Characterization of a Novel Thermostable *O*-Acetylserine Sulfhydrylase from *Aeropyrum pernix* K1

Koshiki Mino and Kazuhiko Ishikawa*

*Special Division for Human Life Technology, National Institute of Advanced Industrial Science
and Technology (AIST, Kansai), Ikeda, Osaka 563-8577, Japan*

An *O*-acetylserine sulfhydrylase (OASS) from the hyperthermophilic archaeon *Aeropyrum pernix* K1, which shares the pyridoxal 5′-phosphate binding motif with both OASS and cystathionine β-synthase (CBS), was cloned and expressed by using *Escherichia coli* Rosetta(DE3). The purified protein was a dimer and contained pyridoxal 5′-phosphate. It was shown to be an enzyme with CBS activity as well as OASS activity in vitro. The enzyme retained 90% of its activity after a 6-h incubation at 100°C. In the *O*-acetyl-L-serine sulfhydrylation reaction, it had a pH optimum of 6.7, apparent K_m values for *O*-acetyl-L-serine and sulfide of 28 and below 0.2 mM, respectively, and a rate constant of 202 s^{-1}. In the L-cystathionine synthetic reaction, it showed a broad pH optimum in the range of 8.1 to 8.8, apparent K_m values for L-serine and L-homocysteine of 8 and 0.51 mM, respectively, and a rate constant of 0.7 s^{-1}. *A. pernix* OASS has a high activity in the L-cysteine desulfurization reaction, which produces sulfide and *S*-(2,3-hydroxy-4-thiobutyl)-L-cysteine from L-cysteine and dithiothreitol.


In bacteria such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium and in plants, L-cysteine is synthesized from L-serine in two steps (16, 27). Serine acetyltransferase (SAT) (EC 2.3.1.30) converts L-serine and acetyl coenzyme A into *O*-acetyl-L-serine (OAS) and coenzyme A (equation 1):

$$\text{L-Serine} + \text{acetyl coenzyme A} \rightleftharpoons \text{OAS} + \text{coenzyme A} \quad (1)$$

O-Acetylserine sulfhydrylase (OASS) (EC 4.2.99.8) then catalyzes the formation of L-cysteine and acetic acid from OAS and sulfide (equation 2):

$$\text{OAS} + \text{sulfide} \rightarrow \text{L-cysteine} + \text{acetic acid} \quad (2)$$
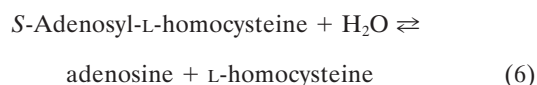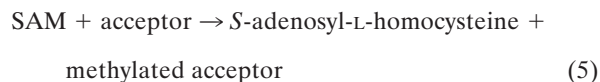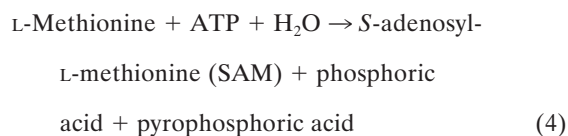
E. coli and *S. enterica* serovar Typhimurium produce two OASS isoenzymes (OASS-A and -B). OASS-B is preferentially used for the biosynthesis of L-cysteine during anaerobic growth conditions (11) and is able to utilize thiosulfate instead of sulfide to produce *S*-sulfo-L-cysteine (33) (equation 3):

$$\text{OAS} + \text{thiosulfate} \rightarrow \text{S-sulfo-L-cysteine} + \text{acetic acid} \quad (3)$$
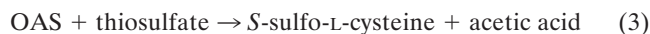
SATs and OASSs have been purified from several bacterial sources, such as *E. coli* and *S. enterica* serovar Typhimurium, as well as from higher plants, and their characteristics have been investigated (16, 27). OASS exists as a dimer and contains pyridoxal 5′-phosphate (PLP).
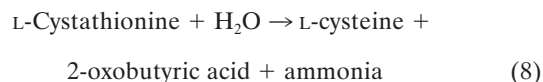
In mammals, on the other hand, L-cysteine is synthesized from L-methionine (15). L-Methionine is converted to L-homocysteine by the actions of methionine adenosyltransferase (EC 2.5.1.6) (equation 4), various *S*-adenosylmethionine methyltransferases (EC 2.1.1.?) (equation 5), and adenosylhomocysteinase (EC 3.3.1.1) (equation 6):

$$\text{L-Methionine} + \text{ATP} + \text{H}_2\text{O} \rightarrow \text{S-adenosyl-}$$
$$\text{L-methionine (SAM)} + \text{phosphoric}$$
$$\text{acid} + \text{pyrophosphoric acid} \quad (4)$$

$$\text{SAM} + \text{acceptor} \rightarrow \text{S-adenosyl-L-homocysteine} +$$
$$\text{methylated acceptor} \quad (5)$$

$$\text{S-Adenosyl-L-homocysteine} + \text{H}_2\text{O} \rightleftharpoons$$
$$\text{adenosine} + \text{L-homocysteine} \quad (6)$$

Cystathionine β-synthase (CBS) (EC 4.2.1.22) is a PLP-dependent enzyme that catalyzes the condensation of L-homocysteine and L-serine to form L-cystathionine (equation 7):

$$\text{L-Homocysteine} + \text{L-serine} \rightarrow \text{L-cystathionine} + \text{H}_2\text{O} \quad (7)$$
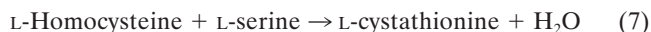
L-Cystathionine is then converted to L-cysteine by cystathionine γ-lyase (EC 4.4.1.1) (equation 8):

$$\text{L-Cystathionine} + \text{H}_2\text{O} \rightarrow \text{L-cysteine} +$$
$$\text{2-oxobutyric acid} + \text{ammonia} \quad (8)$$

CBSs from humans and *Saccharomyces cerevisiae* exist as tetramers and multimers (17, 24). Human CBS contains heme in addition to PLP (22). SAM activates CBSs from human and *S. cerevisiae* (5, 35). CBSs from *S. cerevisiae*, mammals, and *Trypanosoma cruzi* retain activity with respect to L-serine sulfhydration reaction (26, 34, 35, 37) (equation 9):

$$\text{L-Serine} + \text{sulfide} \rightarrow \text{L-cysteine} + \text{H}_2\text{O} \quad (9)$$
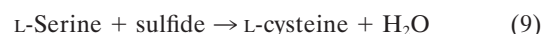
The three-dimensional structures of *S. enterica* serovar Typhimurium OASS-A and human CBS without the C-terminal amino acid sequence from position 414 to 551 indicate that the amino acid residues surrounding the PLP binding site are conserved between OASS and CBS (6, 29).

In *S. cerevisiae*, sulfur is first incorporated into L-homocys-

* Corresponding author. Mailing address: Special Division for Human Life Technology, National Institute of Advanced Industrial Science and Technology (AIST, Kansai), 1-8-31 Midorigaoka, Ikeda, Osaka 563-8577, Japan. Phone: 81-72-751-9526. Fax: 81-72-751-9628. E. mail: kazu-ishikawa@aist.go.jp.

teine by means of the enzyme coded for by *MET17/MET25*, which acts as both *O*-acetylhomoserine sulfhydrylase (EC 4.2.99.10) and OASS in vitro. L-Cysteine is then synthesized from L-homocysteine as well as from L-methionine via reverse a transsulfuration pathway (equations 4 to 8) (7, 36).

In the case of archaea, little is known concerning the pathway of L-cysteine biosynthesis despite the efforts of several investigators (3, 4, 25, 43). By a complementation analysis using a cysteine auxotrophic *E. coli* strain, the gene encoding OASS has been identified in the genome of *Methanosarcina barkeri* (25). The genes encoding SAT and OASS are adjacent to each other in the genomes of both *M. barkeri* (25) and *Methanosarcina thermophila* (3). OASS has been purified from cell extracts of *M. thermophila* and characterized (4). On the other hand, the results of genome sequencing analyses of archaea have shown that the genomes of *Aeropyrum pernix*, *Pyrobaculum aerophilum* (12), *Pyrococcus abyssi*, *Pyrococcus furiosus*, *Sulfolobus solfataricus*, *Sulfolobus tokodaii* (19), *Thermoplasma acidophilum*, and *Thermoplasma volcanium* (21) contain genes that have sequence similarity with OASS (25). Both SAT and OASS are required for the synthesis of L-cysteine, according to equations 1 and 2. However, no genes with a sequence similarity to SAT have yet been identified in these archaea.

A. pernix K1 is a hyperthermophilic archaeon that grows optimally in a temperature range of between 90 and 95°C (38). The putative product of the gene (open reading frame [ORF] APE1586) encoding OASS from *A. pernix* consists of 389 amino acids with a subunit molecular mass of 42 kDa (20). The physiological function of OASS in these archaea, including *A. pernix*, is presently unclear. We have recently attempted to clarify the physiological role of *A. pernix* OASS with respect to L-cysteine biosynthesis in *A. pernix* cells, and the results of this investigation are reported here.

MATERIALS AND METHODS

Materials. The sources of materials used in this study were as follows: L-cysteine, sodium sulfide, and sodium thiosulfate were from Wako Pure Chemical Industries (Osaka, Japan); HiTrap Q HP and Superdex 200 pg were from Amersham Biosciences (Freiburg, Germany); OAS, PLP, L-homocysteine thiolactone, and L-cystathionine were from Sigma-Aldrich (St. Louis, Mo.); and *S*-sulfo-L-cysteine was from Tocris Cookson (Bristol, United Kingdom). All other reagents were of reagent grade.

Cloning of the gene. The gene (ORF APE1586) encoding OASS was amplified from *A. pernix* genomic DNA by PCR with KOD DNA polymerase (Toyobo, Osaka, Japan) with 5′-GCCCTCGCCGACATAAGTGGTTACCTGGAC-3′ as the upper primer and 5′-TAT GGATCC TAGACCGAGTCTCCGGCTCCT-3′ as the lower primer. The *Bam*HI restriction enzyme site in the lower primer is indicated by an underline. To prepare the vector plasmid, pET-3d (Novagen, Madison, Wis.) was digested with *Nco*I and blunt ended with T4 DNA polymerase, followed by digestion with *Bam*HI. The PCR-amplified gene was digested with *Bam*HI and ligated into the vector plasmid. The resultant plasmid was designated pAPE1586. By using this method, the initial codon of the gene APE1586, GTG, was changed to ATG to permit efficient expression of the enzyme in *E. coli*.

Cultivation of the cells. The *E. coli* Rosetta(DE3) strain (Novagen) was transformed with plasmid pAPE1586. Cultivation of the cells was done by a modification of the method of Kery et al. (23). The transformant cells were grown in 1 liter of NZCYM medium (39) containing 100 μg of ampicillin per ml, 34 μg of chloramphenicol per ml, 0.001% pyridoxine, and 0.001% thiamine, with or without 0.3 mM 5-aminolevulinic acid (ALA), with reciprocal shaking at 100 rpm and 37°C. When the absorbance at 600 nm of the culture medium reached around 0.5, isopropyl-β-D-thiogalactopyranoside was added to the medium at a final concentration of 0.01 mM. The cells were allowed to grow for an additional 19 h and were then washed with 0.9% NaCl and frozen at −80°C.

Enzyme purification. The cells (5.9 g [wet weight]) were suspended in 150 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, 1 mM

dithiothreitol, 0.2 mM PLP, and 0.15 M NaCl (buffer A) and then sonically disrupted with a homogenizer (model 450D; Branson Ultrasonics Corp., Danbury, Conn.) at 90 W for 3 min on ice. Cell debris was removed by centrifugation (18,000 × *g* for 30 min at 4°C). PLP was added to the supernatant at a concentration of 1 mM, and the mixture was stirred for 30 min at 4°C. This solution was regarded as the crude extract. The crude extract was heated at 85°C for 20 min. The supernatant was recovered by centrifugation (18,000 × *g* for 20 min). The supernatant was fractionated with ammonium sulfate. The precipitate that formed at 50% ammonium sulfate saturation was recovered by centrifugation and dissolved in 7 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, 1 mM dithiothreitol, and 0.2 mM PLP (buffer B). This solution was dialyzed against buffer B at 4°C. The dialysate was placed on a HiTrap Q HP column (1.6 by 2.5 cm) equilibrated with buffer B and eluted by using a linear increase of NaCl concentration in buffer B from 0 to 1 M at a flow rate of 2 ml/min. The enzyme fractions that eluted between 0.3 and 0.4 M NaCl were collected and concentrated with a Centricon 10 filter (Amicon, Beverly, Mass.). The enzyme solution was placed on a Superdex 200 pg column (2.6 by 61 cm) and eluted with buffer A at a flow rate of 1.5 ml/min. The enzyme fractions were concentrated and dialyzed against buffer A at 4°C. The enzyme solution at a final concentration of 20 mg/ml was stored at −80°C.

Enzyme assays. PLP was added to all assay solutions at a concentration of 0.2 mM. For the OAS sulfhydrylation reaction, the assay mixture (0.3 ml) contained 100 mM potassium phosphate buffer (pH 6.7), 20 mM OAS, 1 mM sodium sulfide, 1 mM EDTA, and an enzyme concentration of 0.0027 mg/ml. For the *S*-sulfo-L-cysteine synthetic reaction, the assay mixture was the same as that used for the OAS sulfhydrylation reaction except that the reaction pH and enzyme concentration were pH 6.1 and 0.01 mg/ml, respectively, and sodium thiosulfate was used instead of sodium sulfide at a concentration of 20 mM. Reactions were carried out for 4 min and then stopped by the addition of 0.15 ml of 20% trichloroacetic acid. The solutions were centrifuged at 18,000 × *g* for 3 min. A 0.3-ml portion of the supernatant was used to determine the amount of L-cysteine or *S*-sulfo-L-cysteine by the method of Gaitonde (14). For the L-cystathionine synthetic reaction, the assay mixture (0.3 ml) consisted of 100 mM sodium carbonate buffer (pH 8.3), 30 mM L-serine, 0.7 mM L-homocysteine, and an enzyme concentration of 0.067 mg/ml. L-Homocysteine was prepared from L-homocysteine thiolactone as described by Drummond et al. (9). Reactions were carried out for 20 min. Thirty-microliter aliquots of solutions were withdrawn and mixed with 6 μl of 6 N HCl. The solutions were centrifuged at 18,000 × *g* for 3 min. A 20-μl portion of the supernatant was dried and treated with phenylisothiocyanate. The resulting phenylthiocarbamoyl-cystathionine was measured by using a Wakosil-PTC column (4 by 150 mm) (Wako) connected to a high-pressure liquid chromatography system (DP-8020; Tosoh, Tokyo, Japan) monitored at 254 nm and 40°C. Elution was done by using a linear increase of acetonitrile concentration from 6 to 46% in 60 mM sodium acetate buffer (pH 6.0) at a flow rate of 1 ml/min. Measurement of the activity of the L-serine sulfhydrylation reaction was done under conditions identical to that used for the OAS sulfhydrylation reaction described above except that OAS was replaced with 20 mM L-serine and that 100 mM sodium carbonate buffer (pH 8.5) and 0.1 mg of the enzyme per ml were used. The L-cysteine desulfurization reaction was done by a modification of the method of Flint et al. (13). The assay mixture (0.6 ml) consisted of 100 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS)–NaOH buffer (pH 8.5), 2.5 mM L-cysteine, 5 mM dithiothreitol, and an enzyme concentration of 0.25 μg/ml. Reactions were carried out for 15 min in a screw-cap vial to avoid leakage of the sulfide vapor produced. The amount of sulfide was measured by the method of Esaki et al. (10).

Reaction mixtures without enzyme were used as blanks. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the formation of 1 μmol of product per min.

Effect of temperature on enzyme activity and stability. The OASS and CBS activities for *A. pernix* OASS were measured in the assay solutions described above in a temperature range of 20 to 90°C. A 0.8-ml portion of the enzyme solution (0.3 mg/ml) was incubated at 4 or 100°C for 6 h in 50 mM potassium phosphate buffer (pH 6.1, 6.7, 7.5, or 8.5) containing 2 mM EDTA and 0.2 mM PLP to examine the thermal stability of the enzyme. The relative remaining *S*-sulfo-L-cysteine synthetic activities were measured at 85°C as described above. The activity prior to incubation was set to 100%.

Kinetic analyses. The *S*-sulfo-L-cysteine synthetic reaction, OAS sulfhydrylation reaction, and L-serine sulfhydrylation reaction were carried out at 85, 60, and 70°C, respectively, in 3 ml of the assay mixtures as described above. For the *S*-sulfo-L-cysteine synthetic reaction, the concentrations of OAS and sodium thiosulfate were varied from 5 to 30 mM. For the OAS and L-serine sulfhydrylation reactions, the concentrations of OAS and L-serine were changed from 5 to 100 mM and from 5 to 120 mM, respectively, at a fixed sodium sulfide concen-

tration of 2 mM. The concentration of sodium sulfide was varied between 0.2 and 2 mM with 20 mM OAS or with 100 mM L-serine. After 40, 80, 120, 160, and 200 s of incubation, 0.22-ml portions of the solutions were withdrawn and mixed with 0.11 ml of 20% trichloroacetic acid. The solutions were centrifuged, and 0.3-ml portions of the supernatants were then used to determine the amount of *S*-sulfo-L-cysteine and L-cysteine by the method of Gaitonde (14). A kinetic analysis of the L-cystathionine synthetic reaction was done at 85°C with the assay mixture described above. The concentrations of L-serine and L-homocysteine were changed from 2.5 to 30 mM at a fixed L-homocysteine concentration of 1.5 mM and from 0.15 to 1.5 mM at a 30 mM concentration of L-serine, respectively. A 30-μl portion of the reaction mixture was withdrawn after 5, 10, and 15 min of incubation. The L-cystathionine produced was determined as described above.

Kinetic constants for the *S*-sulfo-L-cysteine synthetic reaction were determined by using an equation that represents a ping-pong bi-bi mechanism (40). Kinetic constants for the L-cystathionine synthetic reaction and the OAS and L-serine sulfhydrylation reactions were determined by using the Michaelis-Menten equation (40).

Purification and identification of a coproduct in the L-cysteine desulfurization reaction. L-Cysteine (100 mM), dithiothreitol (500 mM), and *A. pernix* OASS (0.08 mg/ml) were allowed to react for 1.5 h at 85°C in 3 ml of 100 mM TAPS-NaOH buffer (pH 8.5). The reaction was stopped by the addition of 70 μl of 6 N HCl. A 35-μl portion of the solution was injected onto a YMC-Pack ODS-A column (20 by 150 mm) (YMC, Kyoto, Japan) connected to a high-pressure liquid chromatography system (Tosoh CCPS). Elution was accomplished with 0.5% acetonitrile containing 0.1% HCl at a flow rate of 7.9 ml/min and monitored by absorbance at 210 nm. This chromatography was repeated for a total of 10 times. Fractions (80 ml) containing a coproduct were evaporated and then lyophilized. The concentration of the product was determined by means of an amino acid analyzer (L-8500; Hitachi, Tokyo, Japan) with the averaged peak area of an amino acid standard solution (type H; Wako). Approximately 2 mg of the coproduct was subjected to one- and two-dimensional ^{1}H nuclear magnetic resonance (NMR) analyses. One-dimensional ^{1}H NMR spectra of L-cysteine and dithiothreitol were also measured as controls. For the matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOFMS) experiment, approximately 1 μg of the purified product was used.

Spectroscopic measurements. Absorption spectra of *A. pernix* OASS were obtained at room temperature with a UV-1600PC spectrophotometer (Shimadzu, Kyoto, Japan). The PLP content of the enzyme was determined by the procedure described by Kaplan and Flavin (18). NMR experiments were done with a JEOL-JNM A-500 spectrometer (500 MHz) (Oxford Instruments, Oxford, United Kingdom). All NMR spectra were measured in D$_2$O solution containing 3-(trimethylsilyl)-1-propanesulfonic acid (Merck, Darmstadt, Germany). MALDI-TOFMS experiments were done with a Voyager DE-STR mass spectrometer (PerSeptive Biosystems, Framingham, Mass.). α-Cyano-4-hydroxy cinnamic acid (Sigma-Aldrich) was used for calibrating the instrument, and 2,5-dihidroxybenzoic acid (Sigma-Aldrich) was used as the matrix.

Sedimentation equilibrium experiment. The molecular mass of the native enzyme was determined by using an Optima XL-A analytical ultracentrifuge (Beckman Instruments, Fullerton, Calif.) at 20°C.

Protein assay. Protein concentrations were determined with a DC protein assay kit (Bio-Rad, Hercules, Calif.) with bovine serum albumin as a standard.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (28) with a 12% gel. Phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa) (Amersham Biosciences LMW electrophoresis calibration kit) were used as markers. The protein bands were stained with Coomassie brilliant blue R-250.

N-terminal amino acid sequence analysis. Approximately 2 μg of the purified enzyme was developed by SDS-PAGE and then blotted electrically onto a Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad). The N-terminal amino acid sequence was determined by the Takara customer service center.

Multiple-sequence alignment. Amino acid sequences of the *A. pernix* proteins were obtained from the database of genomes analyzed at the National Institute of Technology and Evaluation (http://www.bio.nite.go.jp:8080/dogan/MicroTop). The amino acid sequences of known OASSs and CBSs were obtained from the EMBL database. The amino acid sequences were aligned by using the CLUSTAL W software program (42).

RESULTS

Multiple-sequence alignment. The deduced amino acid sequence of *A. pernix* OASS (ORF APE1586) had the following sequence identities: 31% with *S. enterica* serovar Typhimurium

OASS-B, 26% with *M. thermophila* OASS, 24% with *S. enterica* serovar Typhimurium OASS-A, 21% with spinach chloroplast OASS, 20% with human CBS, 15% with *S. cerevisiae* CBS, and 21% with an *A. pernix* protein (ORF APE1223) predicted to be CBS. Alignment of the amino acid sequences of these enzymes showed that the *A. pernix* OASS shares the PLP binding motif (6, 29) with both OASS and CBS (Fig. 1). The *A. pernix* OASS has an additional amino acid sequence of around 75 amino acid residues at the N terminus compared to *S. enterica* serovar Typhimurium OASS-A and -B, *M. thermophila* OASS, and *S. cerevisiae* CBS. The amino acid residues, Cys-52 and His-65, responsible for the binding of heme for human CBS (29) were not conserved in *A. pernix* OASS. The C-terminal region, corresponding to residues 414 to 551 of human CBS and residues 354 to 507 of *S. cerevisiae* CBS, which are required for both the binding of SAM and the dimer-dimer interaction (17, 24), was absent from the *A. pernix* OASS.

Purification and physical properties of the enzyme. *E. coli* Rosetta(DE3) carrying plasmid pAPE1586 was cultivated in medium without ALA, a precursor of heme. *A. pernix* OASS was purified by using the recombinant *E. coli* cells. Table 1 summarizes the purification of *A. pernix* OASS. Approximately 64 mg of *A. pernix* OASS was obtained in a yield of 29% on the basis of the activity of the OAS sulfhydrylation reaction. Purification yields calculated from activities of the *S*-sulfo-L-cysteine and L-cystathionine synthetic reactions and the L-cysteine desulfurization reaction were 30, 29, and 28%, respectively. The purified enzyme was homogeneous on an SDS-polyacrylamide gel, with an apparent subunit molecular mass of 42 kDa (Fig. 2). An N-terminal sequence analysis of the first seven amino acid residues of the purified protein showed that the residues were identical to those obtained from the *A. pernix* genome sequence database except that an initial Met residue was missing, probably due to the action of methionine aminopeptidase from *E. coli* (2). A sedimentation equilibrium experiment with *A. pernix* OASS indicated a molecular mass of 70,583 Da. Considering the subunit molecular mass of *A. pernix* OASS, it is likely that the *A. pernix* OASS exists as a dimer. The absorption spectrum of *A. pernix* OASS exhibited two peaks at 280 and 412 nm. The appearance of the peak at 412 nm indicates the formation of a Schiff base with PLP (30). The peak at 428 nm, typical for the heme observed for human CBS (22), was not present in the absorption spectrum of *A. pernix* OASS. *A. pernix* OASS was prepared from recombinant *E. coli* cells that had been cultivated in medium supplemented with ALA at a purification yield of 24%. The enzyme showed the same spectrum as that of the *A. pernix* OASS purified previously. The ratio of the absorbance at 280 nm to that at 412 nm for *A. pernix* OASS was 6.2. This is high compared with those of OASS-As from *E. coli* (4.0) (13) and *S. enterica* serovar Typhimurium (3.5) (1). The PLP content of *A. pernix* OASS was found to be 1.2 per enzyme subunit. Incubation of the purified *A. pernix* OASS in the presence of PLP at a concentration of 2 mM for 4 h at 4°C failed to increase the activity of the OAS sulfhydrylation reaction. These findings indicate that the purified *A. pernix* OASS is a holoenzyme.

Effect of pH and temperature on enzyme activity and stability. The pH dependencies of the enzyme activities observed for *A. pernix* OASS are shown in Fig. 3. The optimal pHs for the OAS sulfhydrylation and *S*-sulfo-L-cysteine synthesis reac-

```
OASS.Ap(APE1586)  2 ALADISGYLDVLDSVRGFSYLENAREVLRSGEARCLGNPRSEPEYVKALYVIGASRIPVGDGCSHTLEELGVFDISVPGEMVFPSPLDFFERGKPT 97
OASS-B.St         1                                                                            MNTLEQTIGNT 11
OASS.Mt           1                                                                            MGRIFSDITWTIGNT 15
OASS-A.St         2                                                                             SKIYEDNSLTIGHT 15
OASS.sp           1      MASLVNNAYAALRTSKLELREVKNLANFRVGPPSSLSCNNFKKVSSSPITCKAVSLSPPSTIEGLNIAEDVSQLIGKT 78
CBS.human         2    PSETPQAEVGPTGCPHRSGPHSAKGSLEKGSPEDKEAKEPLWIRPDAPSRCTWQLGRPASESPHHHTAPAKSPKILPDILKKIGDT 87
CBS.Sc            1                                                                       MTKSEQQADSRHNVIDLVGNT 21
CBS.Ap(APE1223)   1                                                                       MVGRVRKGLGSGVYGSILDLVGGT 24
                                                                                                    *

OASS.Ap(APE1586)  PLVRSRLQLPN----GVRVWLKLEWYNPFSLSVKDRPAVEIISRLS--RRVEKG-SLVADATSSNFGVALSAVARLYGYRARVYLPGAAEEFGKLL 186
OASS-B.St         PLVKLQRLGPDN----GSEIWVKLEGNNPA-GSVKDRAALSMIVEAEKRGEIKPG-DVLIEATSGNTGIALAMIAALKGYRMKLLMPDNMSQERRAA 102
OASS.Mt           PLVRLNRITKGL---HADVLAKVESFNPM-GSVKDRIGLAMIEEAEREGRIKEG-TTIIEATSGNTGIALACVCAARGYNLILVMPETMTVERRKL 106
OASS-A.St         PLVRLNRIGNG------RILAKVESRNPS-FSVKCRIGANMIWDAEKRGVLKPG-VELVEPTNGNTGIALAYVAAARGYKLTLTMPETMSIERRKL 103
OASS.sp           PMVYLNNVSKGS---VANIAAKLESMEPC-CSVKDRIGYSMIDDAEQKGVITPGKTTLVEPTSGNTGIGLAFIAAARGYKITLTMPASMSMERRVI 170
CBS.human         PMVRINKIGKKFG-LKCELLAKCEFFNAG-GSVKDRISLRMIEDAERDGTLKPG-DTIIEPTSGNTGIGLALAAAVRGYRCIIVMPEKMSSEKVDV 180
CBS.Sc            PLIALKKLPKALG-IKPQIYAKLELYNPG-GSIKDRIAKSMVEEAEASGRIHPSRSTLIEPTSGNTGIGLALIGAIKGYRTIITLPEKMSNEKVSV 115
CBS.Ap(APE1223)   PLLRLGRVERFFRLEGVELYGKLEMFNPG-GSVKDRIGLYMLMDARSRGLVEEG-AVIIEPTAGNTGVGLALAAIHYGFRLVAVMPSKMSVEKELI 118
                  *..              .  * *  ..   *.* *  ..           .  .  .  ..* .* *..*.    *..     .*

OASS.Ap(APE1586)  PRLLGAQVIVDPEAPSTVH---------LLPRVMKDSKNEG-------------------------------FVHVNQFYNDANFEAHMR 236
OASS-B.St         MRAYGAELILVTKEQGMEG---------ARDLALAMSERGE-------------------------------GKLLDQFNNPDNPYAHYT 152
OASS.Mt           LKALGAELVLTSGAEGMRG---------AVNKAEQMAADDSA-------------------------------LYFIPQQFQNPANPEIHRR 158
OASS-A.St         LKALGANLVLTEGAKGMKG---------AIQKAEEIVASDPQ-------------------------------KYLLLQQFSNPANPEIHEK 155
OASS.sp           LKAFGAELVLTDPAKGMKG---------AVEKAEEILKKTP-------------------------------DSYMLQQFDNPANPKIHYE 221
CBS.human         LRALGAEIVRTPTNARFDSPES------HVGVAWRLKNEIPN-------------------------------SHILDQYRNASNPLAHYD 234
CBS.Sc            LKALGAEIIRTPTAAAWDSPES------HIGVAKKLEKEIPG-------------------------------AVILDQYNNMMNPEAHYF 169
CBS.Ap(APE1223)   LRAYGAYVIRTPTAVPPESPLSYYRVAEAVRNLLWMIGGRPGGGEVRKVVEKVQTLVREERVEDLRSILEASVEPTPYAYIPNQYSNPANPRAHEE 214
                  .  **  ..            .   **  ..                                          .*.  *   *   *

OASS.Ap(APE1586)  GTAREIFVQSRRGG--LALRGVAGSLGTSGHMSAAAFYLQS--VDPSIRAVLVQPAQGDSIPGIR---------------R--VETGMLWINMLD 310
OASS-B.St         TTGPEIWRQTSGR-----ITHFVSSMGTTGTITGVSRFLR--EQEKPVTIVGLQPEEGSSIPGIR----------------RWPAEYMPGIFNAS 224
OASS.Mt           TTAAEEIWRDTDGK-----ADVIVAGVGTGGTITGIASAIK--KRRPSFKAIAVEPAESPVLSGG----------SPGPHRIQGIGAGFVPDVLQLE 237
OASS-A.St         TTGPEIWEDTDGQ-----VDVFISGVGTGGTLTGVTRYIKGTKGKTDLITVAVEPTDSPVIAQAL----AGEEIKPGPHKIQGIGAGFIPGNLDLK 242
OASS.sp           TTGPEIWEDTKGK-----VDIFVAGIGTGGTLGVGRYLK--ERNPGVQVIGIEPTESNILSGG----------KPGPHKIQGLGAGFVPSNLDLG 300
CBS.human         TTADEILQQCDGK-----LDMLVASVGTGGTITGIARKLK--EKCPGCRIIGVDPEGSILAEPEELN----QTEQTTYEVEGIGYDFIPTVLDRT 318
CBS.Sc            GTGREIQRQLEDLNLFDNLRAVVAGAGTGTISGISKYLK--EQNDKIQIVGADPFGSILAQPENLN-----KTDITDYKVEGIGYDFVPQVLDRK 258
CBS.Ap(APE1223)   TTGREIWMQTGGR-----VDVVYAGMGTRGTITGVARYLK--RAKPSVRIVGVDPEGSIYSLVKKGMSPHEAAKHARPYKVEGIGEDFIPSTIDLS 303
                  *. **   .        ... ** * ...     ..       . .*  .                          .

OASS.Ap(APE1586)  ISYTLAEVTLEEAMEAVVEVARSDGLVIGPSGGAVKALAKKAAEG--DLEPGDYVVVVPDTGFKYLSLVQNALEGAGDSV           389
OASS-B.St         LVDEVLDIHQNDAENTMRELAVREGIFCGVSSGGAVAGALRVAR----ATPGAIVVAIICDRGDRYLSTGVFGEEHFSQGAGI       303
OASS.Mt           LIDEIIKVTGDEAAATARRLAKEEGILAGISSGAATWAALQVASRE--EMRGKTIVVILPDTGERYLSTDLFD               308
OASS-A.St         LIDKVVGITNEEAISTARRLMEEEVFLAGISSGAAVAAALKLQEDE--SFTNKNIVVILPSSGERYLSTALFADLFTEKELQQ      323
OASS.sp           VMDEVIEVSSEEAVEMAKQLAMKEGLLVGISSGAAAAAAVRIGKRP--ENAGKLIAVVFPSFGERYLSSILFQSIREECENMKPE    383
CBS.human         VVDKWFKSNDEEAFTFARMLIAQEGLLCGGSAGSTVAVAVKAAQ-E--LQEGQRCVVILPDSVRNYMTKFLSDRWMLQKGFLKEEDLTEKKPWWWH  411
CBS.Sc            LIDVWYKTDDKPSFKYARQLISNEGVLVGGSSGSAPTAVVKYCEDHPELTEDDVIVAIFPDSIRSYLTKFVDDEWLKKNNLWDDDVLARFDSSKLE   354
CBS.Ap(APE1223)   LVDEVVVVTDQQAFSMARMLARLEGVLAGGSSGAALYAAVKHIKT---RRVRGVAVVILPDTGRNYINKLYNDRWMGENGFSTRDEEVLGELL      393
                  .        .  .     .   . .. .* *. *..  .          .... .     *..
```

FIG. 1. Multiple-sequence alignment of *A. pernix* OASS with various OASSs and CBSs. Abbreviations: OASS.Ap(APE1586), an *A. pernix* protein (ORF APE1586) predicted to be OASS; OASS-B.St, *S. enterica* serovar Typhimurium OASS-B (EMBL accession no. P29848); OASS.Mt, *M. thermophila* OASS (AAG01804); OASS-A.St, *S. enterica* serovar Typhimurium OASS-A (P12674); OASS.sp, spinach chloroplast OASS (P32260); CBS.human, N-terminal region of human CBS (P35520); CBS.Sc, N-terminal region of *S. cerevisiae* CBS (P32582); CBS.Ap(APE1223), an *A. pernix* protein (ORF APE1223) predicted to be CBS. Hyphens indicate gaps introduced for optimal alignment. Asterisks and dots indicate identical and similar amino acids, respectively. Boldface letters depict the PLP binding motif common to both OASS and CBS.

tions were in a slightly acidic region (Fig. 3A), whereas those for the L-cystathionine synthesis, L-serine sulfhydrylation, and L-cysteine desulfurization reactions were in the alkaline region (Fig. 3B and C). The activity of the L-cystathionine synthetic

TABLE 1. Purification of *A. pernix* OASS

Purification step	Vol (ml)	Total activity[a] (U)	Total protein (mg)	Sp act[a] (U/mg)	Yield (%)
Crude extract	150	11,070	930	11.9	100
Heat treatment	142	7,210	554	13.0	65
Ammonium sulfate	11	6,220	189	32.9	56
HiTrap Q HP	3.9	5,760	164	35.1	52
Superdex 200 pg	3.2	3,250	63.8	50.9	29

[a] The activity of the OAS sulfhydrylation reaction was measured at 60°C as described in the text.

reaction measured at pH 8.3 was unaffected by the addition of SAM at a concentration of 0.5 mM to the assay solution.

A plot of the observed activity for the *S*-sulfo-L-cysteine synthetic reaction against temperature showed that the *A. pernix* OASS exhibited the highest activity at or above 90°C. On the other hand, the activity of the OAS sulfhydrylation reaction showed maxima at both 70 and 80°C. In the case of the L-cystathionine synthetic reaction and L-serine sulfhydrylation reaction, both activities had a maximum at 80°C. The *A. pernix* OASS retained around 90% of its activity after the enzyme solutions had been incubated for 6 h at 100°C at pH 6.1 and 6.7. The relative remaining activities of the enzyme solutions incubated at pH 7.5 and 8.5 were 56 and 11%, respectively. No loss of enzyme activities was observed for enzyme solutions stored at 4°C.
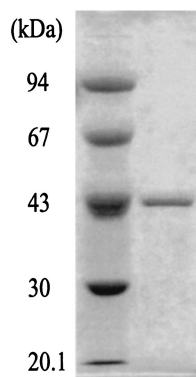
FIG. 2. SDS-PAGE of the purified *A. pernix* OASS. Marker proteins with the indicated molecular masses are shown in the left lane, and the purified *A. pernix* OASS (1 μg) is in the right lane.

Kinetic analyses. Double-reciprocal plots of the initial velocity for the *S*-sulfo-L-cysteine synthetic reaction were represented as a series of parallel lines. This indicates that the *S*-sulfo-L-cysteine synthetic reaction followed a typical ping-pong bi-bi mechanism, which was identical to the kinetic mechanism of the OAS sulfhydrylation reaction for OASS-As from *E. coli* and *S. enterica* serovar Typhimurium (31, 41). In this mechanism, OAS first binds to the enzyme to form a Schiff base with PLP, releasing acetic acid. Second, thiosulfate attacks the α-aminoacrylate intermediate of the enzyme to form *S*-sulfo-L-cysteine. At low concentrations of thiosulfate, a slight inhibition by OAS was observed. The kinetic parameters for the reactions of *S*-sulfo-L-cysteine synthesis and OAS sulfhydrylation for *A. pernix* OASS are summarized in Table 2. The kinetic parameters for the reactions of L-cystathionine synthesis and L-serine sulfhydrylation are shown in Table 3. The kinetic constants obtained for known OASSs and CBSs are also shown in Tables 2 and 3 for comparison. It can be seen that the k_{cat} value for the OAS sulfhydrylation reaction for *A. pernix* OASS differed by two orders of magnitude from those for the L-cystathionine synthetic reaction and L-serine sulfhydrylation reaction for *A. pernix* OASS. The apparent K_m values for sulfide for *A. pernix* OASS were the lowest among those for the substrates in the reactions catalyzed by *A. pernix* OASS.

Identification of a coproduct of the L-cysteine desulfurization reaction. The L-cysteine desulfurization reaction for *A. pernix* OASS was dependent on the concentration of dithiothreitol in the reaction mixture. The activity reached a maximum at a dithiothreitol concentration of 40 mM when L-cysteine was present at a concentration of 2.5 mM in the assay solution. It has been reported that *E. coli* OASS-A and -B catalyze the formation of sulfide from L-cysteine in the presence of dithiothreitol (13). Flint et al. proposed a reaction mechanism for L-cysteine desulfurization for *E. coli* OASS-A and -B (13). In this mechanism L-cysteine first binds to the enzyme to form a Schiff base with PLP. Sulfide is then released from the enzyme-bound L-cysteine. Dithiothreitol then attacks the α-aminoacrylate intermediate to form *S*-(2,3-hydroxy-4-thiobutyl)-L-cysteine. The second product has never been identified. The coproduct of the L-cysteine desulfurization reaction for *A. pernix* OASS was purified and subjected to NMR and MALDI-TOFMS analyses. Figure 4A and B show one-dimen-
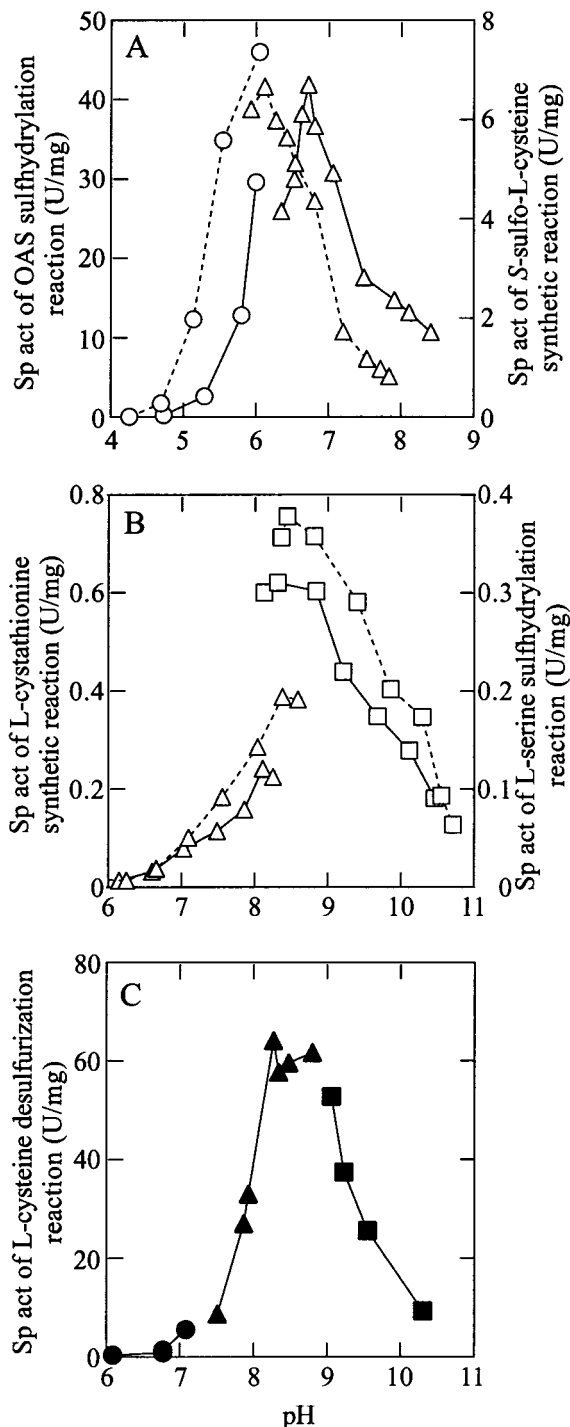

FIG. 3. pH Dependencies of the enzyme activities for *A. pernix* OASS at 85°C. (A) Specific activities for the OAS sulfhydrylation reaction (equation 2) and *S*-sulfo-L-cysteine synthetic reaction (equation 3) are shown by solid and dashed lines, respectively. (B) Specific activities for the L-cystathionine synthetic reaction (equation 7) and L-serine sulfhydrylation reaction (equation 9) are depicted by solid and dashed lines, respectively. (C) Specific activity for the L-cysteine desulfurization reaction. In panels A and B, citrate-NaOH buffer (○), potassium phosphate buffer (△), and sodium carbonate buffer (□) were used. In panel C, 2-morpholinoethanesulfonic acid-NaOH buffer (●), TAPS-NaOH buffer (▲), and cyclohexylaminopropanesulfonic acid-NaOH buffer (■) were used.

TABLE 2. Comparison of kinetic parameters of the OAS sulfhydrylation reaction and S-sulfo-L-cysteine synthetic reaction for *A. pernix* OASS and for known OASSs and CBSs

Enzyme (reference)	OAS sulfhydrylation			S-Sulfo-L-cysteine synthesis		
	K_m for OAS (mM)	K_m for sulfide (mM)	k_{cat} (s^{-1})	K_m for OAS (mM)	K_m for thiosulfate (mM)	k_{cat} (s^{-1})
A. pernix OASS	28	<0.2	202	13	21	24
S. enterica serovar Typhimurium OASS-B (33)				9	2.7	341
E. coli OASS-A (31)	4.8	0.006	2,030			
Spinach chloroplast OASS (8)	1.3	0.25				
T. thermophilus OASS (32)	4.8	0.05	493			
S. cerevisiae CBS (35)	1.27	16.6	19.2			
T. cruzi CBS (34)	4.9	4.1	12.5			

sional ^{1}H NMR spectra of the substrates L-cysteine and dithiothreitol as controls, respectively. The resonances centered around 4.22 and 3.10 ppm arise from the C-1 and C-2 protons of L-cysteine, respectively (Fig. 4A). The resonances centered around 3.70 and 2.67 ppm are assigned to the C-2 and C-1 protons of dithiothreitol (Fig. 4B). The relative ratios for the areas under each envelope were 1.0 and 2.2, respectively, for L-cysteine and 1.0 and 2.0, respectively, for dithiothreitol. Figure 4C shows a one-dimensional ^{1}H NMR spectrum of the coproduct purified from the reaction mixture of the L-cysteine desulfurization for *A. pernix* OASS. Considering the NMR spectra of L-cysteine and dithiothreitol (Fig. 4A and B) and the two-dimensional NMR spectrum of the purified coproduct (data not shown), six envelopes of resonances centered around 3.98, 3.88, 3.70, 3.13, 2.83, and 2.73 ppm were identified as the protons belonging to C-1, C-4, C-5, C-2, C-3, and C-6, respectively, of S-(2,3-hydroxy-4-thiobutyl)-L-cysteine (Fig. 4C). The relative ratios for the areas under each envelope were 1.0, 0.88, 0.81, 0.80, 1.5, and 3.6 (sum of the envelopes of the resonances centered around 2.83 and 2.73 ppm), respectively. MALDI-TOFMS data for the purified coproduct showed a single peak corresponding to a mass of 241.98. This is in good agreement with the mass of the protonated molecular ion for S-(2,3-hydroxy-4-thiobutyl)-L-cysteine, 242.052. These results indicate that the coproduct formed with dithiothreitol in the L-cysteine desulfurization reaction for *A. pernix* OASS was, in fact, S-(2,3-hydroxy-4-thiobutyl)-L-cysteine.

Substrate specificities for the L-cysteine desulfurization reaction. Substrate specificities for the L-cysteine desulfurization reaction were examined at 85°C in the assay mixture described in Materials and Methods, except that various compounds were added in the place of L-cysteine or dithiothreitol. The L-cysteine desulfurization reaction did not proceed when L-cysteine was replaced with D-cysteine or L-homocysteine. On

the other hand, 2-mercaptoethanol, ethanethiol, L-homocysteine, D-cysteine, DL-homocysteine, pyrazole, and glutathione were utilized as nucleophiles instead of dithiothreitol. Their respective relative activities against dithiothreitol were 121, 114, 35, 30, 26, 23, and 8.2%.

DISCUSSION

Little is known concerning the pathway of L-cysteine biosynthesis in archaea, despite several investigations (3, 4, 25, 43). In this study, *A. pernix* OASS was purified from recombinant *E. coli* cells and characterized with respect to its physical and catalytic properties.

The purified *A. pernix* OASS existed as a dimer with one molecule of PLP per subunit of the enzyme, consistent with other OASSs (1, 8, 13, 32). Unlike human CBS, the *A. pernix* OASS lacked heme and existed as dimmer, and the CBS activity for *A. pernix* OASS was unaffected by the addition of SAM. The pH optimum for the OAS sulfhydrylation reaction for *A. pernix* OASS, 6.7 (Fig. 3A), was compatible with that of *Thermus thermophilus* OASS (pH 7) (32) but was in the acidic region compared to those of *E. coli* OASS-A (pH 8 to 9.5) (31) and spinach chloroplast OASS (pH 7.5 to 8.5) (8). The pH optimum for the L-cystathionine synthetic reaction for *A. pernix* OASS, 8.1 to 8.8 (Fig. 3B), was consistent with those for CBSs from humans (pH 8.4 to 9.0) (26) and *S. cerevisiae* (pH ~8.5) (17). The difference between the pH optimum for the OAS sulfhydrylation reaction and that for the L-cystathionine synthetic reaction for *A. pernix* OASS suggests that the catalytic mechanisms of the reactions are different. As is typical of enzymes of hyperthermophiles, the *A. pernix* OASS was most active at or above 90°C for the S-sulfo-L-cysteine synthetic reaction and was highly stable during incubation at 100°C.

The kinetic parameters for the reactions catalyzed by *A.*

TABLE 3. Comparison of kinetic parameters of the L-cystathionine synthetic reaction and L-serine sulfhydrylation reaction for *A. pernix* OASS and for known CBSs

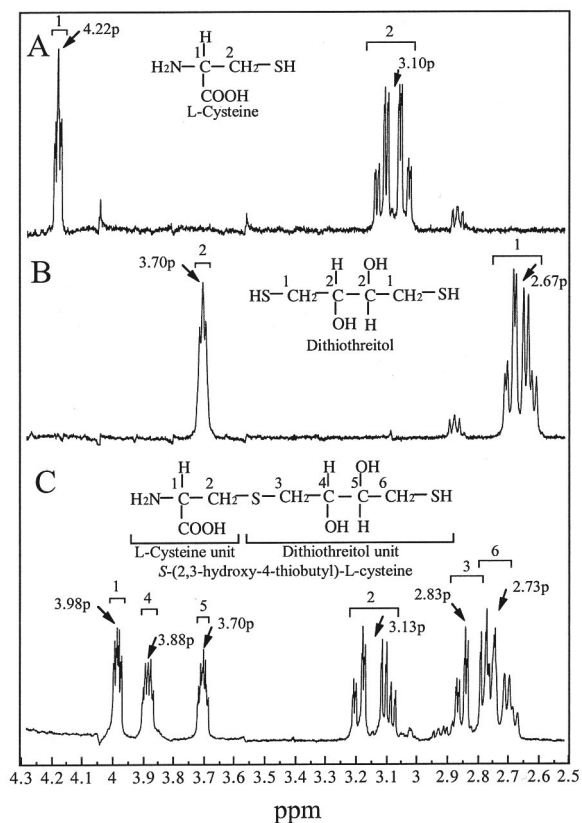| Enzyme (reference)	L-Cystathionine synthesis			L-Serine sulfhydrylation		
	K_m for L-serine (mM)	K_m for L-homocysteine (mM)	k_{cat} (s^{-1})	K_m for L-serine (mM)	K_m for sulfide (mM)	k_{cat} (s^{-1})
A. pernix OASS	8.0	0.51	0.7	31	<0.2	0.56
Human CBS (24)	3.0	1.5	6.0			
S. cerevisiae CBS (35)	2.19	2.25	7.97	4.42	16.8	20.7
T. cruzi CBS (34)	1.0	0.9	21	1.1	3.1	12.8

FIG. 4. ^{1}H NMR spectra. (A) L-Cysteine. (B) Dithiothreitol. (C) The coproduct purified from the reaction mixture of L-cysteine desulfurization for *A. pernix* OASS.

pernix OASS were determined and compared with those for known OASSs and CBSs. The k_{cat} values for the OAS sulfhydrylation and S-sulfo-L-cysteine synthetic reactions for *A. pernix* OASS were lower than those for known OASSs but higher than those for the OAS sulfhydrylation reaction for known CBSs (Table 2). The k_{cat} values for the L-cystathionine synthetic reaction and L-serine sulfhydrylation reaction for *A. pernix* OASS were lower than those for known CBSs (Table 3). For the OAS sulfhydrylation reaction, the apparent K_m value for sulfide for *A. pernix* OASS seems to be compatible with those for known OASSs, but that for OAS for *A. pernix* OASS was high (Table 2). For the S-sulfo-L-cysteine synthetic reaction, the *A. pernix* OASS showed a higher K_m value for thiosulfate than that for *S. enterica* serovar Typhimurium OASS-B, although the deduced amino acid sequence of *A. pernix* OASS had the highest identity with that of *S. enterica* serovar Typhimurium OASS-B. For both the L-cystathionine synthetic reaction and the L-serine sulfhydrylation reaction, the apparent K_m values for L-serine for *A. pernix* OASS were higher than those for known CBSs, whereas that for sulfide for *A. pernix* OASS was lower than those for the CBSs (Table 3). The fact that *A. pernix* OASS had a high affinity for sulfide suggests that *A. pernix* OASS plays a role in the sulfur assimilation pathway. In the genome sequence of *A. pernix*, hypothetical gene products with sequence similarities to methionine adenosyltransferase (ORF APE1596) (equation 4), adenosylhomocysteinase

(APE0624) (equation 6), CBS (APE1223) (equation 7), and cystathionine γ-lyase (APE1226) (equation 8), which are involved in L-cysteine biosynthesis in mammals, have been identified (20). The issues of whether these ORFs have the expected enzyme activities and whether SAT exists in *A. pernix* cells have not been investigated. Further analyses of these points will be necessary to clarify the pathway of L-cysteine biosynthesis in archaea, including *A. pernix*, as well as the physiological meaning of *A. pernix* OASS.

The *A. pernix* OASS used various thiol compounds and pyrazole in lieu of dithiothreitol to release sulfide from L-cysteine. It has been reported that *E. coli* OASS-A utilizes several different nucleophiles instead of dithiothreitol in the L-cysteine desulfurization reaction (13). Flint et al. identified the nonproteineous amino acids formed with the nucleophiles homocysteine, cysteine, azide, and 2-mercaptoethanol for *E. coli* OASS-A by amino acid analysis and mass spectral analysis (13). They also predicted that a nonproteineous amino acid formed with dithiothreitol is S-(2,3-thiohydroxybutyl)-L-cysteine according to the proposed reaction mechanism, but they did not identify it. We first identified the coproduct formed with dithiothreitol as S-(2,3-thiohydroxybutyl)-L-cysteine in the L-cysteine desulfurization reaction for *A. pernix* OASS by NMR and MALDI-TOFMS analyses. These results suggest that the L-cysteine desulfurization reaction for *A. pernix* OASS proceeds in the same manner as that for *E. coli* OASS-A. The rate constant for the L-cysteine desulfurization reaction for *A. pernix* OASS was 84 s^{-1} when the assay was carried out at 2.5 mM L-cysteine and 5 mM dithiothreitol at pH 8.5. This is higher than the rate constants for *E. coli* OASS-A (0.005 s^{-1}) and OASS-B (0.015 s^{-1}) (13).

It has been speculated that OASS and CBS would have diverged from an ancestral enzyme (35). However, the issue of how an ancestral enzyme might have evolved or diverged into the individual enzymes involved in the different pathways of L-cysteine biosynthesis remains unclear. This study revealed that the *A. pernix* OASS had CBS activity as well as OASS activity in vitro. There are, to our knowledge, only two enzymes that exhibit the activities of both OASS and CBS; these are *S. cerevisiae* CBS (35) and *T. cruzi* CBS (34). Based on the findings obtained in this study, it appears that the *A. pernix* OASS retains the properties of an ancestral enzyme that would have diverged later into individual enzymes with narrower substrate specificities and higher rate constants and by reducing the catalytic efficiencies of other reactions. Information concerning the three-dimensional structure of *A. pernix* OASS might be helpful in understanding how an ancestral enzyme might have diverged into the individual enzymes involved in the different pathways of L-cysteine biosynthesis.

REFERENCES

1. **Becker, M. A., N. M. Kredich, and G. M. Tomkins.** 1969. The purification and characterization of *O*-acetylserine sulfhydrylase-A from *Salmonella typhimurium*. J. Biol. Chem. **244:**2418–2427.

2. **Ben-Bassat, A., K. Bauer, S.-Y. Chang, K. Myambo, A. Boosman, and S. Chang.** 1987. Processing of the initiation methionine from proteins: properties of the *Escherichia coli* methionine aminopeptidase and its gene structure. J. Bacteriol. **169:**751–757.

3. **Borup, B., and J. G. Ferry.** 2000. Cysteine biosynthesis in the Archaea: *Methanosarcina thermophila* utilizes *O*-acetylserine sulfhydrylase. FEMS Microbiol. Lett. **189:**205–210.

4. **Borup, B., and J. G. Ferry.** 2000. *O*-Acetylserine sulfhydrylase from *Methanosarcina thermophila*. J. Bacteriol. **182:**45–50.

5. **Bukovska, G., V. Kery, and J. P. Kraus.** 1994. Expression of human cystathionine β-synthase in *Escherichia coli*: purification and characterization. Protein Express. Purif. **5:**442–448.

6. **Burkhard, P., G. S. Jagannatha Rao, E. Hohenester, K. D. Schnackerz, P. F. Cook, and J. N. Jansonius.** 1998. Three-dimensional structure of *O*-acetylserine sulfhydrylase from *Salmonella typhimurium*. J. Mol. Biol. **283:**121–133.

7. **Cherest, H., and Y. Surdin-Kerjan.** 1992. Genetic analysis of a new mutation conferring cysteine auxotrophy in *Saccharomyces cerevisiae*: updating of the sulfur metabolism pathway. Genetics **130:**51–58.

8. **Droux, M., J. Martin, P. Sajus, and R. Douce.** 1992. Purification and characterization of *O*-acetylserine (thiol) lyase from spinach chloroplasts. Arch. Biochem. Biophys. **295:**379–390.

9. **Drummond, J. T., J. Jarrett, J. C. González, S. Huang, and R. G. Matthews.** 1995. Characterization of nonradioactive assays for cobalamin-dependent and cobalamin-independent methionine synthase enzymes. Anal. Biochem. **228:**323–329.

10. **Esaki, N., T. Nakamura, H. Tanaka, and K. Soda.** 1982. Selenocysteine lyase, a novel enzyme that specifically acts on selenocysteine: mammalian distribution and purification and properties of pig liver enzyme. J. Biol. Chem. **257:**4386–4391.

11. **Filutowicz, M., A. Wiater, and D. Hulanicka.** 1982. Delayed inducibility of sulphite reductase in *cysM* mutants of *Salmonella typhimurium* under anaerobic conditions. J. Gen. Microbiol. **128:**1791–1794.

12. **Fitz-Gibbon, S. T., H. Ladner, U.-J. Kim, K. O. Stetter, M. I. Simon, and J. H. Miller.** 2002. Genome sequence of the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*. Proc. Natl. Acad. Sci. USA **99:**984–989.

13. **Flint, D. H., J. F. Tuminello, and T. J. Miller.** 1996. Studies on the synthesis of the Fe-S cluster of dihydroxy-acid dehydratase in *Escherichia coli* crude extract: isolation of *O*-acetylserine sulfhydrylases A and B and β-cystathionase based on their ability to mobilize sulfur from cysteine and to participate in Fe-S cluster synthesis. J. Biol. Chem. **271:**16053–16067.

14. **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.

15. **Griffith, O. W.** 1987. Mammalian sulfur amino acid metabolism: an overview. Methods Enzymol. **143:**366–376.

16. **Hell, R.** 1997. Molecular physiology of plant sulfur metabolism. Planta **202:**138–148.

17. **Jhee, K.-H., P. McPhie, and E. W. Miles.** 2000. Domain architecture of the heme-independent yeast cystathionine β-synthase provides insights into mechanisms of catalysis and regulation. Biochemistry **39:**10548–10556.

18. **Kaplan, M. M., and M. Flavin.** 1966. Cystathionine γ-synthetase of *Salmonella*: structural properties of a new enzyme in bacterial methionine biosynthesis. J. Biol. Chem. **241:**5781–5789.

19. **Kawarabayasi, Y., Y. Hino, H. Horikawa, K. Jin-no, M. Takahashi, M. Sekine, S.-I. Baba, A. Ankai, H. Kosugi, A. Hosoyama, S. Fukui, Y. Nagai, K. Nishijima, R. Otsuka, H. Nakazawa, M. Takamiya, Y. Kato, T. Yoshizawa, T. Tanaka, Y. Kudoh, J. Yamazaki, N. Kushida, A. Oguchi, K.-I. Aoki, S. Masuda, M. Yanagii, M. Nishimura, A. Yamagishi, T. Oshima, and H. Kikuchi.** 2001. Complete genome sequence of an aerobic thermoaciodophilic crenarchaeon, *Sulfolobus tokodaii* strain7. DNA Res. **8:**123–140.

20. **Kawarabayasi, Y., Y. Hino, H. Horikawa, S. Yamazaki, Y. Haikawa, K. Jin-no, M. Takahashi, M. Sekine, S.-I. Baba, A. Ankai, H. Kosugi, A. Hosoyama, S. Fukui, Y. Nagai, K. Nishijima, H. Nakazawa, M. Takamiya, S. Masuda, T. Funahashi, T. Tanaka, Y. Kudoh, J. Yamazaki, N. Kushida, A. Oguchi, K.-I. Aoki, K. Kubota, Y. Nakamura, N. Nomura, Y. Sako, and H. Kikuchi.** 1999. Complete genome sequence of an aerobic hyper-thermophilic crenarchaeon, *Aeropyrum pernix* K1. DNA Res. **6:**83–101.

21. **Kawashima, T., N. Amano, H. Koike, S.-I. Makino, S. Higuchi, Y. Kawashima-Ohya, K. Watanabe, M. Yamazaki, K. Kanehori, T. Kawamoto, T. Nunoshiba, Y. Yamamoto, H. Aramaki, K. Makino, and M. Suzuki.** 2000. Archaeal adaptation to higher temperatures revealed by genomic sequence of *Thermoplasma volcanium*. Proc. Natl. Acad. Sci. USA **97:**14257–14262.

22. **Kery, V., G. Bukovska, and J. P. Kraus.** 1994. Transsulfuration depends on heme in addition to pyridoxal 5′-phosphate: cystathionine β-synthase is a heme protein. J. Biol. Chem. **269:**25283–25288.

23. **Kery, V., D. Elleder, and J. P. Kraus.** 1995. δ-Aminolevulinate increases heme saturation and yield of human cystathionine β-synthase expressed in *Escherichia coli*. Arch. Biochem. Biophys. **316:**24–29.

24. **Kery, V., L. Poneleit, and J. P. Kraus.** 1998. Trypsin cleavage of human cystathionine β-synthase into an evolutionarily conserved active core: structural and functional consequences. Arch. Biochem. Biophys. **355:**222–232.

25. **Kitabatake, M., M. W. So, D. L. Tumbula, and D. Söll.** 2000. Cysteine biosynthesis pathway in the archaeon *Methanosarcina barkeri* encoded by acquired bacterial genes? J. Bacteriol. **182:**143–145.

26. **Kraus, J., S. Packman, B. Fowler, and L. E. Rosenberg.** 1978. Purification and properties of cystathionine β-synthase from human liver: evidence for identical subunits. J. Biol. Chem. **253:**6523–6528.

27. **Kredich, N. M.** 1997. Biosynthesis of cysteine, p. 514–527. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.

28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.

29. **Meier, M., M. Janosik, V. Kery, J. P. Kraus, and P. Burkhard.** 2001. Structure of human cystathionine β-synthase: a unique pyridoxal 5′-phosphate-dependent heme protein. EMBO J. **20:**3910–3916.

30. **Miles, E. W.** 1986. Pyridoxal phosphate enzymes catalyzing β-elimination and β-replacement reactions, p. 253–310. *In* D. Dolphin, R. Poulson, and O. Avramovic (ed.), Vitamin B6, pyridoxal phosphate: chemical, biochemical, and medical aspects, Part B. Wiley InterScience, New York, N.Y.

31. **Mino, K., T. Yamanoue, T. Sakiyama, N. Eisaki, A. Matsuyama, and K. Nakanishi.** 2000. Effects of bienzyme complex formation of cysteine synthetase from *Escherichia coli* on some properties and kinetics. Biosci. Biotechnol. Biochem. **64:**1628–1640.

32. **Mizuno, Y., Y. Miyashita, S. Yamagata, T. Iwama, and T. Akamatsu.** 2002. Cysteine synthase of an extremely thermophilic bacterium, *Thermus thermophilus* HB8. Biosci. Biotechnol. Biochem. **66:**549–557.

33. **Nakamura, T., H. Iwahashi, and Y. Eguchi.** 1984. Enzymatic proof for the identity of the *S*-sulfocysteine synthase and cysteine synthase B of *Salmonella typhimurium*. J. Bacteriol. **158:**1122–1127.

34. **Nozaki, T., Y. Shigeta, Y. Saito-Nakano, M. Imada, and W. D. Kruger.** 2001. Characterization of transsulfuration and cysteine biosynthetic pathways in the protozoan hemoflagellate, *Trypanosoma cruzi*: isolation and molecular characterization of cystathionine β-synthase and serine acetyltransferase from *Trypanosoma*. J. Biol. Chem. **276:**6516–6523.

35. **Ono, B.-I., K. Kijima, T. Inoue, S.-I. Miyoshi, A. Matsuda, and S. Shinoda.** 1994. Purification and properties of *Saccharomyces cerevisiae* cystathionine β-synthase. Yeast **10:**333–339.

36. **Ono, B.-I., T. Hazu, S. Yoshida, T. Kawato, S. Shinoda, J. Brzvwczy, and A. Paszewski.** 1999. Cysteine biosynthesis in *Saccharomyces cerevisiae*: a new outlook on pathway and regulation. Yeast **15:**1365–1375.

37. **Porter, P. N., M. S. Grishaver, and O. W. Jones.** 1974. Characterization of human cystathionine β-synthase. Evidence for the identity of human L-serine dehydratase and cystathionine β-synthase. Biochim. Biophys. Acta **364:**128–139.

38. **Sako, Y., N. Nomura, A. Uchida, Y. Ishida, H. Morii, Y. Koga, T. Hoaki, and T. Maruyama.** 1996. *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100°C. Int. J. Syst. Bacteriol. **46:**1070–1077.

39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

40. **Segel, I. H.** 1993. Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems. John Wiley & Sons, Inc., New York, N.Y.

41. **Tai, C.-H., S. R. Nalabolu, T. M. Jacobson, D. E. Minter, and P. F. Cook.** 1993. Kinetic mechanisms of the A and B isozymes of *O*-acetylserine sulfhydrylase from *Salmonella typhimurium* LT-2 using the natural and alternative reactants. Biochemistry **32:**6433–6442.

42. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.

43. **Zhou, D., and R. H. White.** 1991. Transsulfuration in archaebacteria. J. Bacteriol. **173:**3250–3251.