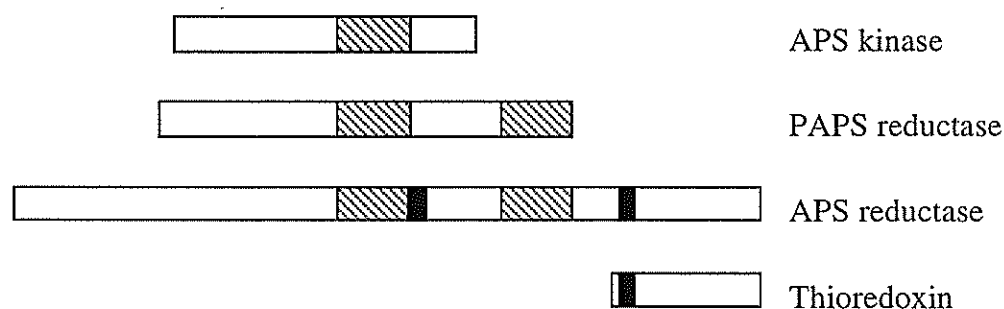


Fig. 15.

Domain structures of APS reducing enzymes in several organisms including bacteria, fungi, and higher plants.

Alignment was done by GENEWORKS. The obliquely lined areas show highly homologous areas. Black boxes show redox-active motif (CXXC).

Table 7 Definition of submotifs for analysis on MOTIFVIEW.

motif	Amino acid sequence	Database
ATP binding	DXXG...GXXXXXGK	(Prosite)
SN	D(X) _N G (N=1-3)	
LN	G(X) _N G (N=2-6)	

Assignment of candidate genes from complete genome database

APS and PAPS binding motifs may be useful for assignment of homologous genes by the homology search. When we surveyed complete genome database of *Bacillus subtilis* and *Synechocystis sp.*, functionally annotated candidates were found; one gene for APS kinase and one gene for PAPS reductase were defined in each species. The functionally unannotated candidates were found in the complete genome sequence of *Archaeoglobus fulgidus*, *Methanobacterium thermoautotrophicum*, *Methanococcus janaschii*, and *Pyrococcus horikoshii*. *Archaeoglobus fulgidus* and *Pyrococcus horikoshii* are hyperthermophilic sulfur reducing archaeon, and *Methanobacterium thermoautotrophicum* and *Methanococcus janaschii* are methane producing archaeon. All of sequences possibly encoding APS kinase had ATP binding motif, and a few candidate genes having both APS and PAPS binding motifs were also found in each species. It is suggested that *Archaeoglobus fulgidus* has two APS kinase genes. APS reductase gene was found in *Archaeoglobus fulgidus*. It is also suggested that *Methanococcus janaschii* and *Pyrococcus horikoshii* has APS reductase gene which has only APS binding motif. From the complete genome sequence of *Haemophilus influenzae*, *Mycoplasma genitalium*, and *Mycoplasma pneumoniae*, occurrence of APS kinase, APS reductase, and PAPS reductase is not suggested.

Grouping of genes related to the sulfate assimilation pathway

MOTIFVIEW can also make it easy to count several motif patterns in genes. I applied MOTIFVIEW to nucleotide binding motifs and redox-active motifs (extraction of cysteine). Several genes encoding APS reductase, APS kinase and PAPS reductase had CXXC or CC pattern in their sequences. Since contents of cysteine residues were different between the genes, I plotted number of cysteine residues vs. minimum distance (n) in each CX_nC motif (Fig. 16A). Since cysteine contents of the genes plotted in the top were one or zero, the distance did not be defined. Each square symbol means each gene; yellow symbols indicate APS kinase, orange ones indicate PAPS reductase, and green ones indicate APS reductase. Bacterial genes had 0 - 6 cysteine residues, plant genes has 5-10 cysteine residues, and sulfur-bacteria and archae have 2 - 16 cysteine residues. Contents of cysteine residues increased from bacteria to higher plants and sulfur reducing bacteria. PAPS reductase I belongs to the bacterial group commonly requires thioredoxin, and sulfur-reducing bacteria and some archaea require ferredoxin for APS reductase reaction. The reason for increased cysteine content is unknown. Sulfur-reducing bacteria and some archaea are usually living in strongly stressed fields and such condition could influence the enzyme structures and design of amino acid sequences. Zero in distance means CC pattern. Since PAPS reductases plotted at 4 to 6 in number of cysteine residues had CC pattern, so they may be classified into a separate group of PAPS reductase (group II). In addition, the genes in group II had CXXC motif although those in group I did not (Table 8). Next, I plotted number of S motif (DX₁₋₃G) vs. number of S2 motif (DXXG) (Fig. 16B). We found three groups, a PAPS reductase I group, and two APS reductase (I or II) (Fig. 16B and Table 8). APS kinase was omitted from the plots. Short-arrows indicate genes encoding PAPS reductase (group II) of *Bacillus subtilis*, *Mycoplasma tuberculosis*, and *Pseudomonas aeruginosa*, which did not belong to the above three groups (PAPS reductase I, and APS reductase I and II). Long-arrows indicate genes encoding APS reductase (group I) of *Methanococcus jannaschii* and *Pyrococcus horikoshii*, APS reductase of which has ferredoxin domain. CLASTALW alignment supports this hypothesis. These results showed that APS kinase, APS reductase and PAPS reductase are all classified into two subgroups, I and II (Table 8). Occurrence of APS binding motif, redox-active motif and CC motif, enzyme activity, and reference or accession number are also shown in Table 8.

Thiocapsa roseopersicina PAPS reductase I has APS binding motif. *Chromatium vinosum* APS reductase I has CXXXC sequence. Summary of Table 8 was shown in Fig. 17. The role of CC motif is still unknown. PAPS reductase I required thioredoxin for the reaction, but PAPS reductase II did not required thioredoxin. If more APS reductase genes are isolated from higher plants, more crucial grouping could be done.

Evolutional model for APS reduction pathway

At last, a model of evolution of APS reduction pathway is shown in Fig. 18. First subset is APS kinase I, PAPS reductase I, and thioredoxin. They are in some gram negative bacteria, yeast and *Penicillium chrysogenum*. The second one is APS kinase I and PAPS reductase II, found in *B. subtilis*. The third one is APS kinase II, APS reductase I, and subunit B (ferredoxin-like protein). It is predicted that the subset of APS kinase I and APS reductase II in plants system evolves from the second subset or the third one. (Fig. 18) More DNA sequence data related to the plant system will be required for further investigation.

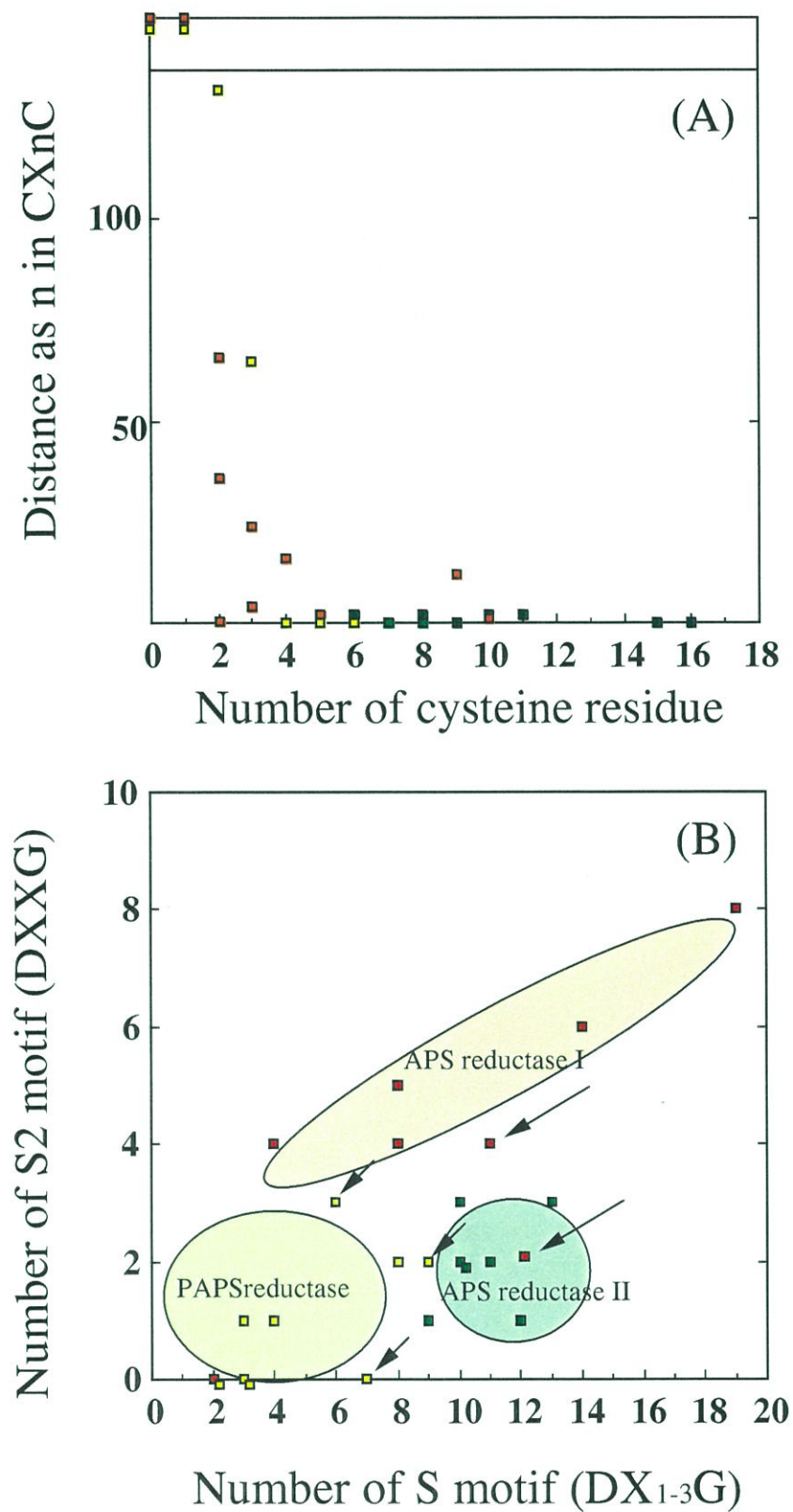


Fig. 16 Plots of genes of enzymes related to APS reducing pathway
Plots of genes of enzymes related to APS reducing pathway as a function of number of cysteine residue in the genes and distance between cysteine residues (A), and of these cysteines (B) number of S and S2 motif. "Arrows" are explained in the text.

Table. 8 List of genes of the enzymes related to APS reducing pathway

Enzymes	Species	motif				Reference or Accession No
		aps_bind	cxxc	cc	activity	
APS reductase I	<i>Archaeoglobus fulgidus</i>	+	+	-	+	Speich et al, 1994
	<i>Chromatium vinosum</i>	+	-	-	+	Hipp et al, 1997
	<i>Desulfovibrio vulgaris</i>	+	-	+	+	Z69372*
	<i>Methanococcus jannaschii</i>	+	++	-		MJ0973**
	<i>Pyrococcus horikoshii</i>	+	++	+		PH0268***
APS reductase II	<i>Arabidopsis thaliana</i>	+	++	+	+	Gutierrez-Marcos et al, 1996 Setya et al, 1996
	<i>Catharanthus roseus</i>	+	++	+		Q39619*
PAPS reductase I	<i>Escherichia coli</i>	-	-	-	+	Krone et al, 1991
	<i>Saccharomyces cerevisiae</i>	-	-	-	+	Thomas et al. 1990
	<i>Salmonella typhimurium</i>	-	-	-	+	Ostrowski et al, 1989
	<i>Schizosaccharomyces pombe</i>	-	-	-		Q10270*
	<i>Synechococcus sp.</i>	-	-	-	+	Niehaus et al, 1992
	<i>Synechocystis sp.</i>	-	-	-		slr1791**
	<i>Thiocapsa roseopersicina</i>	+	-	-		P52672*
PAPS reductase II	<i>Bacillus subtilis</i>	-	+	+		Mansilla and Mendoza 1997
	<i>Mycobacterium tuberculosis</i>	+	+	+		P71752*
	<i>Pseudomonas aeruginosa</i>	+	+	+		O05927*
APS kinase I	<i>Archaeoglobus fulgidus</i>	+	-	-		AF0288**
	<i>Arabidopsis thaliana</i>	+	-	-	+	Arz et al, 1994
	<i>Bacillus subtilis</i>	+	-	-		yisZ**
	<i>Escherichia coli</i>	+	-	-	+	Satishchandra et al, 1992
	<i>Penicillium chrysogenum</i>	+	-	-	+	Foster et al, 1995
	<i>Pseudomonas aeruginosa</i>	+	-	-	+	Satishandran et al, 1993
	<i>Synechocystis sp.</i>	+	-	-		slr0676**
	<i>Saccharomyces cerevisiae</i>	+	-	-	+	Kroch et al, 1991
APS kinase II	<i>Archaeoglobus fulgidus</i>	+	+	-		AF2269**
	<i>Methanobacterium thermoautotrophicum</i>	+	+	-		MTH1176**
	<i>Methanococcus jannaschii</i>	+	++	-		MJ0283**
	<i>Pyrococcus horikoshii</i>	+	+	-		PH0949***

Accession No from, * : GenBank; ** : kegg2 (<http://www.genome.ad.jp/kegg/kegg2.html>); *** : (http://www.bio.nite.go.jp/ot3db_index.html)

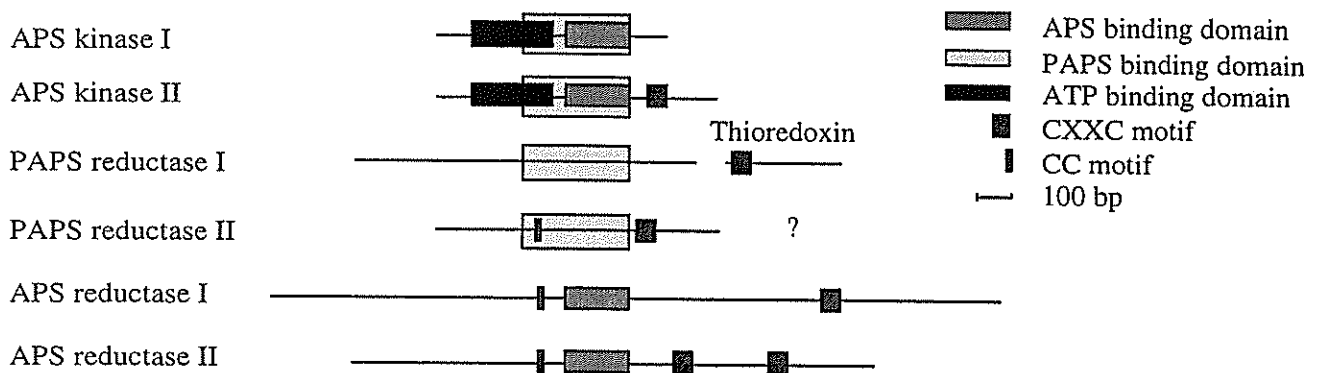


Fig. 17 Summary of the motifs in genes of the enzyme related to APS reducing pathway. Large boxes show the domains and short boxes show motifs.

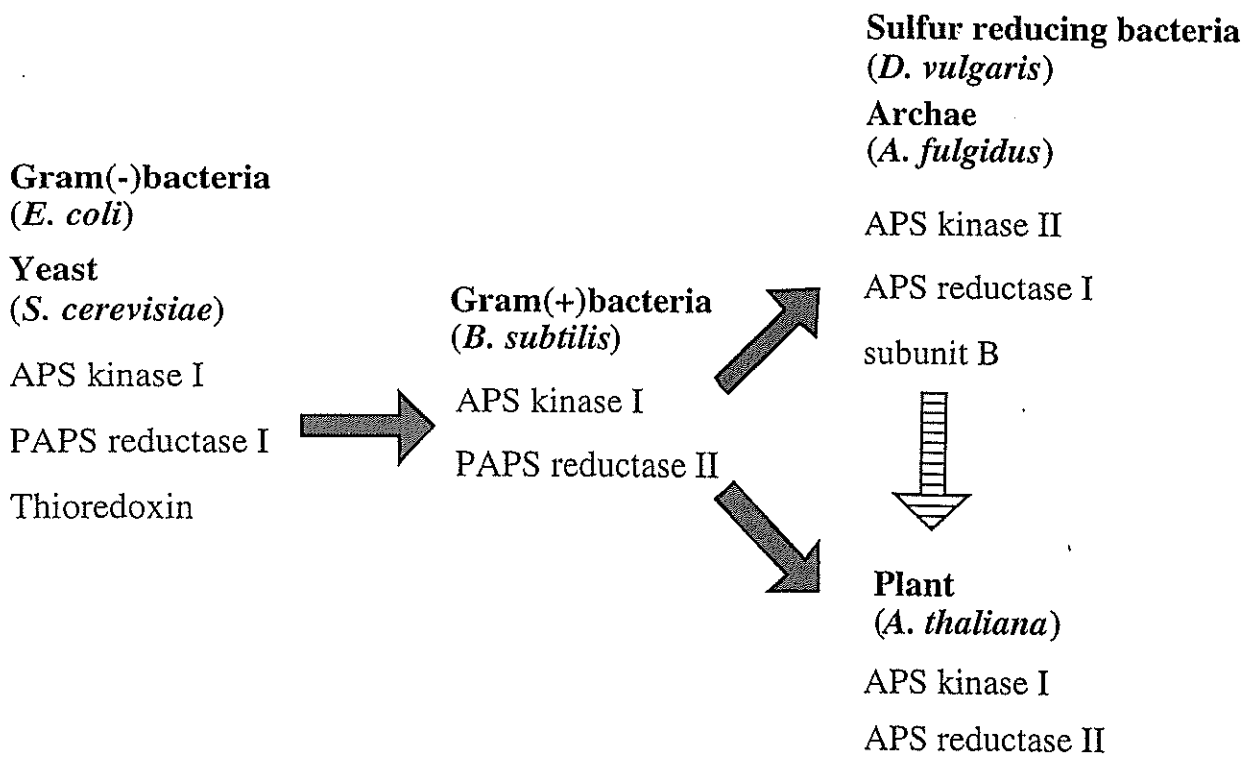


Fig. 18 Model of evolution of APS reducing pathway

Summary

The author developed a rapid, non-radioactive assay for APS sulfotransferase in Chapter 1. Sulfite released by APS sulfotransferase reaction in the presence of excess dithiothreitol was further converted to cysteine by coupling with yeast sulfite reductase and cabbage *O*-acetylserine(thiol)lyase. The cysteine thus formed was measured colorimetrically. By this method, 5 to 300 nmol of sulfite could be assessed. When the method was applied to APS sulfotransferase, the enzyme activity was APS-dependent with the partially purified enzyme. APS sulfotransferase activity was also detected in higher plants by this method.

APS sulfotransferase activity was affected by sulfur nutrition in the medium. Radish APS sulfotransferase activity was enhanced *in vivo* by treatment with 10-25 mM $(\text{NH}_4)_2\text{SO}_4$ for 2 hours. On the other hand, soybean APS sulfotransferase activity decreased to 30% 10 days after sulfur-deficient treatment.

The author purified and characterized APS sulfotransferase in Chapter 2. APS sulfotransferase was first purified from radish seedling to homogeneity by a combination of $(\text{NH}_4)_2\text{SO}_4$ precipitation and column chromatographies with DEAE cellulose, AMP Sepharose and Phenyl Sepharose. Molecular weight of the native enzyme was estimated to be 255,000 by gel filtration and M_r of the subunit was estimated to be 58,000 by SDS-PAGE, suggesting that APS sulfotransferase is a homotetramer. The apparent K_m value for APS was 41 μM , when dithiothreitol was used. 3'-Phospho-APS and adenosine 5'-monosulfate were not utilized as substrates. AMP strongly inhibited APS sulfotransferase activity.

The author analyzed DNA sequences of the genes related to APS reduction pathway and discussed molecular evolution of APS reduction pathway in higher plants in Chapter 3. APS kinase, APS reductase, and PAPS reductase classified into two subgroups based on APS and PAPS binding motifs predicted by MOTIFVIEW program. An evolution model of APS reduction pathway has been proposed.

Reference

- Ananvoranich, S., Varin, L., Gulick, P. and Ibrahim, R. (1994) Cloning and regulation of flavonol 3-sulfotransferase in cell-suspension cultures of *Flaveria bidentis* *Plant Physiol.* **106**, 485-491.
- Ara, T. and Sekiya, J., (1997a) Non-radioactive adenosine 5'-phosphosulfate sulfotransferase assay by coupling with sulfite reductase and O-acetylserine(thiol)lyase, *Biosci. Biotec. Biochem.* **71**, 621-624.
- Ara, T. and Sekiya, J., (1997b) Purification and characterization of APS sulfotransferase from spinach leaves, in "Sulphur Metabolism in Higher Plants: Molecular, Ecophysiological and Nutritional Aspects". Eds. W. J. Cram, L. J. De Kok, I. Stulen, C. Brunold, and H. Rennenberg, SPB Publishers, Amsterdam, The Netherlands. 177-179.
- Arz, H. E., Gisselmann, G., Schiffmann, S., and Schwenn, J. D. (1994) A cDNA for adenylyl sulfate (APS)-kinase from *Arabidopsis thaliana*, *Biochem. Biophys. Acta* **1218**, 447-452.
- Baddiley, J., Buchanan, J. G. and Letters, R., (1957) Synthesis of adenosine-5' sulfatophosphate. A degradation product of an intermediate in the enzymic synthesis of sulphuric esters. *J. Chem. Soc.* **1957**, 1067-1071.
- Bairoch, A. (1992) PROSITE: a dictionary of sites and patterns in proteins. *Nuc. Acid. Res.* **20**, 2013-2018.
- Bell, C. I., Clarkson, D. T. And Cram, W. J. (1995) Partitioning and redistribution of sulfur during S-stress in *Macroptilium atropurpureum* cv. Siratro, *J. Exp. Bot.* **46** 73-81.
- Bogdanova, N. and Hell, R. (1997) Cysteine synthase in plants: protein-protein interactions of serine acetyltransferase from *Arabidopsis thaliana*. *Plant J.* **11**, 251-262.
- Bradford, M. M., (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.* **72**, 248-254.
- Brunold, C., (1976) Regulation of adenosine 5'-phosphosulfate sulfotransferase activity by H₂S in *Lemna minor*, *Planta*, **133**, 85-88.
- Brunold, C., Suter, M., and Lavanchy, P. (1987) Effect of high and low sulfate concentrations on adenosine 5'-phosphosulfate sulfotransferase activity from *Lemna minor*, *Physiol. Plant.* **70**, 168-174.
- Brühl, A., Haverkamp, T., Gisselmann, G. and Schwenn, J. D. (1996) A cDNA clone from *Arabidopsis thaliana* encoding plastidic ferredoxin:sulfite reductase. *Biochem. Biophys. Acta* **1295**, 119-124.
- Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Black, J. A., FitzGerald, L. M., Clayton, R. A., Gocayne, J. D., Kerlavage, A. R., Dougherty, B. A., Tomb, J. -F.,

- Adams, M. D., Reich, C. I., Overbeek, R., Kirkness, E. F., Weinstock, K. G., Merrick, J. K., Glodek, A., Scott, J. L., Geoghagen, N. S. M., Weidman, J. F., Fuhrmann, J. L., Presley, E. A., Nguyen, D., Utterback, T. R., Kelley, J. M., Peterson, J. D., Sadow, P. W., Hanna, M. C., Cotton, M. D., Hurst, M. A., Roberts, K. M., Kaine, B. P., Borodovsky, M., Klenk, H. -P., Fraser, C. M., Smith, H. O., Woese, C. R., and Venter, J. C. (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus janaschii*. *Science* **273**, 1058-1073.
- Burke, J. J., Holloway, P. And Dalling, M. J. (1986) The effect of sulfur deficiency on the organization and photosynthetic capability of wheat leaves, *J. Plant Physiol.* **125**, 371-375.
- Burnell, J. N. and Anderson, J. W. (1973) Adenosine 5'-phosphosulfate kinase activity in spinach leaf tissue. *Biochem. J.* **134**, 565-579.
- Cherniak, R. and Davidson, E. A., (1964) Synthesis of adenylyl sulfate and adenylyl sulfate 3'-phosphate, *J. Biol. Chem.* **239**, 2986-2990.
- Clarkson, D. T., Smith, F. W. and Berg, P. J. V. (1983) Regulation of sulfate transport in a tropical legume, *Macroptilium atropurpureum*, cv. Siratro, *J. Exp. Bot.* **34**, 1463-1483.
- Datko, A.H., Mudd, S. H., Giovanelli, J. and Macnicol, P. K. (1978) Sulfur-containing compounds in *Lemna perpusilla* 6746 grown at a range of sulfur concentrations, *Plant Physiol.* **62**, 629-635.
- Dietz, K. J. (1989) Leaf and chloroplast development in relation to nutrient availability, *J. Plant Physiol.* **134**, 544-550.
- Fleischmann, R. D., Adams, R. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A., Merrick, J. M., McKenney, K., Sutton, G., FitzHugh, W., Fields, C., Gocayne, J. D., Scott, J., Shirley, R., Liu, L. -I., Glodek, A., Kelley, J. M., Weidman, j. F., Phillips, C. A., Spriggs, T., Hedblom, E., Cotton, M. D., Utterback, T. R., Hanna, M. C., Nguyen, D. T., Saudek, D. M., Brandon, R. C., Fine, L. D., Fritchman, J. L., Fuhrmann, J. L., Geoghagen, N. S. M., Gnehm, C. L., McDonald, L. A., Small, K. V., Fraser, C. M., Smith, H. O., and Venter, J. C. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496-512.
- Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., Bult, C. J., Kerlavage, A. R., Sutton, G., Kelley, J. M., Fritchman, J. L., Weidman, J. F., Small, K. V., Sandusky, M., Fuhrmann, J., Nugyen, D., Utterback, T. R., Saudek, D. M., Phillips, C. A., Merrick, J. M., Tomb, J. -F., Dougherty, B. A., Bott, K. F., Hu, P. -C., Lucier, T. S., Peterson, S. N., Smith, H. O., Hutchisn, C. A., and Venter, J. C. (1995) The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**, 397-403.
- Freney, J. R., Spencer, J. R. and Jones, M. B. (1978) The diagnosis of sulfur deficiency in wheat, Aust. *J. Agric. Res.* **29**, 727-738.
- Gaitonde, M. K., (1967) A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids, *Biochem. J.* **104**, 627-633.

- Gutierrez-Marcos, J. F., Roberts, M. A., Campbell, E. I., and Wray, J. L. (1996) Three members of a novel small gene-family from *Arabidopsis thaliana* able to complement functionally an *Escherichia coli* mutant defective in PAPS reductase activity encode proteins with a thioredoxin-like domain and 'APS reductase' activity, *Proc. Natl. Acad. Sci. USA* **93**, 13377-13382.
- Haller, E., Suter, M., and Brunold, C., (1986) Regulation of ATP-sulfurylase and adenosine 5'-phosphosulphate sulfotransferase by the sulfur and the nitrogen source in heterotrophic cell suspension cultures of Paul's scarlet rose, *J. Plant Physiol.* **125**, 275-283.
- Hawkesford, M. J. and Smith, F. W. (1997) Molecular biology of higher plant sulphate transporters. in "Sulphur Metabolism in Higher Plants: Molecular, Ecophysiological and Nutritional Aspects". Eds. W. J. Cram, L. J. De Kok, I. Stulen, C. Brunold, and H. Rennenberg, SPB Publishers, Amsterdam, The Netherlands. 13-25.
- Hell, R. (1997) Molecular physiology of plant sulfur metabolism, *Planta* **202**, p138-148.
- Hell, R., Bork, C., Bogdanova, N., Frolov, I. and Hauschild, R. (1994) Isolation and characterization of two cDNAs encoding for compartment specific isoforms of *O*-acetylserine(thiol)lyase from *Arabidopsis thaliana*. *FEBS Lett.* **351**, 257-262.
- Hell, R., and Schuster, G. and Gruissem, W. (1993) *O*-acetylserine(thiol)lyase cDNA from spinach. *Plant Physiol.* **102**, 1057-1058.
- Himmelreich, R., Hilbert, H., Plagens, H., Pirkel, E., Li, B. C., Herrmann, R. (1996) Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. *Nucleic Acid Res.* **24**, 4420-4449.
- Hipp, W. M., Pott, A. S., Thum-Schmitz, N., Faath, I., Dahl, C., Truper, H. G. (1997) Towards the phylogeny of APS reductases and sirohaem sulfite reductase in sulfate-reducing and sulfur-oxidizing prokaryotes. *Microbiology* **143**, 2891-2902.
- Hodson, R. C. and Schiff, J. A., (1971) Studies of sulfate utilization by algae 9. fractionation of a cell-free system from *Chlorella* into two activities necessary for the reduction of adenosine 3'-phosphate 5'-phosphosulfate to acid-volatile radioactivity, *Plant Physiol.* **47**, 300-305.
- Ideguchi, T., Akashi, T., Onda, Y. and Hase, T. (1995) cDNA cloning and functional expression of ferredoxin-dependent sulfite reductase from maize in *E. coli* cells. in "RESEARCH IN PHOTOSYNTHESIS: from Light to Biosphere II". Ed. P. Mathis, Kluwer, Amsterdam, The Netherlands. 713-716.
- Jain, A. and Leustek, T. (1994) A cDNA clone for 5'-adenylylphosphosulfate kinase from *Arabidopsis thaliana*, *Plant Physiol.* **105**, 771-772.
- Jenni, B. E., Brunold, C., Zrýd, J. -P., and Lavanchy, P. (1980) Properties and regulation of adenosine 5'-phosphosulfate sulfotransferase from suspension cultures of *Nicotiana sylvestris*. *Planta*. **150**, 140-

Kanno, N., Nagahisa, E., Sato, M., and Sato, Y., (1996) Adenosine 5'-phosphosulphate sulfotransferase from the marine macroalga *Porphyra yezoensis* Ueda (Rhodophyta): stabilization, purification, and properties, *Planta* **198**, 440-446.

Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., Tabata, S. (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res.* **3**, 109-136.

Kawarabayasi, Y., Sawada, M., Horikawa, H., Haikawa, y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S., Kosugi, H., Hosoyama, A., Nagai, Y., Sakai, M., Ogura, K., Otsuka, R., Nakazawa, H., Takamiya, M., Ohfuku, Y., Funahashi, T., Tanaka, T., Kudoh, Y., Yamazaki, J., Kushida, N., Oguchi, A., Aoki, K., Yoshizawa, T., Nakamura, Y., Robb, F. T., Horikoshi, K., Masuchi, Y., Shizuya, H., and Kikuchi, H. (1998a) Complete Sequence and Gene Organization of the Genome of a Hyper-thermophilic Archaeobacterium, *Pyrococcus horikoshii* OT3. *DNA Res.* **5**, 55-76.

Kawarabayasi, Y., Sawada, M., Horikawa, H., Haikawa, y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S., Kosugi, H., Hosoyama, A., Nagai, Y., Sakai, M., Ogura, K., Otsuka, R., Nakazawa, H., Takamiya, M., Ohfuku, Y., Funahashi, T., Tanaka, T., Kudoh, Y., Yamazaki, J., Kushida, N., Oguchi, A., Aoki, K., Yoshizawa, T., Nakamura, Y., Robb, F. T., Horikoshi, K., Masuchi, Y., Shizuya, H., and Kikuchi, H. (1998b) Complete Sequence and Gene Organization of the Genome of a Hyper-thermophilic Archaeobacterium, *Pyrococcus horikoshii* OT3 (Supplement). *DNA Res.* **5**, 147-155.

Kennard, O., Issacs, N. W., Motherwell, W. D. S., Coppola, J. C., Wampler, D. L., Larson, A. C. and Watson, D. G. (1971) The crystal and molecular structure of adenosine triphosphate. *Proc. R. Soc. London, Ser. A* **325**, 401-436.

Klenk, H. P., Clayton, R. A., Tomb, J. F., White, O., Nelson, K. E., Ketchum, K. A., Dodson, R. J., Gwinn, M., Hickey, E. K., Peterson, J. D., Richardson, D. L., Kerlavage, A. R., Graham, D. E., Kyrpides, N. C., Fleischmann, R. D., Quackenbush, J., Lee, N. H., Sutton, G. G., Gill, S., Kirkness, E. F., Dougherty, B. A., McKenney, K., Adams, M. D., Loftus, B., Peterson, S., Reich, C. I., McNeil, L. K., Badger, J. H., Glodek, A., Zhou, L., Overbeek, R., Gocayne, J. D., Weidman, J. F., McDonald, L., Utterbavk, T., Cotton, M. D., Sprigges, T., Artiach, P., Kaine, B. P., Sykes, S. M., Sadow, P. W., D'Andrea, K. P., Bowman, C., Fujii, C., Garland, S. A., Mason, T. M., Olsen, G. J., Fraser, C. M., Smith, H. O., Woese, C. R., and Venter, J. C. (1997) The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. *Nature* **390**, 364-370.

Klonus, D., Höfgen, R., Willmitzer, L. and Riesmeier, J. W. (1994) Isolation and characterization of two cDNA clones encoding ATP-sulfurylases from potato by complementation of a yeast mutant. *Plant J.* **6**, 105-112.

- Klonus, D., Riesmeier, J. W. and Willmitzer, L. (1995) A cDNA clone for an ATP-sulfurylase from *Arabidopsis thaliana*. *Plant Physiol.* **107**, 653-654.
- Kroch, C., Mountain, H. A., and Byström, A. S. (1991) Cloning, nucleotide sequence, and regulation of *MET14*, the gene encoding the APS kinase of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **229**, 96-108.
- Krone, F. A., Westphal, G., Schwenn, J.D. (1991) Characterization of the gene *cysH* and of its product phospho-adenyl sulfate reductase from *Escherichia coli*. *Mol. Gen. Genet.* **225**, 314-319.
- Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., Cummings, N. J., Daniel, R. A., Denizot, F., Devine, K. M., Düsterhöft, A., Ehrlich, S. D., Emmerson, P. T., Entian, K. D., Errington, J., Fabret, C., Ferrari, E., Foulger, D., Fritz, C., Fujita, M., Fuma, S., Galizzi, A., Galleron, N., Ghim, S. -Y., Glaser, P., Goffeau, A., Gologhtly, E. J., Grandi, G., Guiseppi, G., Guy, B. J., Haga, K., Haiech, J., Harwood, C. R., Hénaout, A., Hilbert, H., Holsappel, S., Hosono, S., Hullo, M. -F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., Klaerr-Blanchard, M., Klein, C., Kobayashi, Y., Koetter, P., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S. -M., Levine, A., Liu, H., Masada, S., Mauël, C., Médigue, C., Medina, M., Mellado, R. P., Mizuno, M., Moestl, D., Nakai, S., Noback, M., Noone, D., O'Reilly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S. -H., Parro, V., Pohl, T. M., Portetelle, D., Porwollik, S., Prescott, A. M., Presecan, E., Pujic, P., Purnelle, B., Rapoport, G., Rey, M., Reynolds, S., Rieger, M., Rivolta, C., Rocha, E., Rovhe, B., Rose, M., Sadaie, Y., Sato, T., Scanlan, E., Schleich, S., Schroeter, R., Scoffone, F., Sekiguchi, J., Sekowska, A., Seror, S. J., Serror, P., Shin, B. -S., Soldo, B., Sorokin, A., Tacconi, E., Takagi, T., Takahashi, H., Takemaru, K., Takeuchi, M., Tamakoshi, A., Tanaka, T., Terpstra, P., Tognoni, A., Tosato, V., Uchiyama, S., Vandenbol, M., Vannier, K., Vassarotti, K., Viari, A., Wambutt, R., Wedler, E., Wedler, H., Weitzenegger, T., Winters, P., Wipat, A., Yamamoto, H., Yamane, K., Yasumoto, K., Yata, K., Yoshida, K., Yoshikawa, H. -F., Zumstein, E., Yoshikawa, H., and Danchin, A. (1997) The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* **390**, 249-256.
- Lacomme, C. and Roby, D. (1996) Molecular cloning of a sulfotransferase in *Arabidopsis thaliana* and regulation during development and in response to infection with pathogenic bacteria *Plant Mol. Biol.* **30**, 995-1008.
- Laemmli, U. K., (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227**, 680-685.
- Lampreia, J., Moura, I., Teixeira, M., Peck Jr, H. D., Legall, J., Huynh, B. H. and Moura, J. J. G. (1990) The active centers of adenylylsulfate reductase from *Desulfovibrio gigas*: Characterization and spectroscopic studies. *Eur. J. Biochem.* **188**, 653-664.
- Leustek, T., Murillo, M. and Cervantes, M. (1994) Cloning of a cDNA encoding ATP sulfurylase from *Arabidopsis thaliana* by functional expression in *Saccharomyces cerevisiae*. *Plant Physiol.* **105**, 897-

- Li, J. and Schiff, J. A., (1991) Purification and properties of adenosine 5'-phosphosulphate sulfotransferase from *Euglena*, *Biochem. J.* **274**, 355-360.
- Lunn, J. E., Droux, M., Martin, J., and Douce, R., (1990) Localization of ATP sulfurylase and O-acetylserine(thiol)lyase in spinach leaves, *Plant Physiol.* **94**, 1345-1352.
- Mansilla M.C. and de Mendoza D. (1997) L-cysteine biosynthesis in *Bacillus subtilis*: Identification, sequencing and functional characterization of the gene coding for phosphoadenylylsulfate sulfotransferase. *J. Bacteriol.* **179**, 976-981.
- Masada, M., Fukushima, K., and Tamura, K., (1975) Cysteine synthase from rape leaves, *J. Biochem.* **77**, 1107-1115.
- Murillo, M. and Leustek, T. (1995) Adenosine-5'-triphosphate-sulfurylase from *Arabidopsis thaliana* and *Escherichia coli* are functionally equivalent but structurally and kinetically divergent: nucleotide sequence of two adenosine-5'-triphosphate-sulfurylase cDNA from *Arabidopsis thaliana* and analysis of a recombinant enzyme. *Arch. Biochem. Biophys.* **323**, 195-204.
- Niehaus, A., Gisselmann, G. and Schwenn, J. D. (1992) Primary structure of the *Synechococcus* PCC 7942 PAPS reductase gene. *Plant Mol. Biol.* **20**, 1179-1183.
- Nussabaum, S., Shumutz, D., and Brunold, C., (1988) Regulation of assimilatory sulfate reduction by cadmium in *Zea mays* L., *Plant Physiol.* **88**, 1407-1410.
- Ostrowski, J., Wu, J.-Y., Rueger, D. C., Miller, B. E., Siegel, L. M., Kredich, N. M. (1989) Characterization of the *cysJIH* regions of *Salmonella typhimurium* and *Escherichia coli* B. *J. Biol. Chem.* **264**, 15726-15737.
- Pao, S. S., Paulsen, I. T. and Saier, Jr, M. H. (1998) Major facilitator superfamily. *Microbiol. Mol. Biol. Rev.* **62**, 1-34.
- Roberts, M. A. and Wray, J. L. (1996) Cloning and characterization of an *Arabidopsis thaliana* cDNA clone encoding an organellar isoform of serine acetyltransferase. *Plant Mol. Biol.* **30**, 1041-1049.
- Rolland, N., Droux, M., Lebrun, M. and Douce, R. (1993) O-acetylserine(thiol)lyase from spinach (*Spinacia oleacea* L.) leaf: cDNA cloning, characterization, and overexpression in *Escherichia coli* of the chloroplast isoform. *Arch. Biochem. Biophys.* **300**, 213-222.
- Römer, S., d'Harlingue, A., Camara, B., Schantz, R. and Kuntz, M. (1992) Cysteine synthase from *Capsicum annuum* chromoplasts. *J. Biol. Chem.* **267**, 17966-17970.
- Ruffet, M. -L., Lebrun, M., Droux, M. and Douce, R. (1995) Subcellular distribution of serine acetyltransferase from *Pisum sativum* and characterization of an *Arabidopsis thaliana* putative

cytosolic isoform. *Eur. J. Biochem.* **227**, 500-509.

Saito, K., Miura, N., Yamazaki, M., Hirano, H. and Murakoshi, I. (1992) Molecular cloning and bacterial expression of cDNA encoding a plant cysteine synthase. *Proc. Natl. Acad. Sci. USA* **89**, 8078-8082.

Saito, K., Tatsuguchi, K., Murakoshi, I. and Hirano, H. (1993) cDNA cloning and expression of cysteine synthase B localized in chloroplast of *Spinacia oleacea*. *FEBS. Lett.* **324**, 247-252.

Saito, K., Tatsuguchi, K., Takagi, Y. and Murakoshi, I. (1994) Isolation and characterization of cDNA that encodes a putative mitochondrion-localizing isoform of cysteine synthase (*O*-acetylserine(thiol)-lyase) from *Spinacia oleacea*. *J. Biol. Chem.* **269**, 28187-28192.

Saito, K., Yokoyama, H., Noji, M. and Muakoshi, I. (1995) Molecular cloning and characterization of a plant serine acetyltransferase playing a regulatory role in cysteine biosynthesis from watermelon. *J. Biol. Chem.* **270**, 16321-16326.

Satishandran, A., Taylor, J. C., and Markham, G. D. (1993) The ORF1 of the gentamicin-resistance operon (aac) of *Pseudomonas aeruginosa* encode adenosine 5'-phosphosulfate kinase. *Mol. Microbiol.* **9**, 1223-1227.

Satishchandra, C., Hickman, Y. N., and Markham, G. D. (1992) Characterization of the phosphorylated enzyme intermediate formed in the adenosine 5'-phosphosulfate kinase reaction. *Biochemistry.* **31**, 11684-11688.

Schiff, J. A. and Levinthal, M., (1968) Studies of sulfate utilization by algae. 4. Properties of a cell-free-reducing system from *Chlorella*, *Plant Physiol.* **43**, 547-554.

Schiffmann, S. and Schwenn, J. D. (1994) APS-sulfotransferase activity is identical to higher plant APS-kinase (EC 2.7.1.25), *FEBS Lett.* **355**, 229-232.

Schmidt, A. and Schwenn, J. D. (1971) in "On the mechanism of photosynthetic sulfate reduction", ed. by O. Forti, M. Arron and M. Melandri, Junk, The Hague, pp. 507-514.

Schmidt, A., (1975a) Distribution of APS-sulfotransferase activity among higher plants, *Plant. Sci. Let.* **5**, 407-415.

Schmidt, A., (1975b) A sulfotransferase from spinach leaves using adenosine-5'-phosphosulfate. *Planta* **124**, 267-275.

Schmidt, A., (1976) Adenosine 5'-phosphosulphate sulfotransferase from spinach (*Spinacea oleracea* L.). stabilization, partial purification, and properties, *Planta* **130**, 257-263.

Schwenn, J. D. (1989) Sulfate assimilation in higher plants: A thioredoxin-dependent PAPS-reductase from spinach leaves, *Z. Naturforsch.* **44c**, p504-508.

Setya, A., Murillo, M., and Leustek, T. (1996) Sulfate reduction in higher plants: Molecular evidence for a novel 5'-adenylylsulfate reductase, *Proc. Natl. Acad. Sci. USA* **93**, 13383-13388.

Smith, D. R., Doucette-Stamm, L. A., Deloughery, C., Lee, H., Dubois, J., Aldredge, T., Bashirzadeh, R., Blakely, D., Cook, R., Gilbert, K., Harrison, D., Hoang, L., Keagle, P., Lumm, W., Pothier, B., Qiu, D., Spadafora, R., Vicaire, R., Wang, Y., Wierzbowski, J., Gibson, R., Jiwani, N., Caruso, A., Bush, D., Safer, H., Patwell, D., Prabhakar, S., McDougall, S., Shimer, G., Goyal, A., Pietrokovski, S., Church, G. M., Daniels, C. J., Mao, J. -I., Rice, P., Nölling, J., Reeve, J. N., (1997) Complete genome sequence of *Methanobacterium thermoautotrophicum* deltaH: functional analysis and comparative genomics. *J. Bacteriol.* **179**, 7135-7155.

Smith, F. W., Ealing, P. M., Hawkesford, M. J. and Clarkson, D. T. (1995) Plant members of a family of sulfate transporters reveal functional subtypes. *Proc. Natl. Acad. Sci. USA* **92**, 9373-9377.

Speich, N., Dahl, C., Heisig, P., Klein, A., Lottspeich, F., Stetter, K.O. and Trüper, H.G. (1994) Adenylylsulphate reductase from the sulphate-reducing archaeon *Archaeoglobus fulgidus*: cloning and characterization of the genes and comparison of the enzyme with other iron-sulphur flavoproteins. *Microbiology* **140**, 1273-1284.

Suter, M., Tschanz, A., and Brunold, C., (1992) Adenosine 5'-phosphosulphate sulfotransferase from Norway Spruce: Biochemical and Physiological properties, *Bot. Acta.* **105**, 190-196.

Takahashi, H., Saskura, N., Noji, M. and Saito, K. (1996) Isolation and characterization of a cDNA encoding a sulfate transporter from *Arabidopsis thaliana*. *FEBS Lett.* **392**, 95-99.

Takahashi, H., Yamazaki, M., Sasakura, N., Watanabe, A., Leustek, T., De Almeida, E. J., Engler, G., Van Montagu, M. and Saito, K. (1997) Regulation of sulfur assimilation in higher plants: A sulfate transporter induced in sulfate, starved roots plays a central role in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **94**, 11102-11107.

Tsang, M. L. -S. and Schiff, J. A., (1978) Studies of sulfate utilization by algae 18. Identification of glutathione as a physiological carrier in assimilatory sulfate reduction by *Chlorella*, *Plant Sci. Lett.* **11**, 177-183.

Thomas, D., Barbey, R., Surdin-Kerjan, Y. (1990) Gene-enzyme relationship in the sulfate assimilation pathway of *Saccharomyces cerevisiae* *J. Biol. Chem.* **265**, 15518-15524.

Thomas, D., Kuras, L. Cherest, H., Blaiseau, P. -L. and Surdin-Kerjan, Y. (1997) Regulation of gene expression in yeast sulphur metabolism. in "Sulphur Metabolism in Higher Plants: Molecular, Ecophysiological and Nutritional Aspects". Eds. W. J. Cram, L. J. De Kok, I. Stulen, C. Brunold, and H. Rennenberg, SPB Publishers, Amsterdam, The Netherlands. 27-37.

Thomas, M. G. and Jonathan, E. P. (1990) Partial purification and characterization of a 3'-phosphoadenosine 5'-phosphosulfate: desulfoglucosinolate sulfotransferase from cress (*Lepidium sativum*) *Plant*

Physiol. **94**, 811-818.

Varin, L., DeLuca, V., Ibrahim, R. K. and Brisson, N. (1992) Molecular characterization of two plant flavonol sulfotransferase *Proc. Natl. Acad. Sci. USA* **89**, 1286-1290.

Von Arab, C. and Brunold, C., (1983) Measurement of ferredoxin-dependent sulfite reductase activity in crude extracts from leaves using *O*-acetyl-L-serine sulfhydrylase in a coupled assay system to measure the sulfide formed, *Anal. Biochem.* **131**, 198-204.

Von Arb, C. and Brunold, C., (1986) Enzymes of assimilatory sulfate reduction in leaves of *Pisum sativum*: Activity changes during ontogeny and *in vivo* regulation by H₂S and cyst(e)ine, *Physiol. Plant.* **67**, 81-86.

Wyss, H. -R. and Brunold, C., (1979) Regulation of adenosine-5'-phosphosulfate sulfotransferase activity by H₂S and cyst(e)ine in primary leaves of *Phaseolus vulgaris* L., *Planta* **147**, 37-42.

Wyss, H. -R. and Brunold, C., (1980) Regulation of adenosine 5'-phosphosulphate sulfotransferase by sulfur dioxide in primary leaves of beans (*Phaseolus vulgaris*), *Physiol. Plant.* **50**, 161-165.

Yoshimoto, A. and Sato, R., (1968) Studies on yeast sulfite reductase I. Purification and characterization, *Biochem. Biophys. Acta.* **153**, 555-575.

Youssefian, S., Nakamura, M. and Sano, H. (1993) Tobacco plants transformed with the *O*-acetylserine-(thiol)lyase gene of wheat are resistant to toxic levels of hydrogen sulfide gas. *Plant J.* **4**, 759-769.

Publications

Takeshi Ara and Jiro Sekiya: Non-radioactive adenosine 5'-phosphosulfate sulfotransferase assay by coupling with sulfite reductase and *O*-acetylserine (thiol)lyase. (1997) *Biosci. Biotech. Biochem.* **71**, 621-624.

Takeshi Ara and Jiro Sekiya: Purification and characterization of APS sulfotransferase from spinach leaves. (1997) in "Sulphur Metabolism in Higher Plants: Molecular, Ecophysiological and Nutritional Aspects". Eds. W. J. Cram, L. J. De Kok, I. Stulen, C. Brunold, and H. Rennenberg, SPB Publishers, Amsterdam, The Netherlands, 177-179.

Takeshi Ara and Jiro Sekiya: Purification and characterization of adenosine 5'-phosphosulfate sulfotransferase of radish seedlings. (1997) in "Plant Nutrition - for sustainable food production and environment." Eds. T. Ando et al., Kluwer Academic Publishers, Dordrecht, The Netherlands, 855-856.

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Appendix

List of Programs of MOTIFVIEW

1. MOTIFVIEW for redox-active motif

```
#!/usr/bin/perl

print "Content-type: text/html¥n¥n";
print "<html><head><title>z 1</title></head>¥n";
print '<BODY BGCOLOR="#FFFFFF">';

$s = $ENV{QUERY_STRING};
$_ = $s;
s/%0D%0A//g;
s/(+)/ /g;
s/seq=/g;
$a1 = ".";
$a0 = "-";
$a00 = "-----";
$a0a = "=";
$a2 = "===== ";
$a3 = "* ";
s/A/$a1/g;
s/D/$a1/g;
s/E/$a1/g;
s/F/$a1/g;
s/G/$a1/g;
s/H/$a1/g;
s/I/$a1/g;
s/K/$a1/g;
s/L/$a1/g;
s/M/$a1/g;
s/N/$a1/g;
s/P/$a1/g;
s/Q/$a1/g;
s/R/$a1/g;
s/S/$a1/g;
s/T/$a1/g;
s/V/$a1/g;
s/W/$a1/g;
s/Y/$a1/g;
s/\\.\\.\\.\\.\\.\\.\\.\\.\\.\\.\\.\\.\\.\\./$a0/g;
s/$a00/$a0a/g;
s/$a2/$a3/g;
$k = $_;
```

```
print $k;
print "</body></html>";
```

2. MOTIFVIW for nucleotide binding motif

```
#!/usr/bin/perl
```

```
print "Content-type: text/html¥n¥n";
print "<html><head><title>g1</title></head>¥n";
print '<BODY BGCOLOR="#FFFFFF">';
```

```
$s = $ENV{QUERY_STRING};
$_ = $s;
s/%0D%0A//g;
s/(+)/ /g;
s/seq=/g;
$a0 = "-";
$a1 = ".";
$a3 = "...";
$a4 = "....";
$a5 = ".....";
$a9 = ".....";
$a10 = ".....";
$a11 = ".....";
$a12 = ".....";
$a13 = ".....";
$a15 = ".....";
$a16 = ".....";
$a17 = ".....";
$a00 = "-----";
$a00 = "=";
s/A/$a1/g;
s/C/$a1/g;
s/E/$a1/g;
s/F/$a1/g;
s/H/$a1/g;
s/I/$a1/g;
s/L/$a1/g;
s/M/$a1/g;
s/N/$a1/g;
s/P/$a1/g;
s/Q/$a1/g;
s/R/$a1/g;
s/S/$a1/g;
s/T/$a1/g;
```

s/V/\$a1/g;
s/W/\$a1/g;
s/Y/\$a1/g;

&nuc;
&nuc;
&nuc;
s/\.\.\.\.\.\.\.\.\.\.\.\./ \$a0/g;
s/\$a00/\$aa0/g;

sub nuc {
s/\.K\./ \$a3/g;
s/\.KK\./ \$a4/g;
s/\.KKK\./ \$a5/g;
s/\.KD/\.\.D/g;
s/DK\./D\.\./g;
s/DKK\./D\.\.\./g;
s/\.KKD/\.\.\.D/g;
s/\.DKD/\.\.D\.\./g;
s/DKKK\./D\.\.\.\./g;
s/\.KKKD/\.\.\.\.D/g;
s/\.\.\.\.D\.\.\.\./ \$a9/g;
s/\.\.\.\.DD\.\.\.\./ \$a10/g;
s/\.\.\.\.D\.\.\.\.\./ \$a11/g;
s/\.\.\.\.D\.\.\.\.\.\./ \$a12/g;
s/\.\.\.\.DD\.\.\.\.\./ \$a12/g;
s/\.\.\.\.DDD\.\.\.\./ \$a11/g;
s/\.\.\.\.DDD\.\.\.D/\.\.\.\.\.\.\.\.\.\.D/g;
s/D\.\.\.\.DDD\.\.\.\./D\.\.\.\.\.\.\.\.\.\./g;
s/\.\.\.\.D\.\.\.D\.\.\.\./ \$a12/g;
s/\.\.\.\.DDDD\.\.\.\./ \$a12/g;
s/\.\.\.\.D\.\.\.D\.\.\.\./ \$a13/g;
s/\.\.\.\.DDDDD\.\.\.\./ \$a13/g;
s/\.\.\.\.\.\.G\.\.\.\.\.\.\./ \$a15/g;
s/\.\.\.\.\.\.DG\.\.\.\.\.\.\./ \$a15/g;
s/\.\.\.\.\.\.GD\.\.\.\.\.\.\./ \$ a15/g;
s/\.\.\.\.\.\.DGD\.\.\.\.\.\.\./ \$a15/g;
s/\.\.\.\.\.\.GK\.\.\.\.\.\.\./ \$ a15/g;
s/\.\.\.\.\.\.KG\.\.\.\.\.\.\./ \$ a15/g;
s/\.\.\.\.\.\.KGK\.\.\.\.\.\.\./ \$ a15/g;
s/\.\.\.\.\.\.KGD\.\.\.\.\.\.\./ \$ a15/g;
s/\.\.\.\.\.\.DGK\.\.\.\.\.\.\./ \$ a15/g;
s/\.\.\.\.\.\.GG\.\.\.\.\.\.\./ \$ a16/g;
s/\.\.\.\.\.\.GGG\.\.\.\.\.\.\./ \$ a17/g;
s/\.\.\.\.\.\.G\.\.\.\.\.\.\./ \$ a17/g;
s/\.\.\.\.\.\.GGK\.\.\.\.\.\.\./ \$ a17/g;
s/\.\.\.\.\.\.GKG\.\.\.\.\.\.\./ \$ a17/g;
s/\.\.\.\.\.\.KGG\.\.\.\.\.\.\./ \$ a17/g;
s/\.\.\.\.\.\.GKK\.\.\.\.\.\.\./ \$ a17/g;

