A novel *O*-phospho-L-serine sulfhydrylation reaction catalyzed by *O*-acetylserine sulfhydrylase from *Aeropyrum pernix* K1

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Abstract ***O*-Acetylserine sulfhydrylase (OASS), a pyridoxal 5′-phosphate (PLP)-dependent enzyme, catalyzes the synthesis of L-cysteine from *O*-acetyl-L-serine and sulfide. *O*-Acetyl-L-serine is labile at high temperatures at which hyperthermophilic archaea live. Herein, a study of the substrate specificity of OASS from *Aeropyrum pernix* K1 with respect to *O*-acetyl-L-serine in L-cysteine synthesis is described. L-Azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine reacted with *A. pernix* OASS in a PLP-dependent manner. Sulfhydrylation reactions using these substrates reached a maximum in the pH range between 7.3 and 8.1. L-Azaserine and *O*-phospho-L-serine were found to be heat-stable substrates. The presence of $FeCl_3$ or $NiCl_2$ strongly inhibited the *O*-acetyl-L-serine sulfhydrylation reaction, whereas the *O*-phospho-L-serine sulfhydrylation reaction was only slightly inhibited. Kinetic analyses revealed that the *O*-phospho-L-serine sulfhydrylation reaction as well as the *O*-acetyl-L-serine sulfhydrylation reaction for *A. pernix* OASS followed a ping-pong bi-bi mechanism. In the case of the *O*-phospho-L-serine sulfhydrylation reaction at 85°C, the K_m values for *O*-phospho-L-serine and sulfide, and the rate constant were 250 mM, 12.5 mM, and 14 000 s^{-1}, respectively. The reactivity of *O*-phospho-L-serine in the L-cysteine synthetic reaction provides a key for understanding the biosynthesis of L-cysteine by hyperthermophilic archaea. This is the first report of an enzyme that catalyzes the *O*-phospho-L-serine sulfhydrylation reaction.**

Key words: *O*-Acetylserine sulfhydrylase; L-Cysteine biosynthesis; Archaeon; *O*-Acetyl-L-serine; *O*-Phospho-L-serine; *Aeropyrum pernix*

1. Introduction

Serine acetyltransferase (SAT) (EC 2.3.1.30) catalyzes the reaction of acetyl-CoA with L-serine to produce *O*-acetyl-L-serine and coenzyme A (Fig. 1, reaction 1). *O*-Acetylserine sulfhydrylase (OASS) (EC 2.5.1.47), a pyridoxal 5′-phosphate (PLP)-dependent enzyme, catalyzes the formation of L-cysteine and acetic acid from *O*-acetyl-L-serine and sulfide (Fig. 1, reaction 2). OASSs have been purified from several bacterial sources, including *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, as well as higher plants and their characteristics have been investigated [1–5]. SAT and OASS constitute the pathway of L-cysteine biosynthesis from L-serine in bacteria and higher plants (Fig. 1A) [1,2].

In mammals, on the other hand, L-cysteine is synthesized from the essential amino acid L-methionine (Fig. 1B) [6]. L-Methionine is converted to L-homocysteine by the actions of methionine adenosyltransferase (EC 2.5.1.6), various *S*-adenosylmethionine methyltransferases (EC 2.1.1.?), and *S*-adenosylhomocysteinase (EC 3.3.1.1) (Fig. 1, reactions 3–5). Cystathionine β-synthase (EC 4.2.1.22) catalyzes the condensation of L-homocysteine and L-serine to form L-cystathionine (Fig. 1, reaction 6). L-Cystathionine is then converted to L-cysteine by cystathionine γ-lyase (EC 4.4.1.1) (Fig. 1, reaction 7).

In the case of archaea, the L-cysteine biosynthetic pathway remains to be elucidated in spite of the efforts of several groups [7–11]. In the genome sequence of *Aeropyrum pernix*, the open reading frames (ORFs) with sequence similarities to OASS (ORF no. APE1586), methionine adenosyltransferase (APE1596), tRNA (guanine-N^2)-methyltransferase (EC 2.1.1.32) (APE0782), protein-L-isoaspartate(D-aspartate) *O*-methyltransferase (EC 2.1.1.77) (APE1011), uroporphyrin-III *C*-methyltransferase (EC 2.1.1.107) (APE0236), *S*-adenosylhomocysteinase (APE0624), cystathionine β-synthase (APE1223), and cystathionine γ-lyase (APE1226), which are involved in the known L-cysteine biosynthetic pathways, have been identified [12]. Genome-sequencing analyses of several other archaea reveal that archaea, including *Pyrobaculum aerophilum*, *Pyrococcus abyssi*, *Pyrococcus furiosus*, *Sulfolobus solfataricus*, *Sulfolobus tokodaii*, *Thermoplasma acidophilum*, and *Thermoplasma volcanium*, contain ORFs that have sequence similarities with OASS. However, genes that are homologous to SAT are not found in these archaea as well as *A. pernix*. Therefore, the physiological meaning of OASS in thermophilic archaea remains unclear.

A. pernix is a hyperthermophilic archaeon that grows optimally within the temperature range between 90 and 95°C [13]. *O*-Acetyl-L-serine is labile at these temperatures. These facts suggest that hyperthermophilic archaea are able to circumvent the thermal instability of *O*-acetyl-L-serine. We have recently identified *A. pernix* recombinant OASS using *E. coli* cells and characterized the enzyme [11]. The recombinant OASS exists as a dimer with a subunit molecular mass of 42 kDa and contains PLP. In the *O*-acetyl-L-serine sulfhydrylation reaction at 60°C for *A. pernix* OASS, the apparent K_m value for *O*-acetyl-L-serine, 28 mM, is higher and the rate constant, 202

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Abbreviations: SAT, serine acetyltransferase; OASS, *O*-acetylserine sulfhydrylase; PLP, pyridoxal 5′-phosphate; ORF, open reading frame

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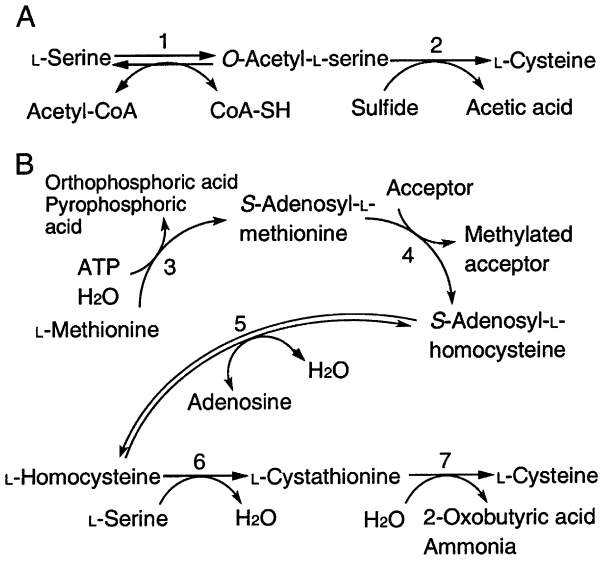

Fig. 1. Known L-cysteine biosynthetic pathways. A: Pathway in bacteria, such as *E. coli* and *S. enterica* serovar Typhimurium, and higher plants. B: Pathway in mammals. Enzymes are: 1, SAT; 2, OASS; 3, methionine adenosyltransferase; 4, various *S*-adenosylmethionine methyltransferases; 5, *S*-adenosylhomocysteinase; 6, cystathionine β-synthase; 7, cystathionine γ-lyase.

s^{-1}, is lower compared to those of other known OASSs. In this study, the substrate specificity of *A. pernix* OASS was examined with respect to *O*-acetyl-L-serine in the L-cysteine synthetic reaction, in an effort to understand the physiological significance of *A. pernix* OASS in *A. pernix* cells and how archaea are able to synthesize L-cysteine at high temperatures.

2. Materials and methods

2.1. Materials

O-Acetyl-L-serine hydrochloride, 3-chloro-L-alanine hydrochloride, *O*-phospho-L-serine, 3-chloro-D-alanine hydrochloride, 3-cyano-L-alanine, *O*-benzyl-L-serine, *O*-*tert*-butyl-L-serine, *O*-phospho-D-serine, *O*-succinyl-L-homoserine, and PLP were obtained from Sigma-Aldrich (St. Louis, MO, USA). L-Azaserine was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were of the highest quality available and were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Enzyme assay

EDTA and PLP were added to all assay solutions at final concentrations of 1 mM and 0.2 mM, respectively. A 0.27-ml portion of 0.111 M potassium phosphate buffer, pH 6.7, was first incubated for 3 min at the assay temperature. A 10-μl aliquot of an appropriate concentration of the enzyme solution, 10 μl of 0.6 M *O*-acetyl-L-serine solution, and 10 μl of 30 mM sodium sulfide solution were each added to the assay solution in that order at intervals of 1 min. The reaction mixture was then incubated for 4 min. 0.15 ml of 20% trichloroacetic acid was then added to the solution, and the amount of L-cysteine produced was determined using the method of Gaitonde [14]. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of L-cysteine per min.

2.3. Enzyme purification

The *A. pernix* OASS was purified from recombinant *E. coli* Rosetta(DE3) cells according to previously described methods [11]. Protein concentrations were measured using a Bio-Rad DC protein assay kit (Hercules, CA, USA) with bovine serum albumin as the standard protein. The purified enzyme solution was stored at −80°C at a final concentration of 14.8 mg/ml in 50 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM PLP, 2 mM EDTA, and 1 mM dithiothreitol. The final specific activity of the enzyme for the *O*-acetyl-L-serine sulfhydrylation reaction at 60°C was 54.5 U/mg, similar to that obtained previously (50.9 U/mg) [11].

2.4. Substrate specificity

In place of *O*-acetyl-L-serine, 10-μl portions of 0.6 M L-azaserine, 3-chloro-L-alanine, 3-chloro-D-alanine, 3-cyano-L-alanine, and L-homoserine, 30-μl portions of 0.2 M *O*-phospho-L-serine, *O*-phospho-D-serine, *O*-*tert*-butyl-L-serine, and *O*-succinyl-L-homoserine, or a 60-μl portion of 0.1 M *O*-benzyl-L-serine were added to assay solutions of *O*-acetyl-L-serine sulfhydrylation, as described above. The final volume of the reaction mixture was adjusted by subtracting deionized water from the assay solution. The reactions were carried out at 60°C as mentioned above. The enzyme concentration in all the reaction mixtures was 1.63 μg/ml. L- and D-cysteines were measured by the procedure of Gaitonde [14]. For the case of the reaction mixture containing L-homoserine or *O*-succinyl-L-homoserine, the reaction product of L-homocysteine was determined according to the method described by Becker et al. [3].

2.5. Spectroscopic analysis

The stock enzyme solution was dialyzed against 50 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM PLP. The enzyme solution was diluted with 50 mM potassium phosphate buffer, pH 7.5, to a final concentration of 0.34 mg/ml. Spectra were recorded for 0.5 ml of the enzyme solution at room temperature using a Shimadzu UV 1600 spectrophotometer (Kyoto, Japan). A 5-μl portion of each of the 0.1 M compounds, as depicted in Table 1 was added to the enzyme solution. A spectrum of the enzyme solution was then recorded. The spectrum of a solution without the enzyme was subtracted from that with the enzyme.

2.6. Effects of reaction pH and temperature on enzyme activities

The pH dependences of the sulfhydrylation reactions of L-azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine for *A. pernix* OASS were examined at 85°C. The assay mixtures were the same as those used for the substrate specificity experiment described above except that the final enzyme concentrations for the sulfhydrylation reactions of L-azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine were 0.46 μg/ml, 1.1 μg/ml, and 0.33 μg/ml, respectively and the following buffers were used: 0.1 M citrate-NaOH buffers, pH 4.6–6.3, 0.1 M potassium phosphate buffers, pH 5.9–8.4, and 0.1 M sodium carbonate buffers, pH 7.4–10.7. Thermal dependences of the activities of the sulfhydrylation reactions of L-azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine were examined by measuring the enzyme activities at 30, 40, 50, 60, 70, 80, and 90°C at the optimal pH of each of the reactions.

2.7. Substrate stability

A 0.27-ml portion of 0.111 M potassium phosphate buffer, pH 6.7, 7.8, or 7.5, and a 0.26-ml portion of 0.12 M potassium phosphate buffer, pH 7.6, were first incubated at 85°C for 3 min. A 10-μl portion of 0.6 M *O*-acetyl-L-serine, L-azaserine, or 3-chloro-L-alanine was added to potassium phosphate buffer at pH 6.7, 7.8, or 7.5, respectively. A 20-μl portion of 0.3 M *O*-phospho-L-serine was added to the potassium phosphate buffer, pH 7.6. These solutions were incubated for 2, 5, 10, and 20 min at 85°C. The reactions at 85°C involved the addition of enzyme and sodium sulfide solutions. The final enzyme

Table 1
Reactivities of *A. pernix* OASS with respect to various compounds

Substrate	Relative activity[a] (%)
O-Acetyl-L-serine	100
L-Azaserine	101
3-Chloro-L-alanine	173
O-Phospho-L-serine	219
3-Chloro-D-alanine	< 1
3-Cyano-L-alanine	< 1
O-Benzyl-L-serine	< 1
O-*tert*-Butyl-L-serine	< 1
O-Phospho-D-serine	< 1
O-Succinyl-L-homoserine	< 1
L-Homoserine	< 1

[a]Ratio of the activities of the sulfhydrylation of various compounds to that of *O*-acetyl-L-serine.

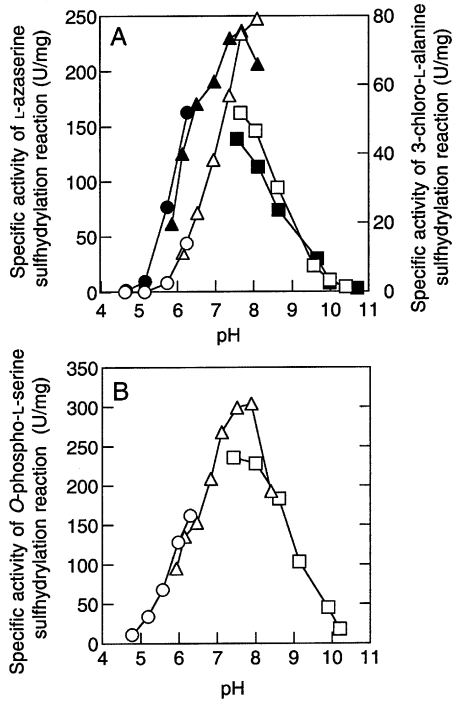

Fig. 2. pH dependences of *A. pernix* OASS activities at 85°C. A: Activities for the sulfhydrylation of L-azaserine (open symbols) and 3-chloro-L-alanine (closed symbols). B: Activity for *O*-phospho-L-serine sulfhydrylation. In panels A and B, circles, triangles, and squares indicate citrate-NaOH buffer, potassium phosphate buffer, and sodium carbonate buffer, respectively.

concentrations in the assay mixtures of sulfhydrylation reactions of *O*-acetyl-L-serine, L-azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine were 2.2 μg/ml, 0.54 μg/ml, 1.1 μg/ml, and 0.54 μg/ml, respectively. For control experiments, reactions were initiated by the addition of *O*-acetyl-L-serine, L-azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine that had been kept on ice. The ratios of the activities to those obtained from the control experiments were plotted against the time required to start the reactions after the addition of *O*-acetyl-L-serine, L-azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine to the reaction mixture.

2.8. Effects of various reagents on enzyme activities

The stock enzyme solution was dialyzed against 50 mM HEPES–NaOH buffer, pH 7.5, containing 0.2 mM PLP. The enzyme solutions were diluted with the same buffer to 0.12 mg/ml and 0.03 mg/ml for measurement of the activities of sulfhydrylation reactions of *O*-acetyl-L-serine and *O*-phospho-L-serine, respectively. Portions of 0.1 ml of the enzyme solutions were mixed with a 0.1-ml portion of 2 mM of each reagent dissolved in 50 mM HEPES–NaOH buffer, pH 7.5 ($CaCl_2$, $CdCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, $GdCl_3$, $HgCl_2$, KCl, $MgCl_2$, $MnCl_2$, NaCl, $NiCl_2$, $Pb(CH_3COO)_2$, $SrCl_2$, $ZnCl_2$, *N*-ethylmaleimide, *p*-chloromercuribenzoic acid, EDTA, dithiothreitol, and 2-mercaptoethanol). A 0.1-ml portion of 50 mM HEPES–NaOH buffer, pH 7.5, was used as a control instead of the reagent solution. The enzyme solutions were incubated at 25°C for 10 min and 10-μl portions were used to measure the relative enzyme activities. The reactions were performed at 60°C in the same manner as described above except that 0.1 M HEPES–NaOH buffers, pH 6.7 and 7.6, were used for the sulfhydrylation reactions of *O*-acetyl-L-serine and *O*-phospho-L-serine, respectively.

2.9. Kinetic analysis

Measurements of the initial rates of the *O*-acetyl-L-serine sulfhydrylation were performed at 60°C in 3 ml of 0.1 M potassium phosphate buffer, pH 6.7, with varying concentrations of *O*-acetyl-L-serine and sodium sulfide from 14 to 60 mM, and from 0.2 to 1.2 mM, respectively. The enzyme concentration was 0.81 μg/ml in the assay mixture.

For measurements of the initial rates of *O*-phospho-L-serine sulfhydrylation, reactions were performed at 60 and 85°C in 3 ml of 0.1 M sodium carbonate buffer, pH 7.6, by varying the concentrations of *O*-phospho-L-serine and sodium sulfide from 14 to 80 mM, and from 0.4 to 3 mM, respectively. The enzyme concentrations in the assay mixtures measured at 60 and 85°C were 0.38 μg/ml and 0.18 μg/ml, respectively. After 30, 60, 90, 120, and 150 s of incubation, 0.2-ml portions of the assay solutions were withdrawn and mixed with 0.1 ml of 20% trichloroacetic acid. The L-cysteine produced was determined by the method of Gaitonde [14]. Kinetic constants of the reactions catalyzed by *A. pernix* OASS were determined by using equation, which represents a ping-pong bi-bi mechanism [15].

3. Results and discussion

3.1. Substrate specificity

Using *A. pernix* OASS, various compounds were examined in place of *O*-acetyl-L-serine as a substrate for production of L-cysteine or L-homocysteine in the presence of sulfide. The results are summarized in Table 1. L-Azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine all served as substrates for the production of L-cysteine, and *O*-phospho-L-serine reacted the best at pH 6.7 and 60°C. It has been reported that OASSs are able to utilize L-azaserine [5,16] and 3-chloro-L-alanine [5,17,18] instead of *O*-acetyl-L-serine to synthesize L-cysteine. However, neither *S. enterica* serovar Typhimurium OASS-B [16] nor OASSs from an alkaliphilic bacterium [4] and *Thermus thermophilus* HB8 [5] are able to utilize *O*-phospho-L-serine. Thus the reactivity of *A. pernix* OASS for *O*-phospho-L-serine appears to be unique among the OASSs isolated to date. Inhibition studies of the *O*-acetyl-L-serine sulfhydrylation reaction were performed in the presence of some of the inactive compounds at a final concentration of 20 mM in the reaction mixture. As a result, 3-chloro-D-alanine, 3-cyano-L-alanine, *O*-benzyl-L-serine, and *O*-*tert*-butyl-L-serine caused 18, 42, 36, and 49% inhibition of the activity of *O*-acetyl-L-serine sulfhydrylation reaction, respectively. No inhibitory effect of *O*-phospho-D-serine was observed.

3.2. Spectroscopic analysis

The absorption spectrum of *A. pernix* OASS exhibits two peaks at 280 and 412 nm [11]. The peak at 412 nm indicates a Schiff base linkage between the active site of the enzyme and PLP [3]. The addition of *O*-acetyl-L-serine to an enzyme solution of *A. pernix* OASS at 1 mM led to a decrease in the

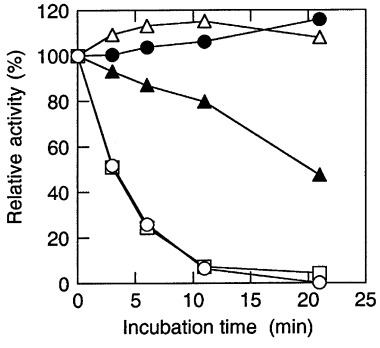

Fig. 3. Effect of incubation time before initiating the reactions for enzyme activities. *O*-Acetyl-L-serine (open circles), L-azaserine (open triangles), 3-chloro-L-alanine (open squares), and *O*-phospho-L-serine (closed circles) were incubated at 85°C. *O*-Acetyl-L-serine was also incubated at 60°C (closed triangles). The activities of the sulfhydrylation reactions were then measured as described in Section 2.

Table 2
Effect of various reagents on *A. pernix* OASS activity

Reagent	Relative activity[a] (%)	
	O-Acetyl-L-serine sulfhydrylation reaction	*O*-Phospho-L-serine sulfhydrylation reaction
None	100	100
$CaCl_2$	92	88
$CdCl_2$	85	74
$CoCl_2$	1.2	39
$CuCl_2$	1.3	21
$FeCl_2$	73	80
$FeCl_3$	3.9	75
$GdCl_3$	106	105
$HgCl_2$	20	1.7
KCl	92	82
$MgCl_2$	92	88
$MnCl_2$	94	90
NaCl	95	95
$NiCl_2$	<1	85
$Pb(CH_3COO)_2$	12	5.0
$SrCl_2$	100	94
$ZnCl_2$	75	77
N-Ethylmaleimide	96	87
p-Chloromercuribenzoic acid	97	88
EDTA	103	101
Dithiothreitol	97	111
2-Mercaptoethanol	94	97

[a]Ratio of the activities of the enzyme treated with different reagents to that obtained for a control experiment.

absorbance at 412 nm and an increase in the absorbance at both 330 and 470 nm. The appearance of the two peaks at 330 and 470 nm is the result of the formation of an α-aminoacrylate intermediate between *O*-acetyl-L-serine and PLP [3]. The addition of L-azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine gave the same spectrum as that observed for the enzyme with *O*-acetyl-L-serine. On the other hand, no spectral changes were detected by the addition of each of the compounds that did not act as a substrate for *A. pernix* OASS (Table 1). These results are consistent with the results obtained from the enzyme activity measurements described above.

3.3. Effects of pH and temperature on the enzyme activities

Results of pH dependences of the activities of the sulfhydrylation reactions of L-azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine at 85°C are depicted in Fig. 2. The sulfhydrylation reactions of L-azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine exhibited maxima in the pH ranges between 7.6 and 8.1, 7.3 and 7.6 (Fig. 2A), and 7.4 and 7.9 (Fig. 2B), respectively. The thermal dependences of the activities of the sulfhydrylation reactions for L-azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine were examined using 0.1 M potassium phosphate buffers at pH 7.8, 7.5, and 7.6, respectively. As a result, the activities for the sulfhydrylation reactions of L-azaserine and *O*-phospho-L-serine reached a maximum at 80 and 90°C, respectively. On the other hand, the activity for the 3-chloro-L-alanine sulfhydrylation reaction showed a maximum at 70°C and the activity measured at 90°C was half of that measured at 70°C.

3.4. Substrate stability

The stabilities of the substrates were also examined. Fig. 3 shows changes in the relative activities of the sulfhydrylation reactions measured using *O*-acetyl-L-serine, L-azaserine, 3-chloro-L-alanine, and *O*-phospho-L-serine as substrates. Not only *O*-acetyl-L-serine, but 3-chloro-L-alanine as well was very labile at 85°C compared to L-azaserine and *O*-phospho-L-serine. The effect of the incubation of a *O*-acetyl-L-serine solution at 60°C on enzyme activity at 60°C is also shown in Fig. 3. These data show that around 90% of the *O*-acetyl-L-serine was viable after 4 min, which corresponds to the reaction time used in the measurement of the enzyme activity.

3.5. Effects of various reagents on enzyme activities

The effects of reagents on the enzyme activity were examined. *A. pernix* OASS was incubated in the presence of various reagents for 10 min at 25°C and the activities of the sulfhydrylation reactions using *O*-acetyl-L-serine and *O*-phospho-L-serine were then measured. The results are shown in Table 2. Both enzyme activities were apparently inhibited by $CoCl_2$, $CuCl_2$, $HgCl_2$, and $Pb(CH_3COO)_2$, but the rates of inhibition were not identical. The activity of the *O*-phospho-L-serine sulfhydrylation reaction was slightly inhibited by $FeCl_3$ and

Table 3
Kinetic parameters for the sulfhydrylation of *O*-acetyl-L-serine and *O*-phospho-L-serine for *A. pernix* OASS

Temperature (°C)	*O*-Acetyl-L-serine sulfhydrylation reaction			*O*-Phospho-L-serine sulfhydrylation reaction		
	K_m for *O*-acetyl-L-serine (mM)	K_m for sulfide (mM)	k_{cat} (s^{-1})	K_m for *O*-phospho-L-serine (mM)	K_m for sulfide (mM)	k_{cat} (s^{-1})
60	21	0.25	156	200	5.0	3 050
85	nd[a]	nd	nd	250	12.5	14 000

[a]Not determined.

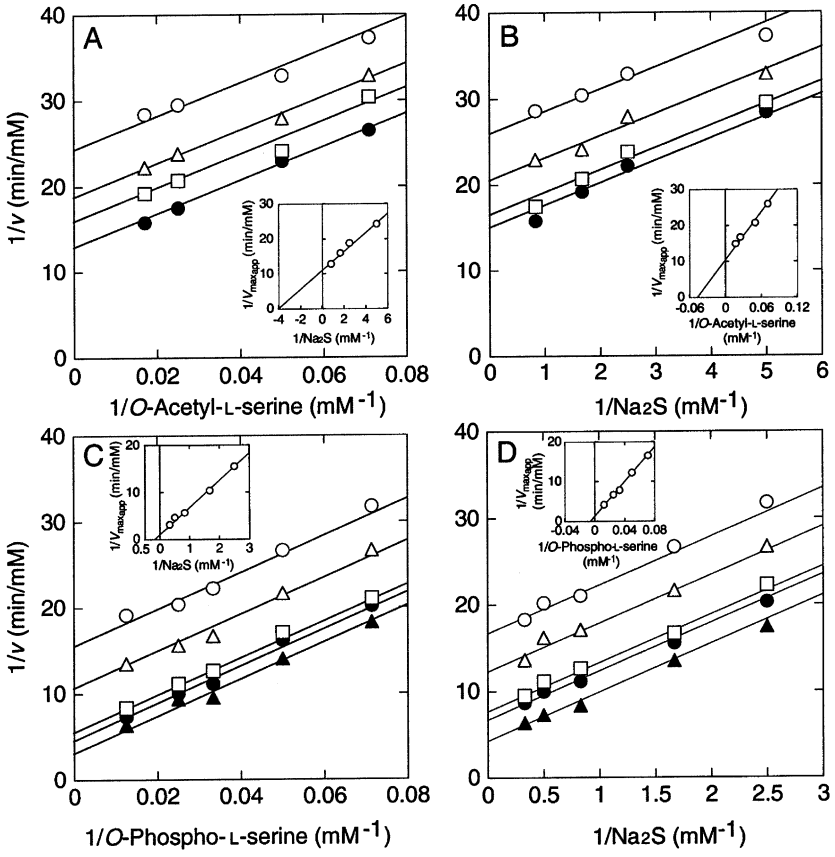

Fig. 4. Double reciprocal plots of the initial rates of the sulfhydrylation reactions of *O*-acetyl-L-serine (A,B) and *O*-phospho-L-serine (C,D) for *A. pernix* OASS at 60°C. A: Sodium sulfide concentrations of 0.2 mM (open circles), 0.4 mM (open triangles), 0.6 mM (open squares), and 1.2 mM (closed circles). B: *O*-Acetyl-L-serine concentrations of 14 mM (open circles), 20 mM (open triangles), 40 mM (open squares), and 60 mM (closed circles). C: Sodium sulfide concentrations of 0.4 mM (open circles), 0.6 mM (open triangles), 1.2 mM (open squares), 2 mM (closed circles), and 3 mM (closed triangles). D: *O*-Phospho-L-serine concentrations of 14 mM (open circles), 20 mM (open triangles), 30 mM (open squares), 40 mM (closed circles), and 80 mM (closed triangles). In insets, $1/V_{max\ app}$ values are plotted against substrate concentrations.

$NiCl_2$ although the activity of the *O*-acetyl-L-serine sulfhydrylation reaction was strongly inhibited by these reagents.

3.6. Kinetic analysis

Fig. 4A,B shows double reciprocal plots of the initial rates of the *O*-acetyl-L-serine sulfhydrylation reaction catalyzed by *A. pernix* OASS at 60°C against substrate concentrations, resulting in a series of parallel lines. Similar patterns were observed for the *O*-phospho-L-serine sulfhydrylation reactions at 60°C (Fig. 4C,D) as well as at 85°C (data not shown). These results indicate that the *O*-phospho-L-serine sulfhydrylation reaction as well as the *O*-acetyl-L-serine sulfhydrylation reaction for *A. pernix* OASS follows a ping-pong bi-bi mechanism as observed for other known OASSs [17,19]. In this mechanism, *O*-acetyl-L-serine or *O*-phospho-L-serine first binds to the enzyme to form the α-aminoacrylate intermediate. The sulfide then reacts with the α-aminoacrylate intermediate to generate L-cysteine. From replots of $1/V_{max\ app}$ against the substrate concentrations, the kinetic parameters for *A. pernix* OASS were determined and these values are summarized in Table 3. The K_m values for *O*-phospho-L-serine and sulfide for the *O*-phospho-L-serine sulfhydrylation reaction at 60°C were 10- and 20-fold higher than those for *O*-acetyl-L-serine and sulfide for the *O*-acetyl-L-serine sulfhydrylation reaction at 60°C, respectively. In addition, the rate constant for the *O*-phospho-L-serine sulfhydrylation reaction at 60°C were 20-fold higher than that for the *O*-acetyl-L-serine sulfhydrylation reaction at 60°C. In the *O*-phospho-L-serine sulfhydrylation reaction, the K_m values of *A. pernix* OASS for sulfide are higher compared with those of *E. coli* OASS-A (6 μM at 25°C) [19], OASS-A (6 μM at 25°C) and -B (10 μM at 25°C) from *S. enterica* serovar Typhimurium [17]. The rate constant for the *O*-phospho-L-serine sulfhydrylation reaction of *A. pernix* OASS at 85°C is significantly higher than that of *E. coli* OASS-A (2030 s^{-1} at 25°C) [19].

3.7. Conclusion

A. pernix OASS catalyzed a novel sulfhydrylation reaction using heat-stable *O*-phospho-L-serine as a substrate. Spectroscopic analyses were consistent with the formation of an α-aminoacrylate intermediate between *O*-phospho-L-serine and PLP as has been observed for the *A. pernix* OASS with *O*-acetyl-L-serine. When *A. pernix* OASS was treated with $FeCl_3$ or $NiCl_2$, the activity of the *O*-acetyl-L-serine sulfhydrylation reaction decreased significantly, whereas the activity of the *O*-phospho-L-serine sulfhydrylation reaction was only slightly decreased. This result implies that the region on *A. pernix* OASS responsible for the binding of the phosphate group of *O*-phospho-L-serine is different from that of the acetyl group of *O*-acetyl-L-serine. Kinetic analyses revealed that

A. pernix OASS had a high rate constant for the *O*-phospho-L-serine sulfhydrylation reaction compared to those for the *O*-acetyl-L-serine sulfhydrylation reactions for *A. pernix* OASS and *E. coli* OASS-A. *O*-Phospho-L-serine is widely distributed in nature and seems to be one of the best substrates of *A. pernix* OASS. The findings presented in this study provide a key to our understanding of the physiological meaning of *A. pernix* OASS as well as the pathway of L-cysteine biosynthesis in hyperthermophilic archaea.

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