Cysteine is the major source of fixed sulfur for the synthesis of sulfur-containing compounds in organisms of the Bacteria and Eucarya domains. Though pathways for cysteine biosynthesis have been established for both of these domains, it is unknown how the Archaea fix sulfur or synthesize cysteine. None of the four archaeal genomes sequenced to date contain open reading frames with identities to either O-acetyl-L-serine sulphhydrylase (OASS) or homocysteine synthase, the only sulfur-fixing enzymes known in nature. We report the purification and characterization of OASS from acetate-grown Methanosarcina thermophila, a moderately thermophilic methanooarchaeon. The purified OASS contained pyridoxal 5'-phosphate and catalyzed the formation of L-cysteine and acetate from O-acetyl-L-serine and sulfide. The N-terminal amino acid sequence has high sequence similarity with other known OASS enzymes from the Eucarya and Bacteria domains. The purified OASS had a specific activity of 129 μmol of cysteine/min/mg, with a K_m of 500 ± 80 μM for sulfide, and exhibited positive cooperativity and substrate inhibition with O-acetyl-L-serine. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a single band at 36 kDa, and native gel filtration chromatography indicated a molecular mass of 93 kDa, suggesting that the purified OASS is either a homodimer or a homotrimer. The optimum temperature for activity was between 40 and 60°C, consistent with the optimum growth temperature for M. thermophila. The results of this study provide the first evidence for a sulfur-fixing enzyme in the Archaea domain. The results also provide the first biochemical evidence for an enzyme with the potential for involvement in cysteine biosynthesis in the Archaea.
pyridoxal 5'-phosphate-dependent OASS from the methanoarchaeon *Methanocarcina thermophila*.

**MATERIALS AND METHODS**

**Cell material.** *M. thermophila* TM-1 was grown on acetate as described previously (46). The medium contained the following constituents in demineralized water at the indicated final percent (wt/vol/wt) concentrations: NH₄Cl, 0.14; KH₂PO₄, 0.13; NaCl, 0.05; MgSO₄, 0.05; Na₂S·9H₂O, 0.027; CaCl₂·2H₂O, 0.006; Fe(NH₄)₂(SO₄)₃, 0.003; cysteine·HCl·H₂O, 0.027; yeast extract (Difco, Detroit, Mich.), 0.01; Trypticase (BBL, Cockeysville, Md.), 0.01; and sodium acetate, 0.41. In addition, the medium contained 1% (vol/vol) each vitamin and trace mineral solutions as described elsewhere (46). The cells were harvested at the end of exponential growth and stored in liquid nitrogen until use.

**Purification of OASS.** OASS was purified by monitoring cysteine production from O-acetyl-L-serine and sulfide in reaction mixtures following each purification step. All procedures were done aerobically and at 20°C unless otherwise indicated. Protein concentrations were determined by the method of Bradford (5), using Bio-Rad dye reagents and bovine serum albumin (Pierce) as a standard.

(i) Preparation of cell extract. Thawed cell paste (20 g [wet weight]) was suspended in 25 ml of buffer A ([50 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-KOH (pH 6.8)] containing 10% [vol/vol] glycerol, DNPase I (0.25 mg) was added to the suspension and then passed twice through a chilled French pressure cell at 20,000 lb/in² (1 lb/in² = 6.9 kPa). The cell lysate was centrifuged at 2,000 × g for 20 min, and the supernatant was recentrifuged at 78,400 × g for 2 h.

(ii) Q-Sepharose chromatography. The supernatant from step i was applied to a Q-Sepharose HP (Pharmacia Biotechnology) column (bed volume = 150 ml) equilibrated with buffer A. The column was washed with 300 ml of buffer A, and a 1-liter linear 0 to 1 M KCl gradient was applied at 7.0 ml/min. Peak fractions were collected with buffer A. The column was washed with 300 ml of buffer A, and at 78,400 × g was centrifuged at 2,000 × g for 10 min. Solutions were made anaerobic and stored under N₂ for sulfide determination. Protein concentrations were determined by the method of Bradford (5), using Bio-Rad dye reagents and bovine serum albumin (Pierce) as a standard.

(iii) Phenyl-Sepharose chromatography. The concentrated protein solution from step ii was raised to a final concentration of 2 M NaCl by addition of 5 M NaCl in 50 mM Tris·Cl (pH 7.5) and loaded onto a phenyl-Sepharose FF HS column (bed volume = 160 ml) equilibrated with buffer B (50 mM Tris·Cl (pH 7.5) containing 2 M KCl). After a 320-ml wash, the column was developed with a 1.1-liter decreasing linear gradient of 2.0 to 0 M NaCl at 2 ml/min. The peak of activity, which eluted between 0.1 and 0.9 M NaCl, was pooled and concentrated as described in step ii.

(iv) Mono Q chromatography I. The pooled samples were dialyzed against 4 liters of buffer C (50 mM Tris·Cl [pH 8.0]) containing 1 mM pyridoxal 5'-phosphate and loaded onto a Mono Q HR 10/10 anion-exchange column (Pharmacia) equilibrated with buffer C. After a 20-ml wash, the column was developed with a 200-ml linear gradient from 0 to 1 M NaCl applied at 2.0 ml/min. The enzyme eluted between 0.3 and 0.4 M NaCl. Fractions with highest specific activity were pooled and stored at −20°C.

(v) Mono Q chromatography II. Step iv was repeated except that buffer A was used, and the column was developed from 0 to 1 M KCl. The purified enzyme, which eluted between 0.4 and 0.5 M KCl, was stored at −20°C.

**Enzyme assay.** OASS activity was measured as described previously (11) except that reactions were performed at 40°C and were allowed to proceed for 1 min. Solutions were made anaerobic and stored under N₂ for sulfide determination to avoid oxidation of the substrate.

**KINETIC ANALYSIS.** The kinetic data were fitted to the indicated rate equations by using the Levenberg-Marquardt algorithm with KaleidaGraph for Windows version 3.09 (Abelbeck Software) and a Pentium Dell XPS M200s computer. The Hill equation (42) is \( v = V_{\text{max}} [S]^{n} / (K_n + [S]^{n}) \), where \( v \) is the velocity of the reaction (rate of product formation), \( V_{\text{max}} \) is the maximum velocity, \( K_n \) is the product of the two kinetic dissociation constants, and \( n \) is the Hill cooperativity constant. At \( K_n = V_{\text{max}} / 2 \) and thus \( K_n = K_{1/2} \).

A modified Hill equation incorporating an ordinate intercept (\( b \)) was proposed by Morgan et al. (27) and called MFM: \( v = \left( k_b + V_{\text{max}} [S]^{n}/(k_b + [S]^{n}) \right) \). At \( [S] = 0 \), \( v = (V_{\text{max}} + b)/2 \), and thus \( K_{1/2} = b/2 \).

The logistic equation (34, 35) is \( v = V_{\text{max}}[1 + \exp (\beta - \gamma[S])] \). At \( [S] = 0 \), \( v = V_{\text{max}}/2 \) and thus \( K_{1/2} = b/\gamma \). Saturation curves included data points from 0 to 7 mM O-acetyl-L-serine to preclude the inhibition portion of the curve (see Fig. 5).

**TABLE 1. Scheme for purification of OASS from *M. thermophila***

<table>
<thead>
<tr>
<th>Step</th>
<th>Vol (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>96</td>
<td>2,891</td>
<td>979</td>
<td>0.339</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>60</td>
<td>105</td>
<td>660</td>
<td>6.30</td>
<td>67</td>
<td>19</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>220</td>
<td>36</td>
<td>525</td>
<td>14.5</td>
<td>54</td>
<td>43</td>
</tr>
<tr>
<td>Mono Q chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>12</td>
<td>6.7</td>
<td>413</td>
<td>61.0</td>
<td>42</td>
<td>180</td>
</tr>
<tr>
<td>II</td>
<td>7</td>
<td>3.5</td>
<td>445</td>
<td>129</td>
<td>45</td>
<td>381</td>
</tr>
</tbody>
</table>

\(^{a}\) Defined as micromoles of Cys produced per minute.

**RESULTS**

**Purification.** The assay of OASS activity in the soluble and membrane fractions obtained by centrifugation of cell extract through a sucrose gradient revealed that 95% of the activity resides in the soluble fraction. A typical purification of OASS is summarized in Table 1. The level of enzyme activity in cell extract was found to be within the range reported for prokaryotes from the *Bacteri* domain (0.03 to 50 U/mg) (9, 15). The *M. thermophila* enzyme could be stored at −20°C for up to 7 days between each purification step with no significant loss of activity. OASS was purified to apparent homogeneity as indicated by SDS-PAGE (Fig. 1). The purified enzyme was stable.
to several freeze-thaw cycles, but activity was completely absent after 2 months of storage at −20°C. However, approximately 10% of the activity could be recovered by incubating the enzyme at 21°C for 30 min in Tris-Cl buffer (pH 8.0) containing 50 mM dithiothreitol and 80 μM pyridoxal 5′-phosphate.

**Physical properties.** A plot of activity versus the assay temperature revealed a broad optimum between 40 and 60°C (data not shown). Significant activity was detected up to 80°C. These results are compatible with the optimum growth temperature of *M. thermophila* (55°C). The N-terminal sequence of the purified OASS from *M. thermophila* (Fig. 2) shows significant identity and similarity to OASS enzymes from a variety of plants and procaryotes from the *Bacteria* domain. The identity extends to residues that are perfectly conserved among all these enzymes.

The UV-visible spectrum (Fig. 3) contained two major peaks with absorbance maxima at 278 and 413 nm, the former due to aromatic amino acids. The absorbance at 413 nm is a property of all OASS enzymes studied in plants and in procaryotes from the *Bacteria* domain. The absorbance is attributed to the aldoxime form of pyridoxal 5′-phosphate (10), produced when a Schiff base is formed between the cofactor and an ε-amino group of a lysine residue in the protein. The OASS purified from *M. thermophila* has an A<sub>280</sub>/A<sub>413</sub> ratio of 4.0, which compares favorably to the ratio found for OASS from *Salmonella* serovar Typhimurium of 3.5 (2). Incubation of OASS with a 1:10 molar ratio of enzyme to pyridoxal 5′-phosphate did not significantly increase activity (data not shown), suggesting that the purified enzyme had a full complement of this cofactor.

![FIG. 1. SDS-PAGE of OASS purified from *M. thermophila*. The 12% gel was loaded with 9 μg of purified OASS. The positions to which the molecular mass markers migrated are shown in kilodaltons at the left.](http://jb.asm.org/)

![FIG. 2. N-terminal amino acid sequence alignment of select OASS enzymes. Completely conserved amino acids are indicated in boldface, partially conserved amino acids with respect to the *M. thermophila* sequence are indicated by gray shading, and gaps introduced by sequence alignment are indicated by dashes. Numbers on the right refer to positions of the right-most residues shown with respect to the entire sequence.](http://jb.asm.org/)

![FIG. 3. UV-visible absorption spectrum of OASS purified from *M. thermophila*. The enzyme (0.13 mg/ml) was in 50 mM Tris-KOH buffer (pH 6.8) containing 350 mM KCl.](http://jb.asm.org/)
A marked change in the spectrum, attributed to pyridoxal 5'-phosphate, occurs upon addition of O-acetyl-L-serine. Titration with increasing amounts of O-acetyl-L-serine produced a shift in absorbance from 413 to 466 nm (Fig. 4) and formation of a broad shoulder at 330 nm (Fig. 4). The increase in absorbance at 466 nm was concentration dependent and was saturated at 10 μM O-acetyl-L-serine (Fig. 4, inset). A similar shift in absorbance was observed by Schnackerz et al. (41) upon binding of L-serine to D-serine dehydratase. The species responsible for this shift in absorbance is the α-aminoacryl acid in the Schiff base with pyridoxal 5'-phosphate. The formation of this α-aminoacrylate intermediate in the OASS from *M. thermophila* was reversible upon addition of sulfide. The original spectrum with its absorption at 413 nm was regenerated upon addition of 1 mM sodium sulfide, consistent with reaction of this second substrate with the intermediate and transfer of the acetyl group of O-acetyl-L-serine to sulfide, forming L-cysteine (10). The observed spectral changes upon addition of O-acetyl-L-serine and reversion upon addition of sulfide demonstrate an active role of pyridoxal 5'-phosphate in the reaction mechanism of the OASS from *M. thermophila*.

Isoelectric focusing determined that the pI of *M. thermophila* OASS is 5.0 ± 0.5, similar to the calculated pI values of the spinach OASS isoenzymes, which range from 5.0 to 6.0 (40). No data are available for any of the procaryotic enzymes. SDS-PAGE analysis of the purified *M. thermophila* OASS revealed a single band at 36 kDa (Fig. 1). The native molecular mass of OASS was determined to be 93 kDa by gel filtration chromatography (data not shown). This is the largest native molecular mass reported for any OASS, the next closest being an OASS from the plant *Datura innoxia*, with a native molecular mass of 86 kDa (23). The SDS-PAGE and native gel chromatography results suggest that the OASS purified from *M. thermophila* is either a homodimer or a homotrimer. All other OASS enzymes purified to date are homodimeric with native molecular masses ranging from 52 to 72 kDa (2, 4, 11, 12, 16, 17, 22, 25, 29, 30, 33, 36, 38, 43, 48, 49), the only exception being a heterodimeric OASS from *D. innoxia* (23).

**Kinetic analyses.** Plots of increasing concentrations of O-acetyl-L-serine versus amount of O-acetyl-L-serine for each spectrum is shown. (Inset) Absorption of enzyme at 466 nm versus amount of O-acetyl-L-serine added.

FIG. 4. Spectroscopic titration of OASS purified from *M. thermophila* with O-acetyl-L-serine. Protein concentration was 0.10 mg/ml in 50 mM Tris-KOH buffer (pH 6.8), containing 350 mM KCl. O-Acetyl-L-serine additions were made in 2 to 5-μl increments in a total volume of 1.0 ml. The amount of O-acetyl-L-serine for each spectrum is shown. (Inset) Absorption of enzyme at 466 nm versus amount of O-acetyl-L-serine added.
TABLE 2. Kinetic constants from substrate saturation experiments with OASS purified from M. thermophila

<table>
<thead>
<tr>
<th>Model</th>
<th>( V_{\text{max}} ) (mM)</th>
<th>( S_{0.5} ) (mM)</th>
<th>( n )</th>
<th>Chi square (10^{-2})</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hill</td>
<td>0.0771</td>
<td>6.667</td>
<td>1.847</td>
<td>4.94</td>
<td>0.9647</td>
</tr>
<tr>
<td>MMF</td>
<td>0.0587</td>
<td>5.597</td>
<td>2.214</td>
<td>4.75</td>
<td>0.9661</td>
</tr>
<tr>
<td>Logistic</td>
<td>0.0408</td>
<td>4.111</td>
<td>NA</td>
<td>4.76</td>
<td>0.9660</td>
</tr>
</tbody>
</table>

\( ^a \) NA, not applicable.

The variations in \( V_{\text{max}} \) are probably due to the restricted data set used to preclude portions of the curve affected by the substrate inhibition.

The genomic sequences of four phylogenetically and metabolically diverse members of the Archaea provide little insight into mechanisms of archaean sulfur fixation. The results presented here provide the first documentation of a sulfur-fixing enzyme, OASS, in the Archaea. The M. thermophila OASS enzyme was identified through its sulfur-fixing activity, the presence of a pyridoxal 5'-phosphate cofactor, and high sequence similarity between the N terminus and those of other known OASS enzymes.

Although OASS is essential for the serine pathway of cysteine biosynthesis in plants and Bacteria, additional functions for OASS enzymes have been proposed. In Eugarya and Bacteria, OASS is involved in the recycling of released sulfur during sulfur starvation (14) and sequestering of sulfide into cysteine to prevent toxic levels in the cell (49). In the Eugarya domain, OASS enzymes can also catalyze the formation of heterocyclic \( \beta \)-substituted alanines from \( O \)-acytetyl-L-serine and heterocyclic compounds (18). Thus, it is possible that M. thermophila OASS has additional functions, or a function unrelated to the serine pathway in the cell, and that another pathway exists for cysteine biosynthesis in the Archaea.

M. thermophila was cultured with acetate as the carbon and energy source in 10-liter fermentors to obtain the large amounts of cell material necessary for purification of OASS. The fermentors require continuous gassing; thus, in addition to volatile sulfide, the presence of cysteine is essential to maintain the reduction potential necessary for growth. The level of OASS activity in cell extracts of M. thermophila was at the lower end of the range reported for procaryotic OASS enzymes. In E. coli and Salmonella serovar Typhimurium, expression of OASS and other enzymes required for cysteine biosynthesis is maximally repressed in the presence of cysteine (21). If a similar regulation was effective in M. thermophila, then cysteine present in the growth medium would repress the synthesis of OASS to constitutive levels; however, this hypothesis could not be tested without the ability to grow M. thermophila in the absence of cysteine. An increase in the levels of OASS when cells are grown in the absence of cysteine would support a role for OASS in cysteine biosynthesis. Positive cooperativity in response to \( O \)-acytetyl-L-serine may be important if OASS functions in the serine pathway and transcription of the genes encoding enzymes of this pathway are regulated by the same mechanism as proposed for E. coli and Salmonella serovar Typhimurium. In the proposed mechanism, the levels of \( O \)-acytetyl-L-serine in the cell influence the expression of serine transacetylase and OASS. Inactivity of the M. thermophila OASS at low concentrations of \( O \)-acytetyl-L-serine may be necessary to allow accumulation of \( O \)-acytetyl-L-serine to levels required for transcription of serine transacetylase and OASS. Clearly, more research is necessary to provide evidence in support of this hypothesis and a role for OASS in cysteine biosynthesis.

The presence of open reading frames in the genomes of M. thermosautotrophicum and P. horikoshii with deduced sequences having identity to homoserine transacetylase and \( \gamma \)-cystathionase sequences is consistent with the homocysteine pathway for cysteine biosynthesis in these Archaea. However, M. thermosautotrophicum and P. horikoshii are classified in different taxonomic orders than the methanosarcinae; thus, M. thermophila may have inherited enzymes of the serine pathway or acquired them by horizontal gene transfer from the Bacteria domain. The complete gene sequence may help to determine the evolutionary relationship to other OASS enzymes and the origin of OASS in the Archaea.

REFERENCES

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