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CYSTEINE BIOSYNTHESIS AND SULFUR FIXATION IN THE ARCHAEA

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by

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ABSTRACT

Cysteine plays a critical role in the structure, stability and catalytic function of many proteins in all domains of life. Cysteine is also the major source of sulfur for the synthesis of sulfur-containing compounds in organisms of the Bacteria and Eukarya domains. Two routes for cysteine biosynthesis in nature have been documented, pathways I and II. Plants and members of the Bacteria domain synthesize cysteine and fix sulfur via pathway I. Fungi fix sulfide and synthesize cysteine using pathway I or II. Although the genomes of seven members of the archaea have been sequenced to date, the data offer little understanding of cysteine biosynthesis in this domain.

Presented here is the purification and characterization of the first archaeal enzyme catalyzing sulfur fixation and cysteine biosynthesis, *O*-acetylserine sulfhydrylase, from the methanoarchaeon *Methanosarcina thermophila*. Evidence was obtained for the involvement of *O*-acetylserine sulfhydrylase in cysteine biosynthesis by pathway I through biochemical, physiological and genetic means. *O*-acetylserine sulfhydrylase from *M. thermophila* exhibits positive co-operativity indicative of involvement in a biosynthetic pathway. *O*-acetylserine sulfhydrylase expression is elevated under growth conditions where the organism must synthesize cysteine, compared to expression levels where cysteine is provided in the media. The two genes involved in pathway I are transcribed in the same direction and possibly form an operon in *M. thermophila*. Activities of enzymes of pathway II are not detected in cell free extract, indicating that the only other pathway known for the synthesis of cysteine is not operable in *M. thermophila* under the conditions tested.

Evidence is presented implicating an additional function for *O*-acetylserine sulfhydrylase. Over 90% of cysteine desulfurase activity in cell extracts of cells grown with cysteine as the sole sulfur source is due to *O*-acetylserine sulfhydrylase, suggesting the possibility that *O*-acetylserine sulfhydrylase provides the primary source of sulfide for biosynthesis. Patterns of expression levels in the presence and absence of sulfide, growth patterns with trimethylamine, inhibition by ammonia of the desulfurase activity,

and changes in sulfide concentration of the media during growth support this interpretation.

Experimental advances were made to obtain genetic knock-out mutants in *M. thermophila* deficient in the genes of pathway I to determine if an additional, novel pathway exists for cysteine biosynthesis in the archaea, and to test the hypothesis for the role of *O*-acetylserine sulfhydrylase in the desulfuration of cysteine to supply sulfur for biosynthesis. In addition a novel assay was developed for sulfide detection in cell extracts that is less prone to high background absorbances than published methods.

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FORWARD

This dissertation focuses on cysteine biosynthesis and sulfur fixation in the methanoarchaea. Chapters 1 and 2 are intended to serve as an introduction to the methanoarchaea and known cysteine biosynthetic pathways in nature. Chapters 3 and 4 describe biochemical and genetic studies for the elucidation of the cysteine biosynthetic pathway and sulfur fixation in the methanoarchaea. A summary of the current research and future directions are presented in Chapter 5. The studies presented in Chapter 3 and 4 have been published as follows:

Birthe Borup and James G. Ferry. 2000. Cysteine Biosynthesis in the Archaea. *Methanosarcina thermophila* utilizes *O*-acetylserine sulfhydrylase for the synthesis of cysteine. FEMS Microbiol. Lett. 189 (2), 205-210.

Birthe Borup and James G. Ferry. 2000. *O*-Acetylserine Sulfhydrylase from *Methanosarcina thermophila*. Journal of Bacteriology 182, 45-50.

Chapter 1

Biochemistry and physiology of the archaea

Archaea – an overview

The domain Archaea was first introduced in 1990 by Woese *et al*. (20), who used rRNA sequence information to determine the evolutionary relatedness of organisms. Life was shown to fall into three domains: Archaea, Bacteria, and Eucarya (Figure 1-1). Until that point kingdoms were the highest taxonomic order, and Archaea and Bacteria were placed in the same kingdom. The new tree of life showed, however, that Archaea were more closely related to Eucarya than to Bacteria.

Woese chose rRNA because its mutation rates are usually not a factor of environmental changes. rRNA is not needed to synthesize a specific protein, but all proteins. Therefore all rRNA should have mutations at a constant rate. Lateral gene transfer, i.e. the transfer of genetic material between distantly related organisms, has been a cause of concern when examining sequences to determine evolutionary relatedness. However, since all organisms have multiple copies of rRNA genes, any lateral gene transfer that may have occurred is assumed to be "diluted out".

In recent years, as more genetic information has become available (especially the information from entire genomes), the structure of the tree of life has come into question again. Through investigation of conserved insertions/deletions in genes Gupta (7) proposed that life falls into two domains, the procaryotes and the eucaryotes. In this proposed tree of life the archaea are related to Gram positive bacteria, and eucaryotes are the result of a fusion between a Gram negative bacterium and an archaeon. Philippe *et al.* (12) on the other hand proposes that the last common ancestor was a eucaryote.

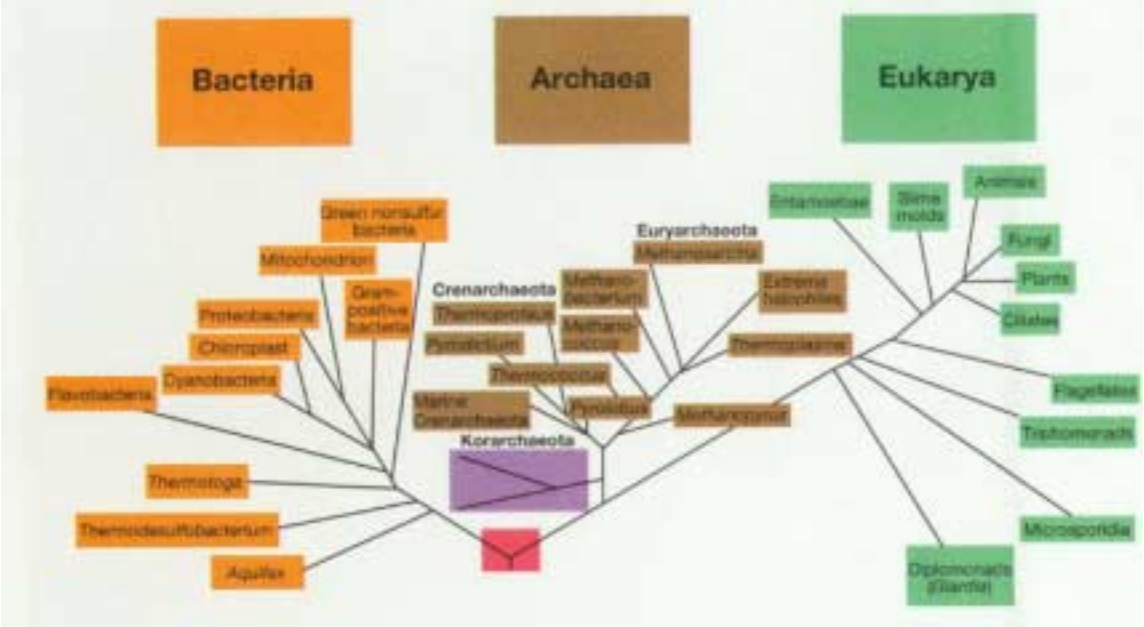

Figure 1-1: Phylogenetic tree of life derived from comparative sequencing of 16S and 18S rRNA. Adapted from (11).

Although the domain Archaea was not introduced until 1990, evidence for the existence of a third form of life was discovered in 1977. Archaea are easily classified by the ecological niches they inhabit, though this does not correspond to their evolutionary relationships. Based on unique aspects of their physiology, all Archaea are members of at least one of three groups. The thermophiles grow at temperatures above 50^{o}C; the halophiles require salt for growth (extreme halophiles require at least 1.5 M NaCl, and can grow at concentrations up to 5.5 M NaCl) and grow in the Dead Sea and in the Great Salt Lake. The methanogens grow under anaerobic conditions and produce methane. Archaea seem to be more widely distributed than believed previously. They can be found in extreme environment, such as hot springs (80 to 100 ºC), deep in the ground under high pressure (40 MPa), or in cold seawater (4 to -15 ºC). Bacteria and Archaea

constitute a major fraction of the planktonik community in the ocean. In fact, up to 30% of the planktonik community consists of unknown (not yet isolated) Archaea (8).

Within the domain Archaea there are two (possibly three) kingdoms. The Crenarchaeota are mostly thermophilic sulfur-dependent organisms. The Euryarchaeota contain mostly methanogens and halophiles, and a few thermophilic sulfur-dependent Archaea. Members of the third kingdom of the Archaea, the Korarchaeota, have never been isolated and grown in the laboratory. Their existence is only known through the sequence of rRNA isolated from the environment.

The Archaea have many features that set them apart from the Bacteria and the Eukarya. Bacterial cell walls contain peptidoglycan (murein). However the Archaea cell walls consist of pseudomurein or only a protein coat. The flagella of Archaea and Bacteria consist of a simple strand of protein subunits that contain a motor at the base; however, the Archaeal flagella proteins are similar to Bacterial pili in protein sequence. Eukarya can also have flagella, but their flagella have a complex tubular structure, and the power of motion is intrinsic to the flagellum itself and does not require a motor.

The Archaeal membranes are quite distinct from those of the other two domains. Bacterial and eukaryal membranes consist of a bilayer phospholipid core studded with bound proteins. The linkage between the polar glycerol and the non-polar fatty acyl of the phospholipids consists of an ester bond. The Archaeal membranes commonly lack phospholipids, and the bond between the polar and the non-polar section of the membrane consists of an ether linkage. The membranes also often lack the bilayer format, and one membrane molecule spans the entire length of the membrane and has a polar section on each end. The non-polar part of the membrane is also usually branched (forming isoprenoids), which is in direct contrast to the unbranched fatty acyl portion of the phospholipids in both the Bacteria and the Eukarya. Membrane fluidity in the thermophiles is decreased by increased numbers of five-membered rings which are part of the hydrophobic portion. The lipids of Archaea from cold environments on the other hand contain double bonds to increase membrane fluidity.

The Archaeal genomes thus far studied are arranged on a circular chromosome like Bacteria, and the genes are also often placed together in operons. Archaea do not

commonly have introns (excised from mRNA before translation) and exons (used for protein encoding), except for DNA encoding ribosomal and transfer RNA. Introns and exons are not found in Bacteria, but they are common in Eukarya. Archaea, like Bacteria, contain plasmids; small circular DNA encoding non-essential DNA that can pass easily from cell to cell.

The structure of the promoter sequence is similar between Eukarya and Archaea, and both domains need transcription factors for the polymerase to bind. Bacteria do not need these transcription factors. Not surprisingly the RNA polymerase of Archaea is structurally similar to the Eukaryal but not the Bacterial RNA polymerase. In regard to ribosomal size, shape and subunit composition, Archaea are intermediate between Bacteria and Eukaryotes. However, certain inhibitors of protein synthesis (e.g. diphtheria toxin) kill Archaea and Eukarya, but not Bacteria, because the elongation factors of Archaea are related to those of Eukarya. On the other hand, some toxins (e.g. kirromycin) kill Bacteria but leave Archaea and Eukarya unharmed. Finally, some heat shock proteins (Hsp 70) from Archaea closely resemble those of Bacteria but not those of Eukarya.

Methanogens

Methanogenesis is carried out only by the Archaea. Methanogens are strict anaerobes that can be found in swamps, sewage digesters, ruminants, and even the cytoplasm of the giant amoeba Pelomyxa where they assist the organism in metabolism. In ruminants, chewed plant material is transferred to the rumen and converted into bacterial and archaeal protein by microbes. The fermentative bacteria also synthesize volatile fatty acids (propionate and butyrate) from the plant material. This material is chewed again before being placed into the digestive system. The ruminants derive their protein from the Bacteria and Archaea, and they derive their energy from the volatile fatty acids.

Methane is a potent greenhouse gas, and methanogens produce about 400 million metric tons of this gas per year (5). The estimated 1% increase in global methane is

mainly attributed to human activities (8). For example, due to the increasing world population, there has been an increase in the number of ruminant animals and thus an increase in the production of methane. According to some estimates the major biological contributors of the amount of methane are methanogens of natural wetlands, rice paddies, and ruminants, in that order (8).

Methanogens are part of at least three interacting metabolic groups decomposing organic matter that has entered the anaerobic environment (Figure 1-2). The carbon is recycled in the form of methane, which enters the aerobic environment again. The first of the three metabolic groups consists of the fermentative bacteria. This group decomposes cellulose and other complex molecules to volatile carboxylic acids (mostly acetate) and hydrogen gas. The acetogenic group decomposes butyrate and propionate to suitable substrates for the methanogens, which constitute the last group.

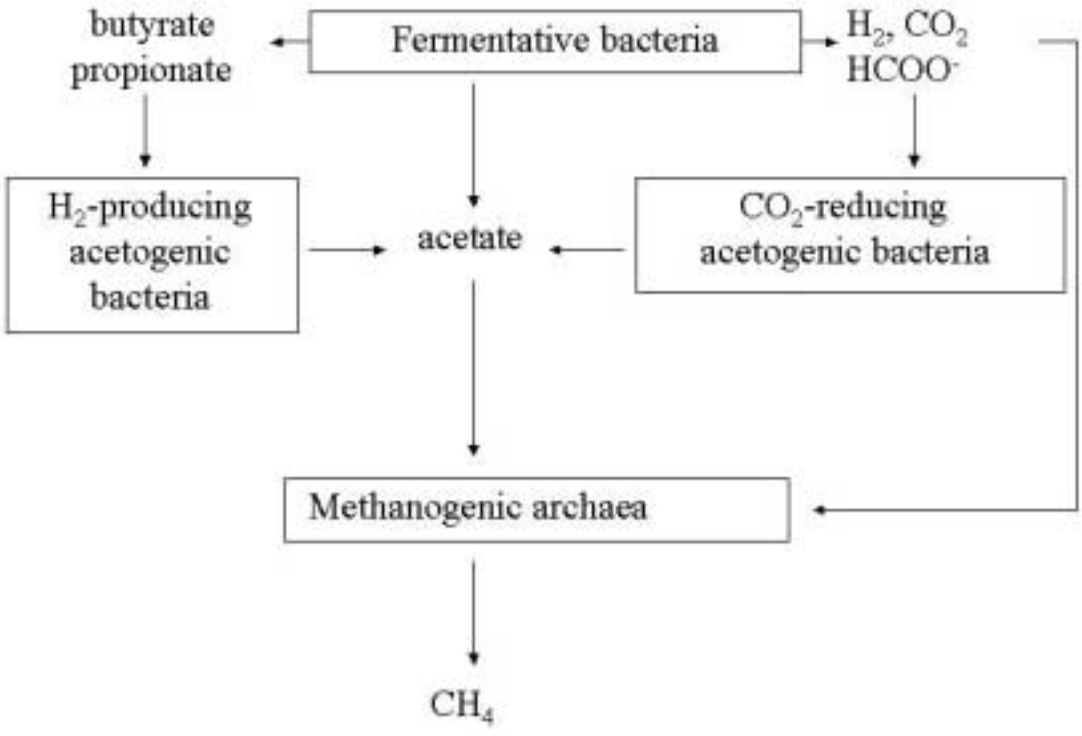

Figure 1-2: Four interacting metabolic groups of anaerobic microorganisms involved in the complete decomposition of organic matter.

Pathways

There are two major pathways for methanogenesis: reduction of CO_2 and fermentation of acetate. Most of all naturally produced methane stems from the methyl group of acetate (5). Several novel enzymes and cofactors are unique to methanogenesis: F_{420}, methanofuran, methanopterin, coenzyme M, HS-HTP (CoB), and F_{430}. Figure 1-3 gives a composite overview of the main methanogenic pathway starting with CO_2.

In the CO_2 reducing pathway, H_2 or formate is oxidized by hydrogenase or formate dehydrogenase, providing the eight electrons necessary to reduce CO_2 to methane. Four two-electron reduction steps then convert CO_2 to CH_4 (4). Formylmethanofuran (formyl-MF) dehydrogenase catalyzes the first reduction in which CO_2 is converted into formyl-MF. Formyltransferase transfers the formyl group from MF to H_4-methanopterin (H_4MPT). Cyclohydrolase converts formyl-H_4MPT to methenyl-H_4MPT. The second and third reductive steps are carried out by F_{420} dependent enzymes; H_4MPT-dehydrogenase reduces methenyl-H_4MPT to methylene-H_4MPT, and H_4MPT-reductase reduces methylene-H_4MPT to methyl-H_4MPT. Methyltransferase transfers the methyl group from H_4MPT to Coenzyme M (S-CoM). Methylreductase, an F_{430} dependent enzyme, catalyzes the demethylation of S-CoM to form methane and a heterodisulfide, CoM-SS-CoB (CoB = 7-mercaptoheptanoylthreonine phosphate). The fourth and final reduction is catalyzed by heterodisulfide reductase (3). During this final reduction, the heterodisulfide is cleaved into CoM-SH and CoB-SH.

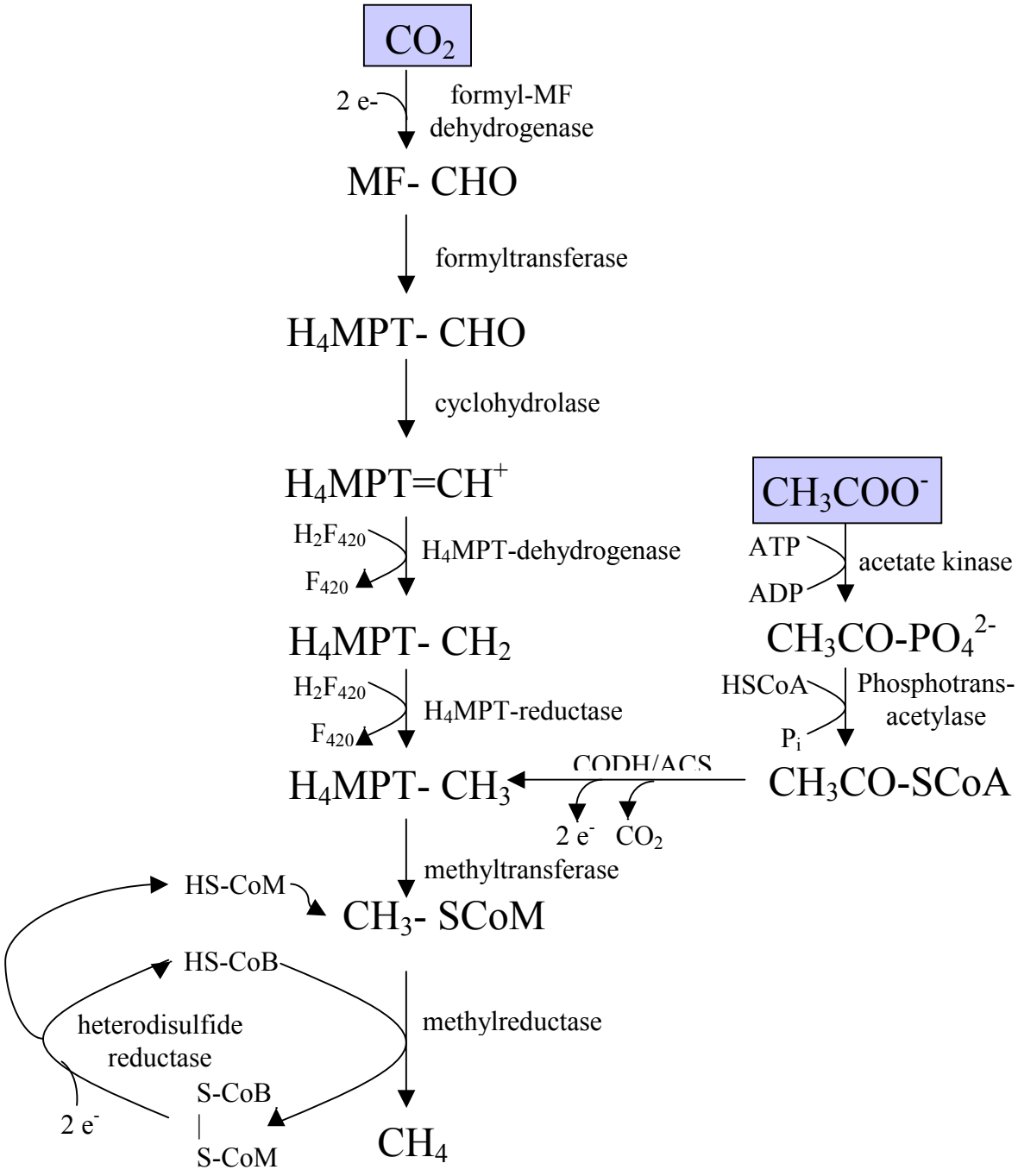

Figure 1-3: Pathways of methanogenesis using carbon dioxide or acetate as substrate.

During acetate fermentation, the methyl group of acetate enters the above pathway at a later stage (as methyl-H_4MPT). Acetate kinase and phosphotransacetylase together activate acetate to acetyl-CoA. Carbon monoxide dehydrogenase/ acetyl CoA synthase (CODH/ACS) then cleaves the carbon carbon and the carbon sulfur bonds, forming a carbonyl group and methyl-H_4sarcinapterin (methyl-H_4SPT). H_4SPT is a structural and functional analog to H_4MPT. The methyl-H_4SPT enters thus the CO_2 reduction pathway before the fourth and final reduction by methylreductase. In the case of acetate metabolism, the electrons for this final reduction originate from the CODH/ACS complex during oxidation of the carbonyl carbon of acetate.

Energetics

The final reduction step in all pathways is the reduction of the heterodisulfide CoM-SS-CoB by heterodisulfide reductase to form CoM-SH and CoB-SH. This is the most important reaction in terms of energy conservation, because the heterodisulfide reductase is the last member of a membrane bound electron transport chain. As electrons pass through this membrane bound electron transport chain, a proton gradient is generated across the membrane (high concentration of protons outside). Movement of the protons back across the membrane to decrease the chemical potential, is coupled to ATP synthesis catalyzed by ATPase (13).

The individual members of the electron transport chain vary according to which substrate is used. During methanogenesis from CO_2, a hydrogenase passes electrons from H_2 through a series of unknown electron carriers to heterodisulfide reductase. During methanogenesis from acetate, electrons are passed from CODH/ACS to a ferredoxin. The electrons are hypothesized to enter the membrane bound electron transport chain through ferredoxin:methanophenazine oxidoreductase. The electrons then pass through cytochrome *b* to an unknown electron carrier (possibly methanophenazine) to cytochrome b_2 and ultimately to the heterodisulfide reductase (13).

Amino Acid Biosynthesis

Amino acid biosynthesis in the methanoarchaea appears to closely resemble that found in the eukarya and the eubacteria. ^{14}C labeling experiments indicate that the carbons of the amino acids alanine, aspartate, and glutamate are derived from pyruvate, oxaloacetate, and α-ketoglutarate, respectively (14). Aspartate aminotransferase, which catalyzes reversible amino group transfer reactions between dicarboxylic amino and oxo acids, was purified from two Methanobacterium species (16, 17). Glutamine is most likely derived from the direct incorporation of ammonia into glutamate, and asparagine is derived from the transamination of aspartate from glutamine.

^{13}C labeling experiments indicate that serine is synthesized from acetate and CO_2 – presumably through the intermediate 3-phosphoglycerate. The enzyme serine transhydroxymethylase catalyzes the formation of glycine from serine. This enzyme has been isolated and characterized from *Methanobacterium thermoautotrophicum* and is very similar to the eukaryotic and bacterial counterparts, except that the archaeal enzyme utilizes tetrahydromethanopterin instead of tetrahydrofolate as carbon acceptor from serine. This might reflect the unique cofactor content of methanogens (14).

The branched chain amino acids valine, leucine and isoleucine are synthesized from pyruvate via the acetohydroxy acid pathway. The α-ketobutyrate needed for isoleucine biosynthesis is derived from pyruvate via a citramalate intermediate instead of the customary threonine intermediate. The enzymes of the acetohydroxy acid pathway (acetohydroxy acid synthase, acetohydroxy acid isomeroreductase, dihydroxy acid dehydratase, and branched-chain aminotransferase) have been isolated and characterized from the methanococci. They are similar to their eubacterial counterparts in kinetic mechanism; however, the archaeal enzymes are more oxygen sensitive (14, 21).

Lysine is probably synthesized from diaminopimelic acid *via* the diaminopimelic acid pathway. Activities of two enzymes involved in this pathway, dihydropicolinate synthase and diaminopimelate decarboxylase, have been detected in *M. thermoautotrophicum* (14). Arginine is probably synthesized from ornithine *via* the ornithine acetyltransferase pathway. Activities of several enzymes of this pathway have

been detected in cell extracts of methanogens. In addition, several genes of enzymes involved in the pathway have been sequenced and show 30-45% sequence similarity on the amino acid level to eubacterial and eukaryotic enzymes (14).

Histidine is probably synthesized from phosphoribosyl-pyrophophate and ATP. Incorporation of stable isotopes into histidine, and sequence similarity between two eubacterial genes whose product is involved in histidine biosynthesis and methanococcal genes confirm this hypothesis (14). Similarly, stable isotope incorporation experiments and genetic analyses indicate that the aromatic amino acids tryptophan, tyrosine, and phenylalanine are synthesized via shikimate and chorismate (14). Enzyme activities of this pathway (shikimate dehydrogenase, chorismate mutase, prephenate dehydratase and prephenate dehydrogenase) were detected in cell extracts of *Methanohalophilus mahii*, and the regulation of these enzymes was similar to that of the bacterial enzymes (6). However, activity of the enzyme catalyzing the first step in aromatic amino acid biosynthesis, DAHP (2-keto-3-deoxyarabinoheptulosonate-7-phosphate) synthase, was not detected (6), and ^{13}C labeling experiments indicate that methanogens use an alternative first step in aromatic amino acid biosynthesis (19).

Finally results of stable isotope incorporation experiments are consistent with threonine and methionine being synthesized from aspartate, and proline being derived from glutamate. Until the present work, very little information was available on the biosynthetic pathway for cysteine; however, this will be addressed in the next chapter.

Protein Biosynthesis

All organisms have tRNAs that are specific to each of the 21 amino acids. Most organisms also have tRNA synthetases that are specific to each of the 21 tRNAs and the appropriate amino acid. However some exceptions do exist. Notably, some Gram-positive eubacteria and some Archaea lack glutaminyl synthetase or asparaginyl synthetase (9, 18). Instead the charged tRNAs are synthesized by attaching glutamate to $tRNA^{Gln}$ and aspartate to the $tRNA^{Asn}$ by glutamate tRNA synthetase and aspartate tRNA

synthetase respectively (9). The precursors Glu-tRNA^{Gln} and Asp-tRNA^{Asn} are converted to the correct forms by transamidation (1, 2, 18).

The genomes of the methanoarchaea *Methanococcus jannaschii* and *M. thermoautotrophicum* both lack cysteinyl-tRNA synthetases. In these organisms, proline tRNA synthetase catalyzes the formation of prolyl-tRNA^{Pro} and cysteinyl-tRNA^{Cys} (10, 15). Incorrect charging of the tRNAs does not occur, indicating that an additional protein might aid in the specificity of the cysteinyl-tRNA synthetase activity (10).

Methanosarcina thermophila

M. thermophila, the organism studied in this thesis, is a thermophilic methanogen. *M. thermophila* was isolated from a sewage digester by Zinder and Mah in 1979 (22). The organism is capable of utilizing acetate, methanol, and trimethylamine as substrates. Like all methanogens *M. thermophila* is a strict anaerobe. At low salt concentrations the optimum growth temperature is 50^{o}C, and the organism grows in distinct, macroscopic clumps. Under high salt (marine) conditions, the cells do not aggregate, and the optimum growth temperature drops to 37^{o}C.

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Chapter 2

Physiological functions and biosynthesis of cysteine

Functions of L-Cysteine

L-Cysteine is one of twenty common protein amino acids. The side chain (-CH_2SH) contains a reactive sulfhydryl as functional group. The pK_a value of the sulfhydryl group of unbound cysteine is 8.2. However, this value can shift within the range of 8-9.5 depending on the protein environment. Once inserted into the protein chain, cysteine usually performs one of three important functions with the sulfhydryl group: stabilization of the proteins overall structure through disulfide bond formation; binding of cofactors such as iron-sulfur clusters; and direct involvement in catalysis. In addition, L-cysteine is the major source of sulfur for the synthesis of sulfur-containing compounds.

Sulfur containing cofactor biosynthesis

Eight sulfur-containing cofactors have been identified in living systems: S-adenosylmethionine, coenzyme M, CoB, coenzyme A, molybdopterin, thiamin pyrophosphate, lipoic acid, and biotin (7) (Figure 2-1). The sulfur for all of these cofactors originates from cysteine, except S-adenosylmethionine where the sulfur derives from methionine (7).

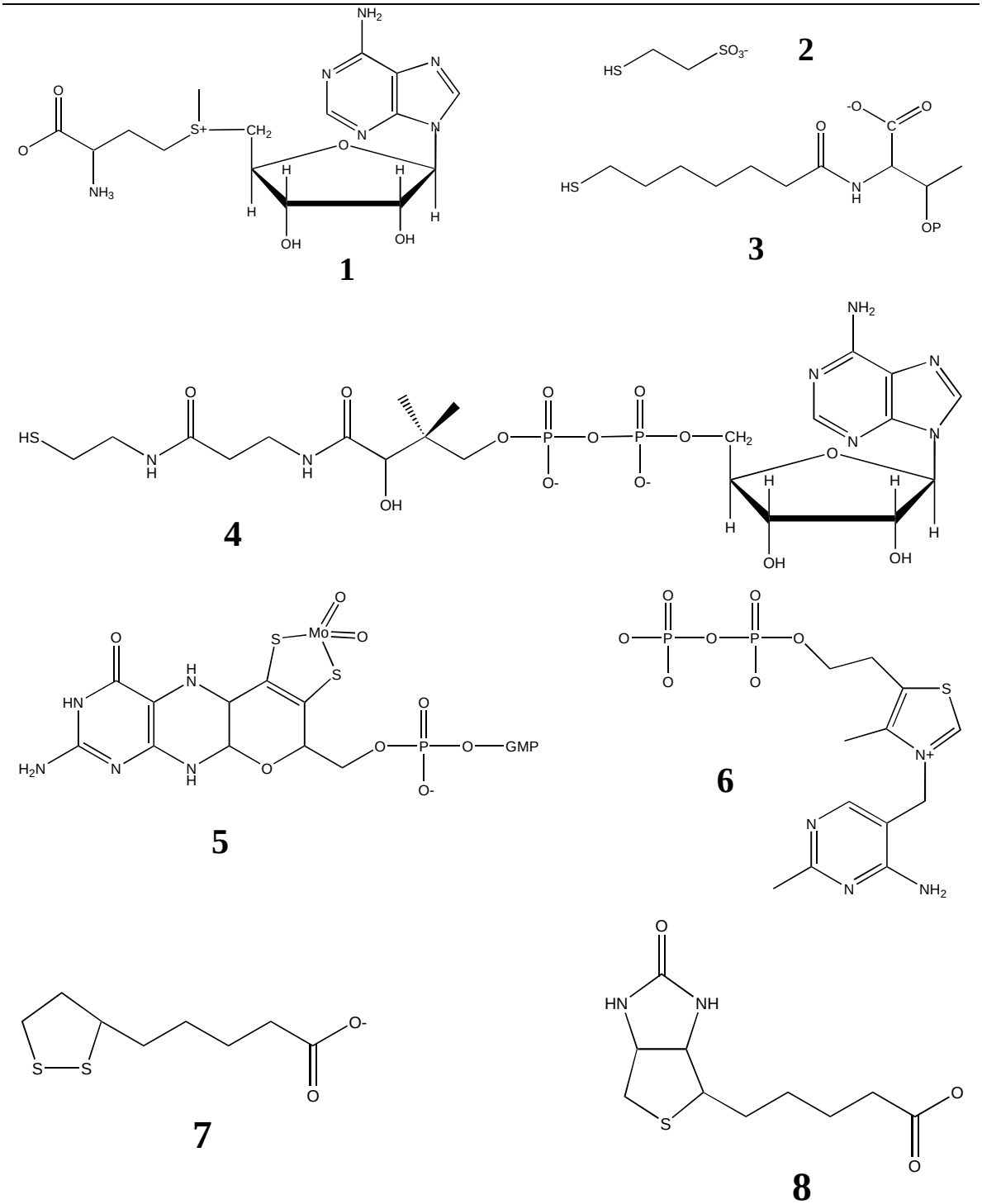

Figure 2-1: Structures of the eight sulfur containing cofactors: (1) S-adenosylmethionine, (2) coenzyme M, (3) N-(7-mercaptoheptanoyl) threonine phosphate, (4) coenzyme A, (5) molybdopterin, (6) thiamin, (7) lipoic acid, (8) biotin.

S-adenosylmethionine is mostly involved in biochemical methylation reactions. The cofactor is also involved in the biosynthesis of biotin and lipoic acid. Biosynthesis of S-adenosylmethionine is straightforward. Methionine is alkylated by ATP with the help of S-adenosylmethionine synthetase (MetK).

Coenzyme M and CoB are unique cofactors used in methanogenesis and, until recently, were believed to be found only in the methanoarchaea. However, coenzyme M was recently discovered in the gram-negative bacterium *Xanthobacter* strain Py2, where the cofactor is the thiol and central cofactor of aliphatic epoxide carboxylation (2). In epoxide carboxylation a toxic C_3 molecule is combined with CO_2 to form acetoacetate, a central C_4 metabolite. Sulfur incorporation into CoB is believed to follow a similar pathway as that of lipoic acid because of their similar structure and because the sulfide derives from cysteine, but the C-S bond in cysteine does not remain intact during incorporation (107). Coenzyme M contains two sulfur moieties. The sulfide sulfur is believed to derive from the direct insertion of cysteine. The sulfite sulfur is believed to derive from direct bond formation between HSO_3^- and phosphoenolpyruvate. The exact mechanism, however, is unknown for these reactions (107).

Coenzyme A functions as an acyl carrier. Coenzyme A is synthesized using the coenzyme pantothenic acid as precursor. Pantothenate is phosphorylated and cysteine is then directly incorporated to yield phosphopantothenoylcysteine, which is then converted to coenzyme A (42).

Molybdopterin is the cofactor that binds molybdenum and tungsten in oxidoreductases (6). Thiamin, involved in acyl transfer, helps stabilize the acyl carbanion intermediate and is very important in carbohydrate metabolism (32). Based on sequence analysis, the sulfur insertion chemistry of molybdopterin and that of thiamin are likely to be similar (80). In thiamin biosynthesis ThiS, a 66-amino acid peptide, is posttranslationally modified by ThiF, ThiI, and ThiJ to form ThiS-thiocarboxylate (ThiS-COSH). The sulfur source in this reaction is cysteine. ThiS-thiocarboxylate is the immediate sulfur source during thiamin biosynthesis. It is possible that this complex sulfide donor is needed to keep small reactive intermediates bound to a protein during thiazole formation. Thiazole is then converted into thiamin (5).

Lipoic acid is involved in acyl group transfer and 2-electron redox reactions. Biotin is involved in bicarbonate-dependent carboxylations. Based on sequence analysis, the sulfur insertion chemistry of lipoic acid is likely to be similar to that of biotin (34, 81). Both biotin synthase (90) and lipoate synthase (18) contain an iron-sulfur cluster. Biotin synthase from *E. coli* is a homodimer and each subunit contains a [2Fe-2S] cluster. When the enzyme is reduced, the clusters dimerize to give a [4Fe-4S] cluster, which is most likely the catalytically active species (26). During catalysis two S-adenosylmethionines activate the 4Fe-4S cluster sequentially and in the process one of the sulfur atoms from the 4Fe-4S cluster is inserted into biotin (7). The 4Fe-4S cluster probably is regenerated by a NifS-like protein.

Iron-sulfur cluster formation

Diverse metalloenzymes in nature contain iron-sulfur clusters. They are found in enzymes needed for photosynthesis, nitrogen fixation, oxidative phosphorylation (8) and cofactor biosynthesis (18, 90). Iron-sulfur clusters are involved in electron transport, catalysis, stabilization of protein structure, and regulation of metabolic pathways. In addition, they play roles in radical formation (30).

Most iron-sulfur clusters contain 2 or 4 irons held together by bridging sulfur atoms (Figure 2-2). These clusters are bound to the protein through cysteine ligands. Since iron-sulfur clusters can form spontaneously under anaerobic conditions, it is believed that they are one of the most ancient biologically active metal cofactors (8). However, the high concentration of sulfur required for spontaneous iron-sulfur cluster formation would be toxic to cells (30). Instead, it is believed that cluster formation *in vivo* is catalyzed by enzymes. It is unclear if iron or if sulfur is inserted first into the apoenzyme. Both types of intermediates, apoprotein containing 4 sulfur (30) and apoprotein containing 4 iron (68), have been observed.

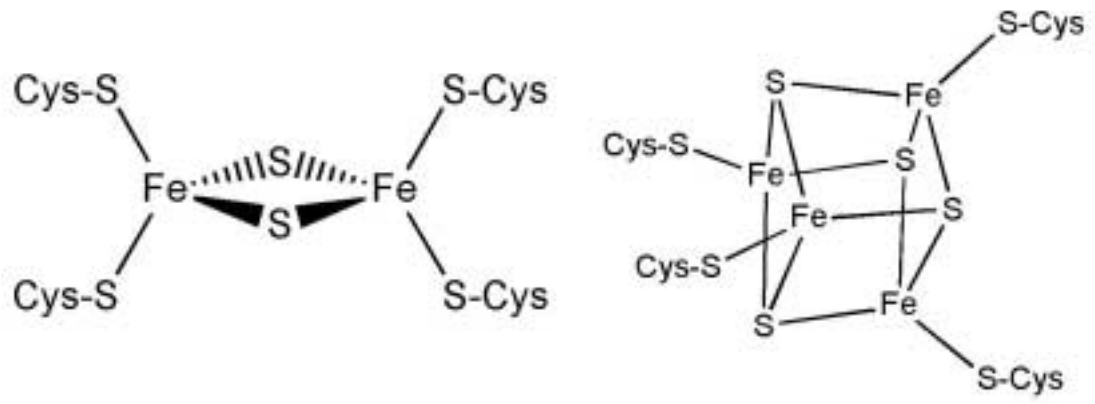

Figure 2-2: Schematic representation of two types of iron-sulfur clusters.

The NifS and NifU proteins are thought to be involved in FeS cluster formation. NifS was first discovered in *Azotobacter vinelandii* as one of a dozen enzymes required for nitrogen fixation, though it is not part of the nitrogenase enzyme (43). Deletion of the *nifS* and *nifU* genes in *Klebsiella pneumoniae* (83) or *A. vinelandii* (43) resulted in a nitrogenase enzyme with very low Fe protein activity and lowered levels of MoFe protein activities. This suggests that NifS and NifU are required for activation or stabilization of the Fe protein of the nitrogenase enzyme. Subsequently, overexpression of the *A. vinelandii* NifS protein in *Escherichia coli* revealed that NifS catalyzes the desulfuration of cysteine (115). In addition, the sulfide in iron-sulfur centers was found to originate from cysteine *in vivo* (106). NifU may function in cluster formation by sequestering inorganic Fe, by aiding in the release of sulfide from NifS (21), or by donating Fe_2S_2 (112). NifS and NifU homologs, IscS and IscU, respectively, have been found in organisms that do not fix nitrogen, suggesting a universal role for these enzymes in Fe-S cluster formation (113). This was confirmed through an IscS knock-out mutant in *E. coli*, which was found to have decreased specific activities for proteins containing Fe_4S_4 clusters (93).

Recently, it has been shown that NifS interacts with NifU, and that one labile, transient Fe_2S_2 cluster is formed per NifU dimer. This Fe_2S_2 cluster is rapidly released upon reduction (112). Similar work with IscS and IscU determined that as the ratio of IscS vs IscU is increased from 1:460 to 1:28, the amount of Fe_2S_2 clusters on IscU in the

presence of Fe^{3+} and cysteine increased from one to two clusters per dimer. Within 10 hours of incubation the two clusters combined to form one Fe_4S_4 cluster per IscU homodimer. These results indicate that IscU can potentially donate both Fe_2S_2 and Fe_4S_4 clusters to other proteins (1).

NifS and IscS catalyze the desulfuration of cysteine to form alanine and sulfur (Figure 2-3). The sulfur can then be transferred to NifU or IscU, respectively, for incorporation into FeS clusters. The crystal structure of the NifS-like protein from *Thermotoga maritima* gives good insight into the reaction mechanism of NifS (47).

In the absence of substrate, NifS and IscS have a characteristic absorbance at 392nm, indicative of a pyridoxal phosphate bound to the enzyme through a lysine residue (Figure 2-3, intermediate 1) (115). This lysine residue is also the most likely residue for α-hydrogen elimination from the substrate, due to its location in the active site (47).

The histidine residue, which is strictly conserved in the NifS-like protein family, seems to play an important role in catalysis. The residue is stacked against the enzymes cofactor and thus stabilizes the pyridoxal 5'-phosphate ring. Because of electrostatic forces the protonated (acidic) histidine favors the quinonoid form of the co-factor, while a deprotonated (basic) histidine stabilizes the aromatic form (47). Owing to its location in the active site, the histidine is also the most likely candidate to perform most of the protonation and deprotonation steps, except for the elimination of the α-hydrogen of the substrate (47).

Upon reaction of the substrate L-cysteine to the enzyme, the absorbance of the enzyme shifts to 416 nm in addition to a new peak at 370 nm (115). This spectral change was not observed with the other L-amino acids and D-cysteine, indicating that the enzyme is specific to L-cysteine. Similar spectral changes were observed upon incubation of NifS-like proteins from *E. coli* (29), *Synechocystis* PCC 6803 (44) and *T. maritima* (47).

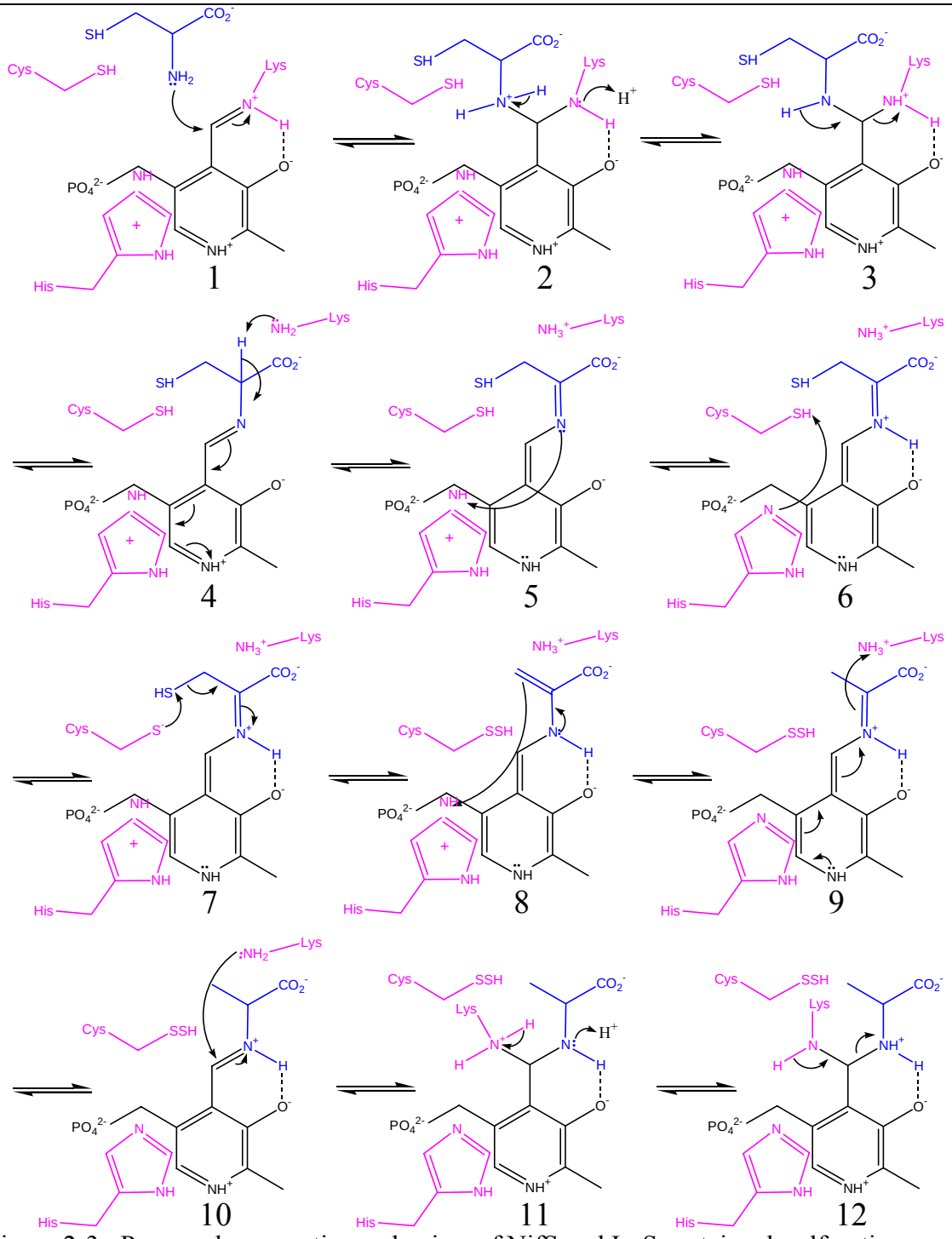

Figure 2-3: Proposed enzymatic mechanism of NifS and IscS cysteine desulfuration (47)(114). After sulfane sulfur is removed from the cysteine residue in [12], the enzyme returns to [1]. Intermediates: internal [1] and external [4] aldimine; geminal diamine [2, 3, 11, 12]; substrate [5, 6, 7] and product [9] ketimine quinonoid; α-aminoacrylate quinonoid [8]; and eneamine [10].

Substrate quinonoid ketimine (Figure 2-3, structure 7). Upon incubation of NifS from *T. maritima* with cysteine an absorption peak at 510 nm was noted (47). Absorption at 510 nm is indicative of a quinonoid intermediate. This peak was not observed upon incubation of the NifS-like protein from *Synechocystis* (44). One possible explanation is that the depletion of the quinonoid intermediate is fast, resulting in no significant accumulation of cofactor in the quinonoid form compared to the geminal diamine state, and that the quinonoid intermediate was therefore not detected in UV-VIS spectroscopy. A different explanation for the lack of absorbance at 510 nm could be that the NifS-like protein from *Synechocystis* catalyzes cysteine desulfuration by a different mechanism in which no quinonoid intermediate is formed. Further data is necessary to determine which explanation is correct.

Product quinonoid enimine (Figure 2-3, structure 8). A reactive cysteine residue in the active site was found to play an active role in catalysis, because NifS was easily inactivated by alkylating reagents, such as *N*-ethylmaleimide (114). Through cross-linking experiments with the fluorescent marker 1,5-I-AEDANS, which reacts with thiols, it was discovered that the reactive cysteinyl forms a persulfide bond during catalysis. In the absence of cysteine, 1,5-I-AEDANS reacted with the thiol group of the reactive cysteinyl of NifS and thus inactivated the enzyme. In the presence of cysteine, 1,5-I-AEDANS reacted with the persulfide formed on the reactive cysteine residue on NifS, and 81% of the total fluorescent marker was released upon addition of DTT (115). The identity of the reactive cysteine residue was determined by reaction of NifS with the suicide inhibitors L-allylglycine and L-vinylglycine and identification of the altered residue (114). This cysteine residue is conserved among NifS and IscS enzymes (115). The identity of the reactive cysteinyl was confirmed through site-directed mutagenesis in which the cysteine residue was replaced by alanine. Replacement of the cysteine residue by alanine resulted in an inactive enzyme (114). However the enzyme could still react with cysteine to form the substrate ketimine adduct (Figure 2-3, structure 7) as was determined by spectral analysis of the Cys to Ala mutant NifS enzyme upon incubation with cysteine (44).

Formation of the disulfide bond seems to be the slow step in the desulfuration of cysteine by NifS. After incubation of NifS in deuterated water with cysteine, it was found that all remaining cysteine in the reaction mix was deuterated at the α-position (114). Thus all cysteine had reacted with NifS to form the quinonoid intermediate (Figure 2-3, structure 5), but not all cysteine was desulfurated to form the eneamine intermediate (Figure 2-3, structure 8).

Product ketimine quinonoid (Figure 2-3, structure 9). During formation of alanine from cysteine in the presence of deuterated water, all three β hydrogens of alanine were exchanged (114), suggesting a rapid exchange between the eneamine and ketimine quinonoid intermediates (Figure 2-3, structures 8 and 9).

Eneamine (Figure 2-3, structure 10). During formation of alanine from cysteine in the presence of deuterated water, all α-hydrogens were also exchanged, providing evidence for the formation of the enamine intermediate (114). Binding and dissociation of alanine with NifS is another slow step in the overall reaction of cysteine to alanine. Millimolar amounts of alanine do not inhibit NifS (114). In addition, when NifS was incubated with alanine in the presence of deuterated water, a small fraction of the alanine had their α-hydrogen, and all three β-hydrogens exchanged; whereas the rest of the alanine did not contain any deuterium. Thus only a small fraction of the alanine bound to the pyridoxal 5'-phosphate cofactor of NifS, yet once bound, the rapid equilibrium between the eneamine and ketimine intermediates resulted in complete exchange of all α– and β–hydrogens (114).

In the absence of a reductant in the reaction mix, the sulfane sulfur formed on the reactive cysteinyl of NifS is released in the form of sulfur. In the presence of a reductant such as DTT the sulfane sulfur is released from the enzyme as sulfide (29). The rate of release of the sulfur increases from a k_{cat} of 0.5 min^{-1} in the absence of DTT to a k_{cat} of 3 min^{-1} in the presence of DTT. Considering these slow rates of release, it has been hypothesized that under cellular conditions the sulfane sulfur remains on the enzyme until direct transfer to a second enzyme, such as NifU, can occur (29). The reactive cysteine residue is part of a highly conserved series of small and hydrophilic residues, which form

a highly flexible loop in *T. maritima* (47). The function of this loop could potentially be to deliver the sulfane sulfur directly into sites distant from the NifS active site.

Apart from NifS and IscS, two other enzymes have been found to desulfurate cysteine and participate in Fe-S cluster formation *in vitro*: *O*-acetylserine sulfhydrylase and cystathionine-β-lyase (30). However, desulfuration is not considered to be the primary physiological role for either of these enzymes.

Sulfur assimilation

In anaerobic environments H_2S accumulates, and some strict anaerobes, including methanogens, can only use this as a sulfur source. The most common sulfur source for bacteria living in aerobic environments is organic compounds. Cysteine and methionine are directly incorporated into the cell. All other organic sulfur is metabolized to sulfate. Sulfate is then reduced to sulfide via four enzymatic steps (Figure 2-4, reactions 1-4) in which 3 high energy phosphate bonds are broken, and 4 NADPH are oxidized (69).

Sulfur reduction requires initial "activation" of the sulfur to a phosphosulfate. ATP sulfurylase combines sulfate and ATP to form adenosine-5'-phosphosulfate (APS) and pyrophosphate. Plants, yeast and some bacteria can then reduce APS to sulfite or a sulfite derivative. However, in other organisms such as *Escherichia coli* and *Salmonella*, APS must be further activated. APS kinase phosphorylates APS to form 3'-phosphoadenosine 5'-phosphosulfate (PAPS). PAPS is then reduced to sulfite by PAPS reductase with thioredoxin serving as the electron donor. Sulfite is finally reduced to sulfide by sulfite reductase using NADPH as the electron donor (54).

O-acetylserine sulfhydrylase and homocysteine synthase are the only two known enzymes in nature to fix sulfur (Figure 2-4, reactions 6 and 8). This fixed sulfur then enters the metabolism of the organism as cysteine (the product of the *O*-acetylserine sulfhydrylase reaction), or as homocysteine (the product of the homocysteine synthase reaction). Homocysteine is then converted to cysteine or methionine.

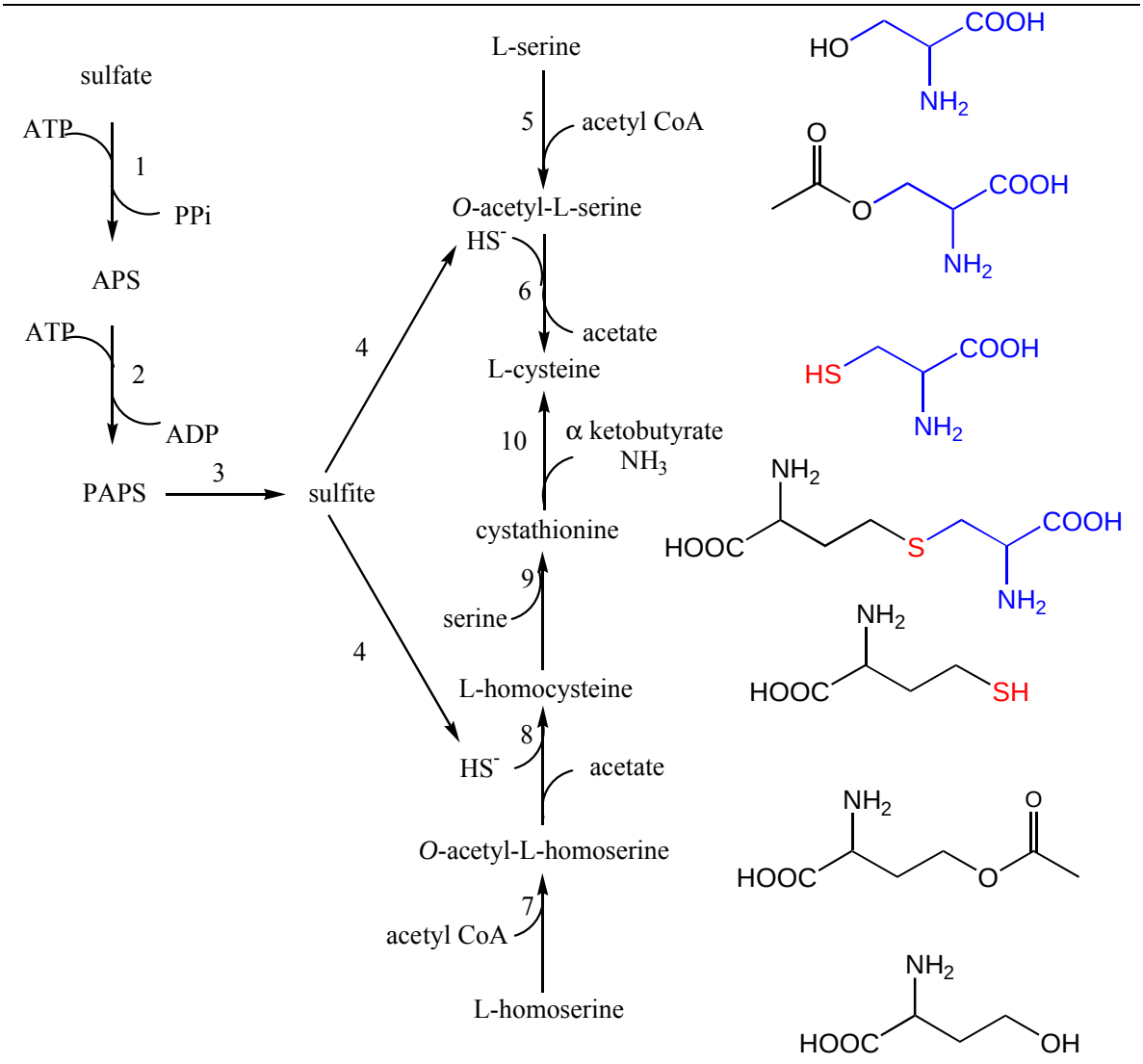

Figure 2-4: Known cysteine biosynthetic pathways. Enzymes catalyzing the steps are: 1, ATP sulfurylase; 2, APS kinase; 3, PAPS reductase; 4, sulfite reductase; 5, serine transacetylase; 6, *O*-acetylserine sulfhydrylase; 7, homoserine transacetylase; 8, homocysteine synthase; 9, cystathionine β-synthase; 10, cystathionine γ-lyase.

Methionine synthesis and degradation

Methionine synthase catalyzes the formation of methione from homocysteine and methyl-tetrahydrofolate. Methionine γ-lyase degrades methionine to α-ketobutyrate, ammonia, and methanethiol (101). In aerobic environments, methanethiol is most likely converted to methanesulfonate, and the methanesulfonate is desulfonated by alkanesulfonatase to release sulfite, which enters the sulfur assimilation pathway (105). In anaerobic environments methanethiol is directly degraded to methane, carbon dioxide, and sulfide by methanogens (61, 118).

The released sulfide can reenter the cysteine biosynthetic pathway through either of the two sulfur fixation enzymes. However, a second route exists for movement of sulfide from methionine to cysteine. In this route, methionine is converted to the cofactor S-adenosylmethionine which is then degraded to form homocysteine. Homocysteine enters the reverse transsulfuration pathway and is converted into cysteine.

The two Archaea *Sulfolobus acidocaldarius* and *Halobacterium marismortui* were able to transfer the sulfide from ^{34}S-labeled methionine into protein-bound cellular cysteine (116); however, it remains unclear if these organisms used the reverse transsulfuration pathway or the pathway *via* methanethiol to transfer the sulfide.

Cysteine biosynthesis

Two routes for cysteine biosynthesis in nature have been documented. Serine transacetylase and O-acetylserine sulfhydrylase catalyze steps in pathway I (Figure 2-4, reactions 5-6) (64). Cystathionine β-synthase and cystathionine γ-lyase catalyze steps of pathway II (Figure 2-4, reactions 9-10) (64). Pathway II is also known as the reverse transsulfuration pathway. Plants and members of the Bacteria domain synthesize cysteine and fix sulfur *via* pathway I, and also fix sulfur by synthesizing homocysteine; however, most cannot utilize homocysteine for cysteine biosynthesis (66, 72). One major exception are the filamentous bacteria, the Actinomycetaceae, which do contain both enzymes of the reverse transsulfuration pathway (66).

Fungi fix sulfide and several can synthesize cysteine using pathways I and II. *Saccharomyces lipolytica* seems to utilize both pathways for cysteine biosynthesis, and only when both pathways are simultaneously impaired does the organism become a cysteine auxotroph (64). *Neurospora crassa* can also synthesize cysteine using both pathways; however, pathway II is unlikely to play an important physiological role as long as synthesis of cysteine from acetylserine is not impaired (76). Similarly, in *Aspergillus nidulans* pathway I must be impaired (e.g. through a mutation) before the organism uses pathway II for cysteine biosynthesis (77). On the other hand, although enzymes for both pathways are present in the yeast *Saccharomyces cerevisiae*, only pathway II is used for cysteine biosynthesis (17). The yeasts *Shizosaccharomyces pombe* (11), *Pichia membranofaciens* and *Candida valida* (78, 79) lack cystathionine β-synthase and cystathionine γ-lyase, indicating that these organisms must synthesize cysteine through pathway I.

Although the genomes of seven members of the Archaea have been sequenced to date, the data offer little understanding of cysteine biosynthesis. The *M. jannaschii* (12), *M. thermoautotrophicum* (95) and *Archaeoglobus fulgidus* (52) genomes contain no open reading frames (ORFs) having a deduced sequence with significant identity to enzymes of either pathway for cysteine biosynthesis. The genome of *Aeropyrum pernix* (49) contains ORFs with deduced sequence similarity to cystathionine β-synthase and cystathionine γ-lyase. The genomes of *Halobacterium* sp. NRC-1 (70), *Pyrococcus horikoshii* (50) and *Thermoplasma acidophilum* (86) contain ORFs with deduced sequence similarity to cystathionine γ-lyase. *Halobacterium* sp. NRC-1 also contains ORFs with deduced sequence similarity to *O*-acetylserine sulfhydrylase and serine acetyltransferase. However, it is not known whether these genes are expressed or if the gene products have the expected enzyme activities. No ORFs are present in the genomes of *A. pernix*, *P. horikoshii* and *T. acidophilum* with a deduced sequence having significant identity to enzymes of pathway I for cysteine biosynthesis.

Enzymes involved in cysteine biosynthesis

Serine acetyltransferase (SAT)

SAT catalyzes the formation of *O*-acetylserine and CoA from serine and acetylCoA (Figure 2-4, reaction 5). SAT has been purified and characterized from plants (67, 89), and bacteria (56). From the bacteria, one isoform was purified, while in the plants multiple isoforms localized in the cytoplasm, mitochondria and chloroplast exist (87). SAT from *Escherichia coli* (38) is a loosely packed dimer of trimers and all SATs purified have a monomer subunit molecular mass of 30-35 kDa. The N-terminus is important for binding of monomers in SAT (10). For all purified SATs the K_m for serine ranges from 0.56 mM to 5.1 mM , and the K_m for acetylCoA ranges from 0.1 mM to 0.26 mM.

The bacterial and some of the plant SATs form a complex with *O*-acetylserine sulfhydrylase (OASS); which catalyzes the formation of cysteine from *O*-acetylserine and sulfide (117). It has been suggested that the dimers of OASS are located at opposite ends of the stacked SAT trimers (38). The reason for complex formation is unclear, especially since *O*-acetylserine synthesized by SAT is not channeled to OASS (19). Instead, *O*-acetylserine is released to the environment and must bind to another OASS for conversion (59). In bacteria, and in the cytosolic isoform of SAT in plants, the SAT/OASS complex dissociates in the presence of 1 mM *O*-acetylserine. Binding of SAT to OASS decreases activity of OASS to 5%, but does not affect SAT activity (63). Deletion experiments suggest, that the C-terminus of *E. coli* SAT is involved in complex formation with OASS.

Bacterial and plant cytosolic SATs are feedback inhibited by micromolar concentrations of cysteine (56), though the enzyme is not inhibited by serine – which is astonishing since the two structures are so similar. There seems to be no relationship between complex formation and inhibition by cysteine (63), even though the C-terminus, which is involved in complex formation, is also involved in feedback inhibition by

cysteine (63). SAT and the product of the reaction, *O*-acetylserine, are the major regulatory factors in the biosynthesis of cysteine. Availability of *O*-acetylserine seems to be the rate-limiting step for cysteine synthesis *in vivo* (33). OASS is always in excess of SAT; sometimes as high as 345 fold (10). *O*-acetylserine not only serves as metabolite in cysteine biosynthesis, but the metabolite also controls the pathway itself, by inducing sulfur transport enzymes (59), and increasing activity of OASS by dissociating SAT from OASS. Overexpression of OASS has no affect on cysteine biosynthesis (88); however, increasing the level of SAT expression (33), or decreasing cysteine feedback inhibition (100) leads to increased amounts of cysteine in the cell.

_O_-acetylserine sulfhydrylase (OASS)

OASS catalyzes the formation of cysteine and acetate from O-acetylserine and sulfide (Figure 2-4, reaction 6). OASS is a pyridoxal phosphate dependent enzyme, which has been found in both the Eukarya and Bacteria domains of life. The enzyme is widely distributed in the Bacteria domain (16, 36). OASS has been purified from plants (*Spinacia oleracea* (25, 65, 110), *Capsicum annuum* (84), *Citrullus vulgaris* (39), *Pisum sativum* (41), *Phaseolus* (9), and *Datura inoxia* (57)), algae (*Chlamydomonas reinhardtii* (58)), fungi (*Cephalosprium acremonium* (23)), and Bacteria (*Salmonella typhimurium* (4, 55), *E. coli* (30), *Rhodospirillum tenue* (37), and *Paracoccus denitrificans* (15)). In most of these organisms, multiple isoforms have been purified. OASS is usually a homodimer with subunit molecular mass ranging from 30 to 35 kDa, though one OASS purified from *D. inoxia* is a heterodimer (57). The K_m for sulfide ranges from 0.02 to 2.7 mM, and the K_m for *O*-acetylserine ranges from 1.25-50 mM. Of the enzymes purified, *S. typhimurium cysM* (55) exhibites positive cooperativity with respect to *O*-acetylserine when bound to serine transacetylase, and one isoform from *S. oleracea* exhibites positive cooperative with respect to *O*-acetylserine in the presence of sulfide. The enzyme from *D. inoxia* exhibits positive cooperativity with respect to both substrates (57), and the enzyme from *Phaseolus* exhibits positive cooperativity with respect to sulfide (9).

Cysteine is the major source of sulfide in the cell, and is the first organic sulfur metabolite. Thus cysteine plays an important role in the assimilation of sulfide during sulfur starvation. Under sulfur starvation conditions, the enzyme is upregulated in *Arabidopsis thaliana* (35), cultured tobacco, *Chlamydomonas reinhardtii* cells and maize leaves (3). In *Synechococcus* sp. PCC the *cysK* and *cysE* genes are encoded on a plasmid. The function of the enzymes encoded on the plasmid seems to be to compete for available sulfur. The genes are expressed under sulfur limiting conditions (71). In phototrophic Bacteria it was found that OASS is repressed when the organism is grown on cysteine, sulfide, and thiosulfate as sulfur sources (36).

However, the catalytic capacity of plant OASS enzyme exceeds sulfur assimilation needs by several 100-fold (111). Therefore, different isoforms of OASS from several plant species have been implicated in the process of detoxification, especially of gaseous pollutants, such as SO_2, H_2S and HCN (40). Tobacco plants transformed with the OASS gene of wheat are resistant to toxic levels of H_2S (111). In addition, the catalytic capacity of this enzyme exceeds sulfur assimilation needs by several hundred fold (111).

Apart from cysteine biosynthesis, sulfur assimilation, and cell detoxification, some OASS enzymes also have additional functions such as formation of selenocysteine (15), formation of beta-substituted alanines (39, 41, 65) and formation of L-quisqualic acid and L-mimosine (41). OASS from *Phaseolus* and *E. coli* are also capable of catalyzing the desulfuration of cysteine, though this reaction is 100-1000 fold slower than the cysteine biosynthetic reaction (9, 30).

The reaction mechanism of OASS from *S. typhimurium* has been characterized extensively. Using steady state kinetics it was determined that the enzyme catalyzes a Bi Bi Ping Pong reaction with competitive substrate inhibition by both *O*-acetylserine and sulfide (20). The first half reaction consists of *O*-acetylserine reacting with the pyridoxal phosphate, and the β-elimination of acetate (Figure 2-5, intermediates 1 - 6) to form the α-aminoacrylate (Figure 2-5, intermediate 6). This half reaction is reversible, though only at high acetate concentrations (0.4 M acetate) (20). The second half reaction consists of the reaction of sulfide with the α-aminoacrylate to form cysteine (Figure 2-5,

intermediate 6 - 11). This half reaction is also reversible (20). However, the reaction is practically irreversible in the direction of conversion of cysteine to *O*-acetylserine.

Rapid-scanning stopped flow experiments suggested that the first half reaction is the overall rate limiting step in the formation of cysteine from *O*-acetylserine and sulfide (108). The second half reaction proceeds at a rate near the diffusion limit (98). Sulfide gives only partial competitive substrate inhibition suggesting that a pathway in which sulfide binds prior to *O*-acetylserine is allowed (20, 98). The crystal structure of OASS indicates the presence of a hole behind pyridoxal 5'-phosphate where sulfide could bind before *O*-acetylserine (13), thus accounting for the observed inhibition pattern with respect to sulfide. A variety of substrates can substitute for the sulfide; and substrate specificity appears to depend more on the pK_a of the nucleophile than its structure (99).

Internal aldimine (Figure 2-5, structure 1). In the absence of substrate the pyridoxal 5'-phosphate is bound to the enzyme *via* the ε-amino group of a conserved lysine group (K41 in *S. typhimurium* OASS), forming an internal aldimine. The internal aldimine tautomerizes between the enolimine and the ketoenamine forms (Figure 2-6). The enolimine absorbs at 310-340 nm. The ketoenamine can exist in two resonance forms and absorbs at 400-430 nm (99). The large absorbance at 412 nm of OASS indicates the predominance of the ketoenamine form over the enolimine form of the internal aldimine of this enzyme. However, the slight absorbance at 330 nm indicates that some of the internal aldimine is in the enolimine tautomer conformation (4, 20).

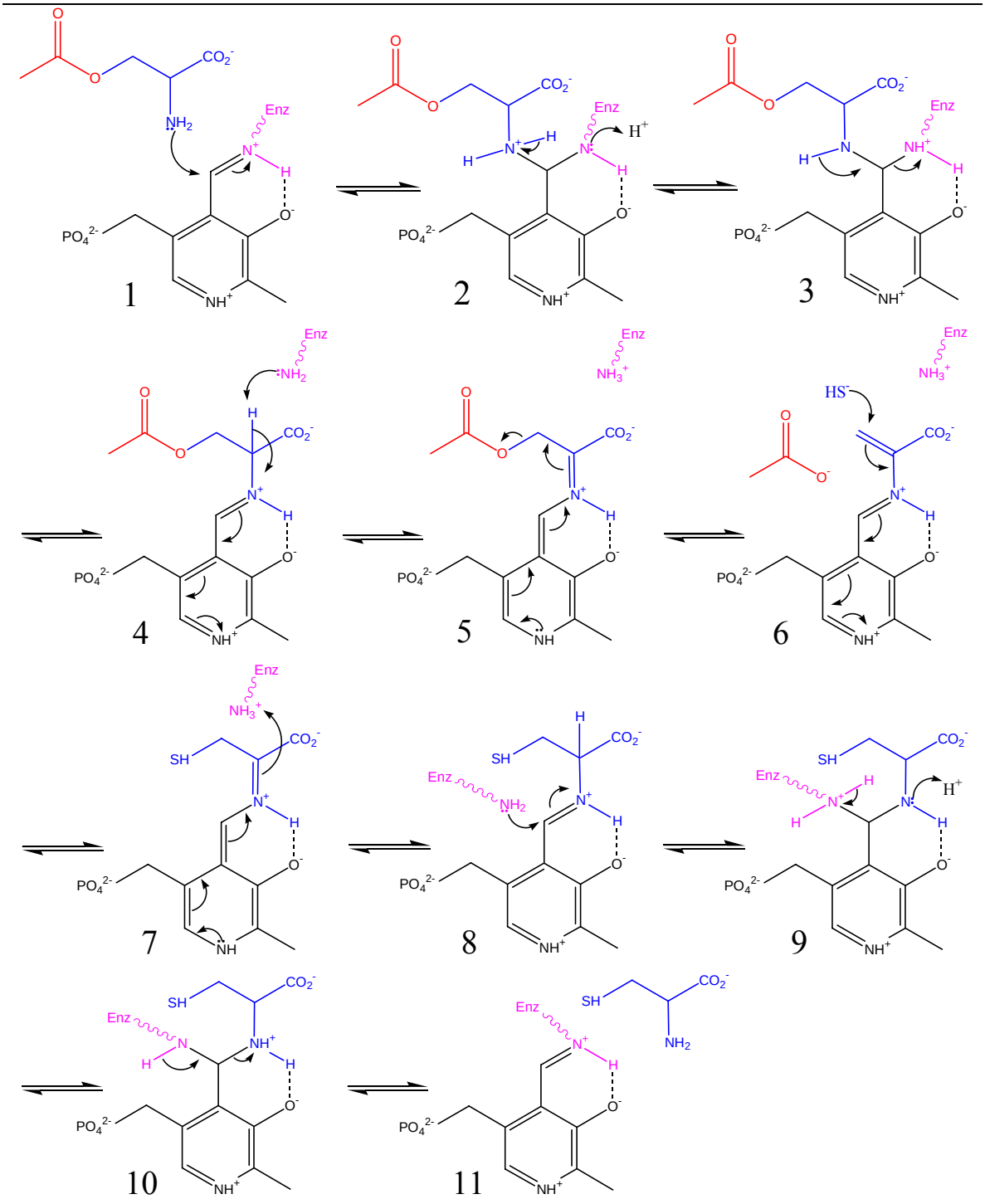

Figure 2-5: Proposed enzymatic mechanism of *O*-acetylserine sulfhydrylase (99). Intermediates: internal [1, 11] and external [4, 8] aldimine; geminal diamine [2, 3, 9, 10]; substrate [5] and product [7] ketimine quinonoid; and α-aminoacrylate [6].

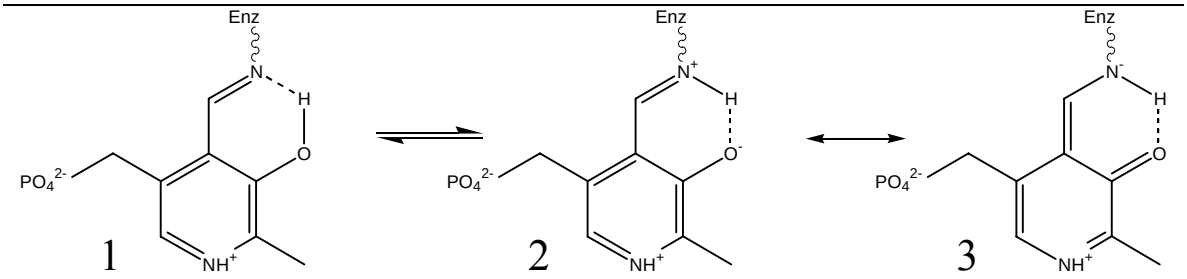

Figure 2-6: Tautomeric and resonance forms of the internal aldimine. Structures: 1, enolimine; 2 and 3, ketoeneamine resonance forms.

Geminal diamine (Figure 2-5, structures 2 and 3)**.** The geminal diamine intermediate is formed *via* nucleophilic attack of the amino group of *O*-acetylserine at the C4' of the pyridoxal 5'-phosphate imine of the internal aldimine. After an intermolecular proton transfer from the ε-amine of the lysine to the amino group of *O*-acetylserine, the C4-C4' bond rotates to place the leaving ε-amino group of the lysine orthogonal to the pyridine ring (99) (Figure 2-7). Bonds that are orthogonal to the pyridoxal 5'-phosphate are inherently labile (99). The geminal diamine absorbs at 320-340nm (99), however, no spectral evidence has been obtained for a gem-diamine with OASS.

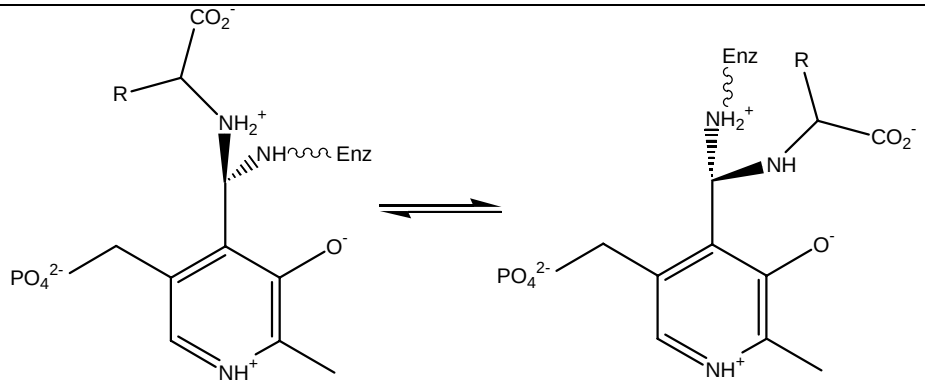

Figure 2-7. The gem-diamine structures. The left structure shows attack by incoming α-amine of amino acid; the right structure is rearranged in order to expel the Schiff base lysine of the internal amine.

External aldimine with *O*-acetylserine (Figure 2-5, structure 4)**.** Upon collapse of the geminal diamine, an external aldimine between *O*-acetylserine and pyridoxal 5'-phosphate is formed. The external aldimine has similar tautomer and resonance structures as the internal aldimine, and the absorption due to these structures is similar (99).

Although the exact *O*-acetylserine binding in external aldimine form has not been determined, an analog of the structure has been obtained by crystallizing a K41A mutant of *S. typhimurium* in the presence of methionine (14). K41 is the lysine that forms the internal Schiff base. Analysis of the crystal structure provided evidence that upon binding of *O*-acetylserine the enzyme apparently undergoes a conformational change, resulting in closure of the active site from the surrounding environment. The conformational change seems to be triggered by the acetyl group of the substrate.

Quinonoid intermediate (Figure 2-5, structure 5)**.** A quinonoid intermediate is formed upon abstraction of the α-proton of bound *O*-acetylserine, and delocalization of the electrons into the pyridine ring. For this abstraction to occur the α-proton must be orthogonal to the pyridoxal 5'-phosphate bond. Quinonoid structures absorb at about 500 nm (99). Although spectral evidence for a quinonoid intermediate is available for other pyridoxal 5'-phosphate enzymes (24), no spectral evidence was obtained for such intermediate in OASS. Considering the excellent nature of acetate as a leaving group and the absence of spectral evidence for a quinonoid intermediate, it seems plausible that the reaction mechanism follows through a concerted α-β elimination (Figure 2-8), and a full quinonoid intermediate does not form.

The ε-amine of K41 (the lysine that forms the internal aldimine with the pyridoxal phosphate) acts as the general base during α-elimination of the proton. This has been determined through pH studies (97) and further substantiated with the K41A mutant in which catalysis ends with the formation of the external Schiff base with *O*-acetylserine (82).

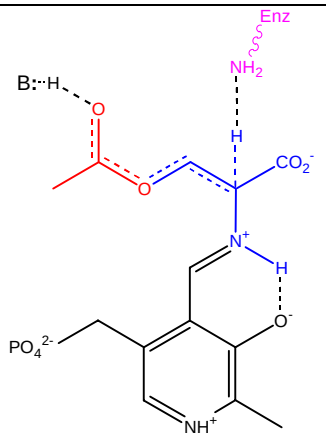

Figure 2-8. Transition state of concerted α-β elimination in the OASS mechanism.

α-aminoacrylate (Figure 2-5, structure 6)**.** An α-aminoacrylate in Schiff base with the pyridoxal 5'-phosphate is formed upon β–elimination of acetate. Again the leaving group (acetate) is aligned orthogonally to the π-bond to be formed, before abstraction can occur. The α-aminoacrylate mimics the tautomers and resonance structures of the internal and external aldimine. However, because of the additional π-bond, the absorption of the enolimine and ketoenamine shift slightly to longer wavelengths, 330 nm and 470 nm, respectively. In this intermediate a mixture of both the enolimine and the ketoenamine tautomers Figure 2-6 are present in OASS (20, 92).

The conformation of the enzyme seems to revert to an open form since addition of *O*-acetylserine to OASS does not result in a change in the far-UV CD, indicating that there is no change in the overall protein structure upon reaction with the first substrate (91). However, the open conformation of the α-aminoacrylate is distinct from the open conformation with the internal aldimine since the α-aminoacrylate intermediate is a mixture of the enolimine and ketoenamine structures, whereas the internal aldimine is most prevalent in the ketoenamine conformation (99).

External aldimine with cysteine (Figure 2-5, structure 8)**.** The α-aminoacrylate is very susceptible to nucleophilic attack in solution, though it is quite stable on some pyridoxal 5'-phosphate enzymes owing to the environment in which the active site resides. In OASS, sulfide in the HS^- form (97) attacks the α-aminoacrylate at the β-

position to form cysteine. Addition of the α-hydrogen seems to occur concurrently, since no evidence for a quinonoid intermediate (Figure 2-5, structure 7) at this stage was found (99). Finally cysteine is released into the environment by reversal of the geminal diamine intermediates (Figure 2-5, structures 9 – 10).

Addition of cysteine to OASS results in a significant change in the far-UV CD, suggesting a possible closing of the active site upon binding of the product (91). This conformational change was confirmed through fluorescence studies (62). Addition of cysteine to OASS also results in a shift in absorbance maxima to 418 nm (91).

Cystathionine β-synthase

Cystathionine β-synthase catalyzes the formation of cystathionine from serine and homocysteine (Figure 2-4, reaction 9); the first, and rate limiting (85), step of the transsulfuration pathway. Cystathionine β-synthase has been purified from fungi (73) and mammals (51, 53), and is a pyridoxal phosphate dependent homotetramer with subunit size ranging from 55 to 63 kDa. However, the enzyme undergoes proteolysis in the cells at the C-terminus to a more active dimer with a subunit size ranging from 39 to 48kDa (45). The C-terminus is also the site of S-adenosylmethionine binding (104), an allosteric activator of the mammalian and possibly the fungal enzymes (45, 73); once the C-terminus is removed the enzyme becomes unresponsive to allosteric regulation. Binding of S-adenosylmethionine to the enzyme decreases the K_m for homocysteine eight-fold (85). The N-terminal region forms the catalytic domain. The K_m for serine ranges from 0.67 to 8.3 mM, and the K_m for homocysteine ranges from 0.59 to 25 mM.

The activity and expression of cystathionine β-synthase does not change in response to sulfur starvation in the cells of *Neurospora* (28) and *S. cerevisiae* (74). However, cysteine does repress the level of activity in cells in *A. nidulans* (76).

The mammalian (51), but not the fungal (46), cystathionine β-synthase is a heme-dependent enzyme. Heme is incorporated into the protein during folding and is essential for the activity of the enzyme; heme incorporation is a prerequisite for pyridoxal

phosphate binding (51). It has also been suggested that the heme plays a catalytic role, by activating homocysteine (103). The heme-independent enzyme from yeast seems to bind homocysteine in a different manner, although the mechanism is yet unknown (45).

Cystathionine β-synthase has been studied extensively in humans because of the vital role the enzyme plays in detoxification of the cells of homocysteine. Marked deficiency of cystathionine β -synthase acitivity is the most common cause for homocystinuria, which causes dislocation of the optic lenses, vascular disorders, skeletal abnormalities and mental retardation. Cystathione β-synthase and methionine synthase are the only two pathways known for human cells to decrease the concentration of the toxic metabolite homocysteine. Both enzymes are redox-sensitive. Methionine synthase activity is enhanced at lower cellular redox potentials, whereas cystathionine β-synthase activity is increased under oxidizing conditions. Increased levels of cystathionine β–synthase activity leads indirectly to an increase in the antioxidant glutathionine. Therefore, a potential role of the heme in human cystathionine β-synthase could be to help regulate the redox potential within the cell (102).

Cystathionine β-synthase and *O*-acetylserine sulfhydrylase seem to be evolutionarily related, and still retain evolutionary vestigial catalytic activities (73). Cystathionine β-synthase of *S. cerevisiae* and *N. crassa* both had *O*-actylserine sulfhydrylase like activity (73). In addition, the human (53), rat (31), and *S. cerevisiae* (73) cystathionine β-synthase catalyze the formation of cysteine from serine and sulfide, and the rat enzyme also catalyzes the desulfurization of cysteine to form serine and sulfide (31).

Cystathionine γ-lyase

Cystathionine γ-lyase catalyzes the formation of cysteine, α-ketobutyrate and ammonia from cystathionine (Figure 2-4, reaction 10); the last step of the reverse transsulfuration pathway. Cystathionine γ-lyase has been purified and characterized from bacteria (66, 94), fungi (22, 27, 109), and mammals (96). Cystathionine γ-lyase is a

pyridoxal phosphate dependent homotetramer with subunit size ranging from 35 to 48 kDa. Cystathionine γ-lyase is a member of the γ-family of pyridoxal phosphate dependent homotetramers. The tetramers are a dimer of dimers, where two monomers are in close association and share residues to form two pyridoxal phosphate containing active sites. Each monomer consists of three distinct domains: (i) the N-terminal domain forms part of the active site of the partner monomer; (ii) the pyridoxal phosphate binding domain which contains most of the enzymes active site residues; and (iii) the C-terminal domain (60). The K_m for cystathionine ranges from 0.20 to 3.5 mM.

Cystathionine γ-lyase, cystathionine γ-synthase, cystathionine β-lyase, and homoserine sulfhydryase seem to be evolutionarily related (22, 72). Most cystathionine γ-lyase enzymes can catalyze one or more of the above reactions, except for human and rat cystathionine γ-lyase which are more specific (96). Cystathionine γ-synthase catalyzes the formation of cystathionine from cysteine and *O*-succinyl- or *O*-acetyl-homoserine. Cystathionine β-lyase catalyzes the formation of homocysteine, pyruvate and ammonia from cystathionine. Together these enzymes catalyze the transfer of sulfur from cysteine to homocysteine. Homoserine sulfhydrylase catalyzes the formation of homocysteine from *O*-succinyl- or *O*-acetyl-homoserine and sulfide.

Cystathionine γ-lyase from *S. cerevisiae* (75) and from *Streptomyces* (48) exhibit cystathionine γ-synthase activity. The enzyme from *Streptomyces* has a higher affinity towards *O*-succinyl-homoserine than for cystathionine; however, the K_m value is so high (40 mM) that the cystathionine γ-synthase reaction is unlikely to be of physiological significance (48). In *S. cerevisiae*, *O*-succinyl-homoserine is not available in the cells, and the enzyme can therefore not catalyze the cystathionine γ-synthase reaction *in vivo* (75).

Cystathionine γ-lyase from *Streptomyces phaeochromogenes* (66) and *S. cerevisiae* (109) exhibit cystathionine β-lyase activity. *S. phaeochromogenes* catalyzes the cystathionine β-lyase reaction at 15% of the rate of the cystathionine γ-lyase activity (66). The enzyme from *S. cerevisiae* actually catalyzes the cystathionine β-lyase reaction

at twice the rate of the cystathionine γ-lyase reaction (109); however, the enzyme does not catalyze the former reaction *in vivo* (75).

Cystathionine γ-lyase from *S. cerevisiae* also catalyzes homoserine sulfhydrylation and *O*-succinyl-homoserine sulfhydrylation (109); however, the latter reaction does not seem to occur at a physiological relevant rate (75). Cystathionine γ-lyase from *S. cerevisiae* (109) and *Lactobacillus fermentum* (94) are also able to desulfurize cysteine; the yeast enzyme does so at 10% of the cystathionine γ-lyase rate (109).

The activity and expression of cystathionine γ-lyase is regulated by the availability of cysteine and other organic and inorganic sulfur sources. In *Neurospora* the activity of the enzyme itself is inhibited by the presence of cysteine (27). In *A.nidulans* the presence of cysteine in growth media represses the activity of cystathionine γ-lyase (76), and in *S. cerevisiae* (74) and *Neurospora* (28) cystathionine γ-lyase activity increased by 15 and 30 fold, respectively, when cells are starved for sulfur during growth.

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Chapter 3

O-acetylserine sulfhydrylase from *Methanosarcina thermophila*

Abstract

Cysteine is the major source of fixed sulfur for the synthesis of sulfur-containing compounds in organisms of the Bacteria and Eukarya 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 sulfhydrylase (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 methanoarchaeon. 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 Eukarya and Bacteria domains. The purified OASS had a specific activity of 129 μmoles 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. SDS-PAGE revealed a single band at 36 kDa, and native gel filtration chromatography indicated a molecular mass of 93 kDa suggesting the purified OASS is either a homodimer or a homotrimer. The optimum temperature for activity was between 40 and 60 ºC consistent with the optimum growth temperature for *M. thermophila*. The results of this study provide the first evidence, of any kind, 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.

Introduction

Pathway I and pathway II are the two major routes for cysteine biosynthesis in nature (Figure 2-4). Serine transacetylase and *O*-acetylserine sulfhydrylase (OASS) catalyze steps in pathway I (reactions 1 and 2) (Figure 2-4, reactions 5 and 6) (28).

L-serine + acetyl CoA → *O*-acetyl-L-serine + CoA [1]

O-acetyl-L-serine + sulfide → L-cysteine + acetate [2]

Homoserine transacetylase, homocysteine synthase, cystathionine β-synthase, and γ-cystathionase catalyze steps of pathway II (reactions 3-6) (Figure 2-4, reactions 7-10) (28).

L-homoserine + acetylCoA → *O*-acetyl-L-homoserine + CoA [3]

O-acetyl-L-homoserine + sulfide → L-homocysteine + acetate [4]

L-homocysteine + serine → cystathionine [5]

cystathionine → L-cysteine + α-ketobutyrate [6]

Cysteine is the major source of fixed sulfur for the synthesis of sulfur-containing compounds in organisms from the Bacteria and Eucarya domains; thus, the sulfur fixing enzymes catalyzing reactions [2] and [4] are key enzymes in sulfur metabolism (13). Plants and members of the Bacteria domain synthesize cysteine and fix sulfur using the serine pathway. Plants and procaryotes from the Bacteria domain also fix sulfur by synthesizing homocysteine; however, they cannot utilize homocysteine for cysteine biosynthesis (21). Fungi fix sulfide and synthesize cysteine using both pathways (26). Although in yeast the homocysteine synthase also has OASS activity (28), pathway II appears to be the major route for cysteine biosynthesis (21). Many members of the Archaea are autotrophic and do not require cysteine or other forms of fixed sulfur for growth; however, it is unknown how the Archaea fix sulfur or synthesize cysteine.

The genomes of four Archaea have been sequenced. The *Methanococcus jannaschii* (6) and *Archaeoglobus fulgidus* (20) genomes contain no open reading frames having a deduced sequence with significant identity to any enzymes known to be involved in the fixation of sulfur or in cysteine biosynthesis. The methanoarchaeal genomes are also void of any open reading frames with deduced sequence identity to any

known cysteinyl-tRNA synthetases. The genome of *Pyrococcus horikoshii* (19) contains an open reading frame with sequence similarity to γ-cystathionase, and the genome of *Methanobacterium thermoautotrophicum* (44) contains an open reading frame with sequence similarity to homoserine transacetylase. However, these putative genes have not been expressed and it is unknown whether the gene products have the expected enzyme activities. It is also possible, that *O*-acetyl-L-homoserine is an intermediate only for the biosynthesis of methionine and not for L-cysteine (3). Furthermore, there are no open reading frames in either the *P. horikoshii* or *M. thermoautotrophicum* genomes with a deduced sequence having significant identity to other enzymes of pathway II, or any enzymes in pathway I for cysteine biosynthesis. Remarkably, none of the four archaeal genomes sequenced to date contain open reading frames with deduced sequence identities to either OASS or homocysteine synthase, the only sulfur fixing enzymes known in nature. We present here the first purification from an archaeon of an enzyme catalyzing sulfur fixation and cysteine biosynthesis, the pyridoxal 5'-phosphate-dependent OASS from the methanoarchaeon *Methanosarcina thermophila*.

Materials and Methods

Cell material. *M. thermophila* TM-1 was grown on acetate as described (46). The medium contained the following constituents in demineralized water at the final percent concentrations (wt/vol): NH_4Cl, 0.14; K_2HPO_4, 0.13; KH_2PO_4, 0.133; NaCl, 0.05; $MgSO_4$, 0.05; $Na_2S \cdot 9H_2O$, 0.027; $CaCl_2 \cdot 2H_2O$, 0.006; $Fe(NH_4)_2(SO_4)_2$, 0.001; cysteine-$HCl \cdot H_2O$, 0.027; yeast extract (Difco, Detroit, MI), 0.01; trypticase (BBL, Cockeysville, MD), 0.01; sodium acetate, 0.41. In addition, the medium contained 1% (vol/vol) each of vitamin and trace mineral solutions as described (46). The cells were harvested at the end of exponential growth and stored in liquid nitrogen until use.

Purification of OASS. *O*-acetylserine sulfhydrylase 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 21°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 [20g (wet weight)] was suspended in 25 ml of buffer A (50 mM TES-KOH (pH = 6.8)), containing 10% glycerol (v/v). DNase I (0.25 mg) was added to the suspension and then passed twice through a chilled French pressure cell at 20,000 psi (1 psi = 6.9 kPa). The cell lysate was centrifuged at 2,000 x *g* for 20 min, and the supernatant was recentrifuged at 78,400 x *g* for 2 hours.

(*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 0-1 M KCl gradient was applied at 7.0 ml/min. Peak fractions containing OASS activity, which eluted between 0.28 and 0.35 M KCl, were pooled and stored at - 20^{o}C. The procedure was repeated twice. The pooled peak fractions were combined and concentrated in dialysis tubing (3.5 kDa cutoff, Spectrum) imbedded in dry PEG (M_r = 8000, Sigma) at 4^{o}C.

(*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 (Pharmacia Biotechnology) column (bed volume = 160 ml) equilibrated with buffer B [50 mM Tris-Cl (pH 7.5) containing 2M NaCl]. After a 320 ml wash, the column was developed with a 1.1-liter decreasing linear gradient of 2.0-0 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^{o}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 reactions were performed at 40 ºC and were allowed to proceed for 1 min. Solutions were made anaerobic and stored under N_2 for sulfide K_m determination to avoid oxidation of the substrate.

Kinetic data analysis. The kinetic data were fitted to the indicated rate equations using the Levenberg-Marquardt algorithm with KaleidaGraph™ for Windows program version 3.09 (Abelbeck Software) and a Pentium Dell XPS M200s computer. The Hill equation (42) is

$$v = V_{max} [S]^n / (K_h + [S]^n) \qquad \text{(Eq. 1)}$$

where v is the velocity of the reaction (rate of product formation), V_{max} is the maximum velocity, K_h is the product of the two kinetic dissociation constants (dissociation of first and second substrate from enzyme), and n is the Hill cooperativity constant. At $S_{0.5}$, $v = V_{max} / 2$ and thus $S_{0.5} = K_h^{1/n}$.

A modified Hill equation incorporating an ordinate intercept (b) was proposed by Morgan *et al*. (27) and called MMF.

$$v = (bK + V_{max} [S]^n) / (K + [S]^n) \qquad \text{(Eq. 2)}$$

At $S_{0.5}$, $v = (V_{max} + b) / 2$, and thus $S_{0.5} = K^{1/n}$.

The logistic equation (34, 35) is

$$v = V_{max} / (1 + \exp (\beta - \gamma[S]) \qquad \text{(Eq. 3)}$$

At $S_{0.5}$, $v = V_{max} / 2$ and thus $S_{0.5} = \beta / \gamma$. Saturation curves included data points from 0-7 mM *O*-acetyl-L-serine to preclude the inhibition portion of the curve (Figure 3-9).

N-terminal analysis. Purified enzyme was subjected to SDS-PAGE and electroblotted onto Immobilon-P PVDF membrane (Millipore Bedford, MA) at 23V for 14-16 hours at 4 ºC with 10 mM CAPS-Na (pH 11) containing 10% MeOH (v/v). The N-terminal sequence was determined with a PE Biosystems 477A Sequencer coupled to a 120A Analyzer (PE Biosystems, Foster City CA). The BLAST blastp program (1) was

used to search the non-redundant sequence databases at the National Center for Biotechnology Information (Bethesda, MD) for enzymes with a similar N-terminus, and later for OASS sequences. Representative OASS enzymes from the eukaryotic group [Spinach CSaseA (38) CSase B (39) and CSase C (40), *Citrullus vulgaris* CSase A (32), Wheat (49), *Arabidopsis thaliana* CSase A and CSase B (14), *Capsicum annuum* CSase B (36), and *Entamoeba histolytica* isozymes (33)] and the prokaryotic group [*E. coli* cysK (8) and cysM (43), *S. typhimurium* cysK (8), *Synechococcus* sp. PCC 7942 CysK (31), *Flavobacterium* K3-15 CysK (29), *Campylobacter jejuni* CysM (12), and *Aspergillus nidulans* CysM (47)] were aligned by using the Protein-Induced (local) Multiple Alignment 1.4 from the Baylor College of Medicine Search Launcher (45).

Molecular mass determination. SDS-PAGE was performed as described (24). The molecular mass of the native enzyme was determined with a Superose 12 HP (Pharmacia) gel filtration column. After equilibration with buffer D (50 mM Tris-Cl (pH 7.5), 100 mM KCl), 0.2 ml of sample were injected, and the column was developed at a flow rate of 0.3 ml/min. The column was calibrated with a molecular weight kit (Sigma) containing cytochrome C (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa).

UV-visible absorption spectroscopy. The UV-visible absorption spectrum was recorded at 21^{o}C with a Beckman, DU 640. The concentration of *O*-acetyl-L-serine in solution was increased stepwise by addition of 2-5 µl from 0 to a final concentration of 0.5, 1.0, 2.0, 5.0, 7.5, 10 and 15 µM. Spectra were taken at each individual concentration. Sodium sulfide was added to a final concentration of 1 mM and a final spectrum recorded.

Isoelectric focusing. Isoelectric focusing was performed in a Rotofor System (Bio-Rad).

Materials. All chemicals were of reagent grade from Sigma or Fisher. Molecular mass standards were from Sigma.

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 3-1. The level of enzyme activity in cell extract was found to be within the range reported for procaryotes from the Bacteria domain (0.03 - 50 U/mg) (9, 15). The *M. thermophila* enzyme could be stored at –20 ºC 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 *(*Figure 3-1). The purified enzyme was stable to several freeze-thaw cycles, but activity was completely absent after 2 months storage at –20 ºC. However, approximately 10% of the activity could be recovered by incubating the enzyme at 21 ºC for 30 min in Tris-Cl buffer (pH 8.0) containing 50 mM DTT and 80 µM pyridoxal 5' - phosphate.

Table 3-1: Purification scheme for OASS from *M. thermophila*.

Step	Volume	protein	total activity	specific activity	recovery	purification
	ml	mg	U[a]	U/mg	%	fold
Cell Extract	96	2891	979	0.339	100	1
Q-Sepharose	60	105	660	6.30	67	19
Phenyl Sepharose	220	36	525	14.5	54	43
Mono Q I	12	6.7	413	61.0	42	180
Mono Q II	7	3.5	445	129	45	381

U[a] = µmol Cys/min

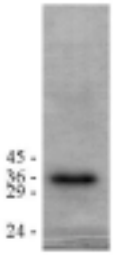

Figure 3-1: SDS-PAGE of OASS purified from *M. thermophila*. The 12% gel was loaded with 9 μg of purified OASS. The position to which the molecular weight markers migrated are shown to the left.

Physical properties. A plot of activity *versus* the assay temperature revealed a broad optimum between 40 and 60 ºC (Figure 3-2). Significant activity was detected up to 80 ºC. These results are compatible with the optimum growth temperature of *M. thermophila* (55 ºC). A plot of activity versus the assay pH revealed an optimum at pH 7.5 (Figure 3-3). This result is similar to that found for OASS from Spinach (11, 48). No data are available for any of the prokaryotic enzymes.

The N-terminal sequence of the purified OASS from *M. thermophila* (Figure 3-4) 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.

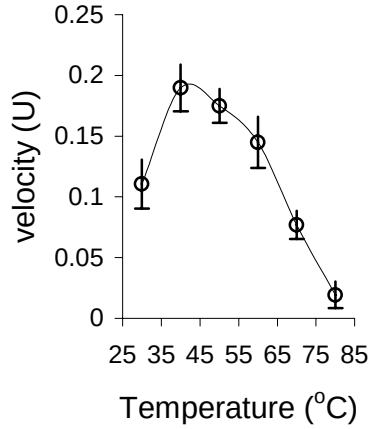

Figure 3-2: Effect of temperature on the activity of OASS from *M. thermophila*. Standard assays were used except that the temperature was varied as indicated. Each experiment was carried out in triplicate one or more times, and values are recorded as the average of all experiments. Error bars are the standard deviation of all data from the average. The line is drawn to guide the eye.

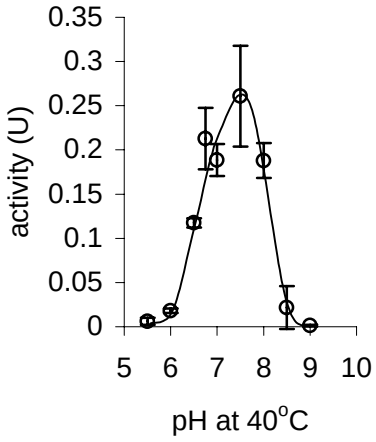

Figure 3-3: Effect of pH on the activity of OASS from *M. thermophila*. Standard assays were used, except that the pH was varied as indicated. Each experiment was carried out in triplicate one or more times, and values are recorded as the average of all experiments. Error bars are the standard deviation of all data from the average. The line is drawn to guide the eye.

Spinacia oleracea CSase A	EKAFIAKDVT-EL**IG** K**T**PLVYLNTV----A	28
Citrullus vulgaris CSase A	AKSTIAKDVT-EL**IG** N**T**PLVYLNRV----V	28
Triticum aestivum	SSPAIAKDVT-EL**IG** N**T**PLVYLNKV----T	29
Arabidopsis thaliana CSase A	---RIAKDVT-EL**IG** N**T**LLVYLNNV----A	25
Spinacia oleracea CSase B	-GLNIAEDVS-QL**IG** K**T**PMVYLNNV----S	87
Capsicum annuum CSase B	-GLNIAEDVT-QL**IG** N**T**PMVYLNTI----V	85
Arabidopsis thaliana CSase B	-ALNIADNAA-QL**IG** K**T**LMVYLNNV----R	95
Spinacia oleracea CSase C	-GTNIKTNVS-QL**IG** R**T**PLVYLSKI----S	69
Escherichia coli CysK	----IFEDNS-LT**IG** H**T**PLVRLNRI-----	23
Salmonella typhimurium CysK	----IYEDNS-LT**IG** H**T**PLVRLNRI-----	23
Synechococcus CysK	----FFADNS-QT**IG** K**T**PLVRLNRI----V	27
Entamoeba histolytica	PRKRIYHNIL-ET**IG** G**T**PLVELHGVTEHPR	37
Escherichia coli CysM	-----MSTLE-QT**IG** N**T**PLVKLQRM----G	20
Flavobacterium CysK	---MKFQNAL-ET**IG** N**T**PVVKINNL-----	21
Campylobacter jejuni CysM	---MKVHEKVSEL**IG** N**T**PIIHLKKF-----	22
Aspergillus nidulans CysM	QAQGFVNGLTEKA**IG** N**T**PLIRLKRL----S	53
Methanosarcina thermophila	--GRIFSDIT-WT**IG** N**T**PLVRLN-------	20

Figure 3-4: N-terminal amino acid sequence alignment of select OASS enzymes. Completely conserved amino acids are indicated by boldface type. 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. The numbering on the right side refers to the position of the right most residues shown, with respect to the entire sequence.

The UV-visible spectrum (Figure 3-5) 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 internal aldimine 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 (Figure 2-5, structure 1). The OASS purified from *M. thermophila* has an A_{280}/A_{413} nm absorbance ratio of 4.0 which compare`s favorably to the ratio found for OASS from *S. 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 (Figure 3-6), suggesting that the purified enzyme had a full complement of this cofactor.

A marked change in the spectrum, attributed to pyridoxal 5'-phosphate in Schiff base with a lysine residue of the enzyme, occurs upon addition of *O*-acetyl-L-serine (refer to Chapter 2 for a review). Titration with increasing amounts of *O*-acetyl-L-serine produced a shift in absorbance from 413 to 466 nm (Figure 3-7), and formation of a broad shoulder at 330 nm (Figure 3-7). The increase in absorbance at 466 nm was concentration dependent, and saturated at 10 μM *O*-acetyl-L-serine (Figure 3-7 inset). A similar shift in absorbance was observed by Schnackerz *et al.* (41) upon binding of D-serine to D-serine dehydratase. The species responsible for this shift in absorbance is the α-aminoacrylic acid in Schiff base with pyridoxal 5'-phosphate (Figure 2-5, structure 6). 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 serine 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*.

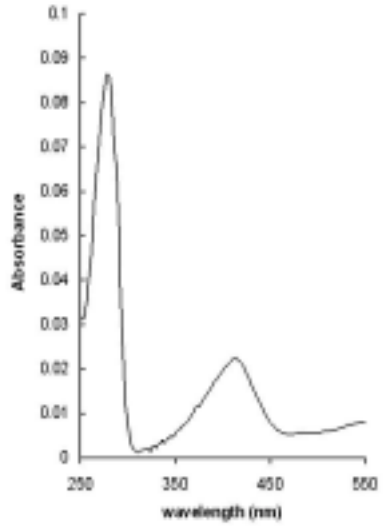

Figure 3-5: UV-visible absorption spectrum of OASS purified from *M. thermophila*. The enzyme (0.13 mg/ml) was in 50 mM Tris-KOH buffer (pH 6.8), containing 350 mM KCl.

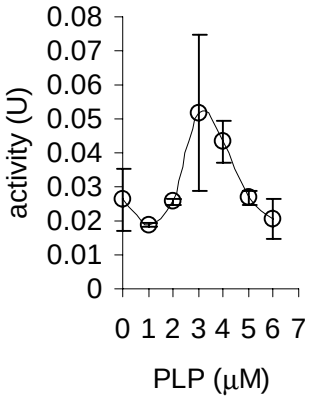

Figure 3-6: Incorporation of pyridoxal 5'-phosphate into OASS from *M. thermophila*. Standard assays were used except that the enzyme was preincubated for 5 minutes in varying amounts of pyridoxal 5'-phosphate at 40 ^{o}C. Each experiment was carried out in triplicate one or more times, and values are recorded as the average of all experiments. Error bars are the standard deviation of all data from the average. The line is drawn to guide the eye.

Isoelectric focusing determined that the pI of *M. thermophila* OASS is 5.0 ± 0.5, a result similar to the calculated pI values of the spinach OASS iso-enzymes, 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 (Figure 3-1). The native molecular mass of OASS was determined to be 93 kDa by gel filtration chromatography (Figure 3-8). 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).

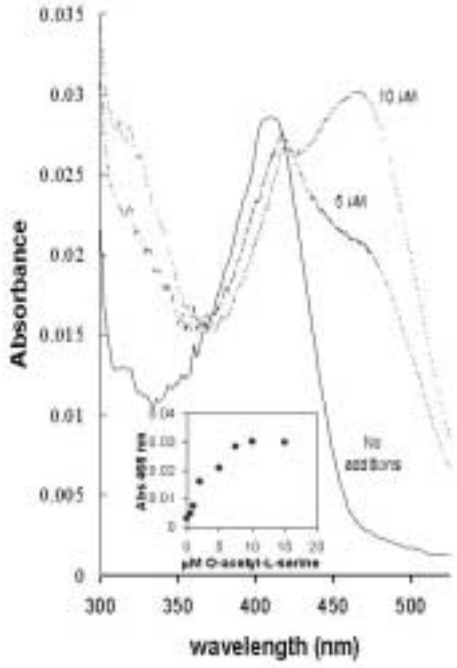

Figure 3-7: Spectroscopic titration of OASS purified from *M. thermophila* with *O*-acetyl-L-serine. Protein concentration was 0.10 mg/ml in 50 mM Tris-KOH buffer (pH = 6.8), containing 350 mM KCl. *O*-acetyl-L-serine additions were made in 2-5 µl increments in a total volume of 1.0 ml. The amount of *O*-acetyl-L-serine for each spectrum are shown on the figure. Inset: Absorption of the enzyme at 466 nm *versus* amount of *O*-acetyl-L-serine added.

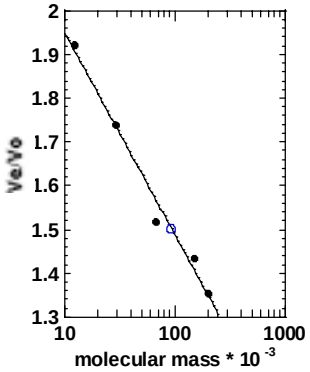

Figure 3-8: Native molecular mass determination of OASS from *M. thermophila*. Symbols: • molecular mass standards; **o** OASS from *M. thermophila*.

Kinetic analyses. Enzyme activity was determined over time, and found to be linear from 0 to 5 minutes. All kinetic data was derived from one minute assays. Plots of increasing concentrations of *O*-acetyl-L-serine *versus* the initial velocity of the reaction were sigmoidal (Figure 3-9B), a result suggesting the OASS purified from *M. thermophila* displays positive kinetic cooperativity at low concentrations of this substrate. Similar positive cooperativity has been noted in some plant enzymes (23, 37), and in *S. typhimurium* when OASS type B (CysM) is bound to serine transacetylase, the first enzyme in pathway I (22). However, in *S. typhimurium* no positive cooperativity is observed when the enzyme is not associated with serine transacetylase. The activity of the purified *M. thermophila* OASS was inhibited at concentrations of *O*-acetyl-L-serine above 10 mM (Figure 3-9A). Inhibition of activity has also been reported for the OASS from *S. typhimurium* at concentrations above 7.5 mM *O*-acetyl-L-serine (10) and in *Phaseolus* OASS at concentrations above 10 mM (4). Analysis of the data in Figure 3-9B using three different equations all gave similar fits, indicated by the similar Chi Square error and the coefficient of determination (R^2), the best fit being provided by the MMF model (Table 3-2). The MMF model is a generalized form of the Hill equation, which allows for a non-zero intercept. No data were available close to zero; thus, it might be erroneous to assume that the curve is strictly sigmoidal from zero to the first data point. The V_{max} and $S_{0.5}$ values obtained by the logistic equation, describing a very general growth curve, were slightly lower than those obtained by the other two methods. Both models allowing for a non-zero intercept had better fits than the Hill equation. The $S_{0.5}$ values obtained were similar to the ones found for the *D. innoxia* enzymes (23). The variations in V_{max} are most likely due to the restricted data set used to preclude portions of the curve affected by the substrate inhibition.

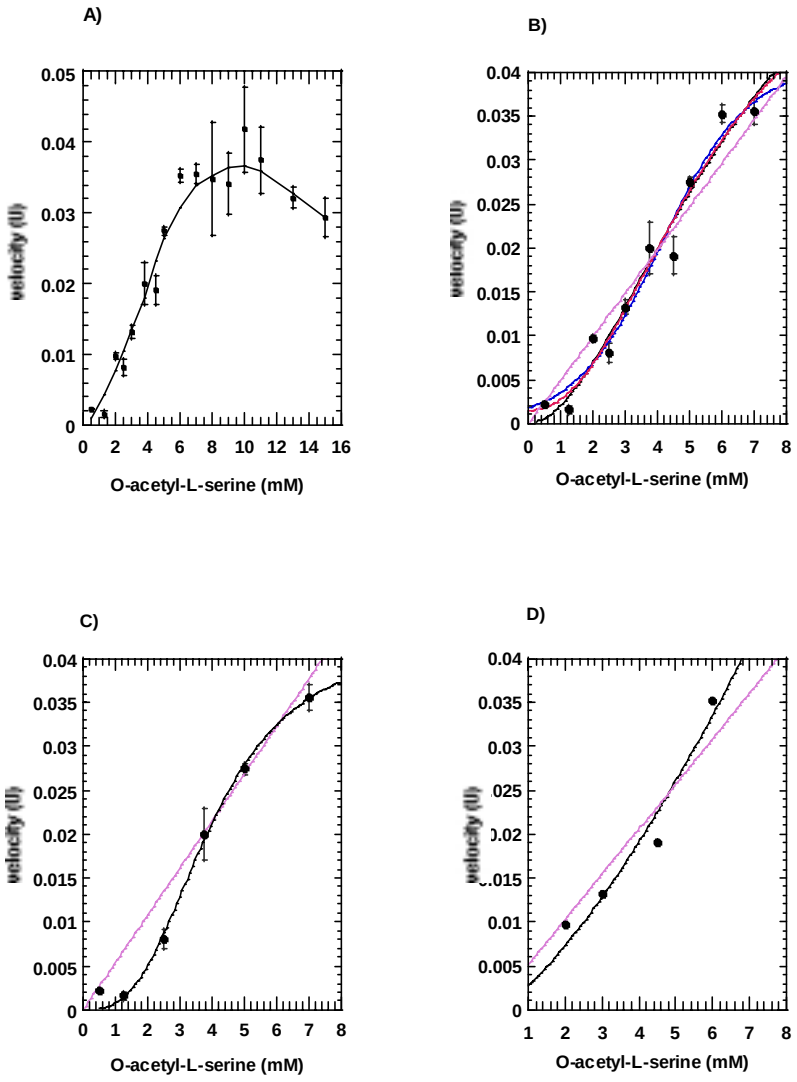

Figure 3-9: A. Dependence of activity of purified OASS from *M. thermophila* on *O*-acetyl-L-serine concentration. Standard assays were used, except that *O*-acetyl-L-serine was varied as indicated, and 0.1 µg of enzyme were used per assay over 2 days. B. At low *O*-acetyl-L-serine concentrations the data was fit using the Hill equation (—), the logistic equation (—), the MMF equation (—), and the Michaelis Menten equation (—). C. Data points recorded on day 1 of experiment. D. Data points recorded on day 2 of experiment. Each experiment was carried out in triplicate one or more times, and values are recorded as the average of all experiments. Error bars are the standard deviation of all data from the average.

Table 3-2: Kinetic constants from substrate saturation experiments with OASS purified from *M. thermophila*.

model	V_{max}	$S_{0.5}$ mM	n	ChiSquare	R^2
Hill	0.0711	6.667	1.847	$4.94x10^{-5}$	0.9647
MMF	0.0587	5.597	2.214	$4.75x10^{-5}$	0.9661
logistic	0.0408	4.111	N/A	$4.76x10^{-5}$	0.9660

SDS-PAGE and native gel chromatography of the OASS purified from *M. thermophila* suggested that the enzyme is either a homodimer or a homotrimer. Consistent with all OASS enzymes studied, it is anticipated that each subunit of the *M. thermophila* OASS contains one active site with a single pyridoxal 5'-phosphate. The values of *n* determined by the Hill and the MMF model are also consistent with two or more active sites. Generally the Hill equation underestimates the amount of cooperativity (42), whereas the MMF model tends to overestimate it (23). The Hill model revealed a K_h value of 33 ± 12 mM *O*-acetyl-L-serine. The K_h value is the product of the two kinetic dissociation constants; however, substrate inhibition precluded an accurate determination of the individual constants.

Data points in Figure 3-9B did not closely fit any of the models used although better correlation was seen with a cooperative model for the enzyme than with a non-cooperative model (Michaelis Menten kinetics). It was impossible to record all data points in one day because of inherent limitations of the assay; therefore, data points for Figure 3-9 were recorded on consecutive days. Variations in enzymatic activity were noted between days, which could account for the problems in obtaining a good fit of the data with any of the cooperativity models. The variations in activity are most likely due to the inherent problems of the assay. These problems include the instability of the Ninhydrin used to detect cysteine, however, the reagent must be incubated at room temperature for at least one day before use to obtain reproducible assay results. This raises the possibility that the apparent cooperativity was an artifact caused by the assay problems, and not due to cooperativity of the enzyme. Therefore, data points of Figure

3-9B were separated by the individual days that they were obtained, and the data were analyzed using the Michaelis-Menten and the Hill equations (Figure 3-9C and Figure 3-9D). The data from the first day (Figure 3-9C) closely fit the Hill model and not the Michaelis-Menten model. The Hill coefficient determined is 2.9 and the K_h is 55 mM. However the calculated V_{max} is 10 fold higher (0.34 U) than observed in Figure 3-9A for the enzyme. Owing to substrate inhibition it is impossible to determine the true V_{max} for this enzyme. The increased Hill coefficient indicates strong cooperativity of this enzyme towards *O*-acetyl-serine and further validates the above conclusions. Neither model fits the data from day 2 (Figure 3-9D). However the graph does not contain enough data points for a good fit to any model. One method to circumvent the above problems is to repeat all data points. However, due to the limitations of the assay each data point can only be analyzed once and not in triplicate as has been done for the previous data.

Plots of increasing concentrations of sodium sulfide *versus* the initial velocity of the reaction exhibited normal Michaelis-Menten kinetics (Figure 3-10). The K_m value for sulfide is 500 ± 80 μM. This value falls within the range of K_m values reported for sulfide for other OASS enzymes purified (20 - 2700 μM) (7, 15, 36).

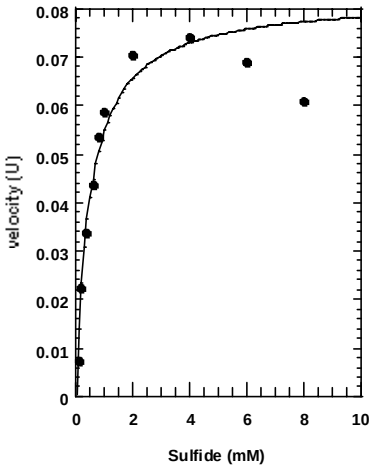

Figure 3-10: Dependence of activity of purified OASS from *M. thermophila* on sulfide concentrations. Standard assays were used except that sulfide was varied as indicated. At low sulfide concentrations (0-5 mM) the data were fit using the Michaelis-Menten equation.

Discussion

The genomic sequences of four phylogenetically and metabolically diverse members of the Archaea provide little insight into mechanisms of archaeal 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 that of other known OASS enzymes.

Although OASS is essential for pathway I of cysteine biosynthesis in plants and Bacteria, additional functions for OASS enzymes have been proposed. In Eukarya 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 Eukarya domain, OASS enzymes can also catalyze the formation of heterocyclic β-substituted alanines from *O*-acetyl-L-serine and heterocyclic compounds (18). Thus it is possible that *M. thermophila* OASS might have additional functions, or a function unrelated to pathway I 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 *S. typhimurium* expression of OASS and other enzymes required for cysteine biosynthesis are 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*-acetyl-L-serine may be important if OASS functions in pathway I and transcription of the genes encoding enzymes of this pathway are regulated by the same mechanism proposed for *E. coli* and *S.typhimurium*. In the proposed mechanism, the levels of *O*-acetyl-L-serine in the cell influence the expression of serine transacetylase and OASS. Inactivity of the *M. thermopila* OASS at low concentrations of *O*-acetyl-L-serine may be necessary to allow accumulation of *O*-acetyl-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. thermoautotrophicum* and *P. horikoshii* with deduced sequences having identity to homoserine transacetylase and γ-cystathionase is consistent with pathway II for cysteine biosynthesis in these Archaea. However, *M. thermoautotrophicum* and *P. horikoshii* are classified in different taxonomic orders than the methanosarcina; thus, *M. thermophila* may have inherited enzymes of pathway I 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.

Acknowledgments

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Chapter 4

Cysteine biosynthesis in the Archaea: *Methanosarcina thermophila* utilizes *O*-acetylserine sulfhydrylase for the synthesis of cysteine.

Abstract

Two pathways for cysteine biosynthesis are known in nature; however, it is not known which, if either, the Archaea utilize. Enzyme activities in extracts of *Methanosarcina thermophila* grown with combinations of cysteine and sulfide as sulfur sources indicated this archaeon utilizes the pathway found in the Bacteria domain. The genes encoding serine transacetylase and *O*-acetylserine sulfhydrylase (*cysE* and *cysK*) are adjacent on the chromosome of *M. thermophila* and possibly form an operon. When *M. thermophila* is grown with cysteine as the sole sulfur source, *O*-acetylserine sulfhydrylase activity is maximally expessed suggesting alternative roles for this enzyme apart from cysteine biosynthesis.

Introduction

Cysteine plays a critical role in the structure, stability and catalytic function of many proteins in all domains of life. Cysteine is also the major source of sulfur for the synthesis of sulfur-containing compounds in organisms of the Bacteria and Eucarya domains. Two routes for cysteine biosynthesis in nature have been documented. Serine transacetylase and *O*-acetylserine sulfhydrylase catalyze steps in pathway I (Figure 2-4, reactions 5-6) [1]. Cystathionine β-synthase and cystathionine γ-lyase catalyze steps of

pathway II (Figure 2-4, reactions 9-10) [1]. Plants and members of the Bacteria domain synthesize cysteine and fix sulfur via pathway I, and also fix sulfur by synthesizing homocysteine (Figure 2-4, reactions 7-8); however, most cannot utilize homocysteine for cysteine biosynthesis. Fungi fix sulfide (Figure 2-4, reactions 6 and 8) and can theoretically synthesize cysteine using pathways I and II. Although enzymes for both pathways are present in the yeast *Saccharomyces cerevisiae*, only pathway II is used for cysteine biosynthesis [2]. On the other hand, the yeasts *Schizosaccharomyces pombe* [3], *Pichia membranofaciens*, and *Candida valida* [4,5] lack cystathionine β-synthase and cystathionine γ-lyase, indicating these organisms must synthesize cysteine through pathway I.

Although the genomes of five members of the Archaea have been sequenced to date, the data offer little in understanding of cysteine biosynthesis. The *Methanococcus jannaschii* [6], *Methanobacterium thermoautotrophicum* [7], and *Archaeoglobus fulgidus* [8] genomes contain no open reading frames (ORFs) having a deduced sequence with significant identity to enzymes of either pathway for cysteine biosynthesis. The genome of *Aeropyrum pernix* [9] contains ORFs with deduced sequence similarity to cystathionine β-synthase and cystathionine-γ-lyase. The genome of *Pyrococcus horikoshii* [10] contains an ORF with deduced sequence similarity to cystathionine γ-lyase. However, it is not known whether these genes are expressed or if the gene products have the expected enzyme activities. No ORFs are present in the genomes of *Pyrococcus horikoshii*, and *Aeropyrum pernix* with a deduced sequence having significant identity to enzymes of pathway I for cysteine biosynthesis.

In *Methanosarcina barkeri* two genes were identified that had significant sequence similarity to *cysK* (encoding *O*-acetylserine sulfhydrylase) and *cysE* (encoding serine transacetylase) [11]. The *cysK* gene was found to complement a cysteine auxotrophic *Escherichia coli* strain, indicating that the gene encodes *O*-acetylserine sulfhydrylase. However, *cysK* expression was not demonstrated in *M. barkeri* and no other data was reported supporting *cysK* involvement in cysteine biosynthesis. Notably in *S. cerevisiae* both pathway I enzymes are present but they have an undefined role distinct from cysteine biosynthesis [12]. However, it was recently shown that *O*-

acetylserine sulfhydrylase is expressed in *M. thermophila* and the enzyme has properties suggesting involvement in cysteine biosynthesis [13]. Here we provide further evidence that pathway I is used for cysteine biosynthesis and pathway II is not present.

Materials and methods

***M. thermophila* growth media and cell extract preparation.** General anaerobic methods for the growth of *M. thermophila* and preparation of extracts were as described [13,14]. Minimal media [14] contained the following constituents in demineralized water at the final percent concentrations (wt/vol): NH_4Cl, 0.13; K_2HPO_4, 0.11; KH_2PO_4, 0.09; $CaCO_3$, 0.0002; *p*-aminobenzoic acid 0.000004; sodium acetate • $3H_2O$, 1.4; $Fe(NH_4)_2(SO_4)_2$ • $6H_2O$, 0.001; Na_2CO_3, 0.25; resazurin, 0.0001; and 1% (vol/vol) each of vitamin and trace mineral solutions as described [15]. The following were added individually or in combination to supply the sulfur source (final concentration in the media (wt/vol)): Na_2S • $9H_2O$, 0.025; cysteine-HCl • H_2O, 0.025.

Cultures were transferred during late-log phase 10 successive times into fresh media containing either cysteine or sulfide or both (1 ml into 60 ml) before growth was recorded, to ensure no carryover of cysteine or sulfide from the original inoculum.

***S. cerevisiae* w303*a* growth media and cell extract.** *S. cerevisiae* w303*a* was a gift from Robert J. Durso. YPD medium contained 2 % glucose, 2 % peptone, and 1 % yeast extract [16,17]. Growth was at 30°C. Cell homogenates were prepared as described previously [16]. Cells were harvested during late logarithmic phase, and washed twice with STE (10 mM Tris-Cl [pH 7.4], 1 mM EDTA, 100 mM NaCl). Washed cells were resuspended 1:1 in WCE (20 mM potassium phosphate [pH 7.2], 100 nM pyridoxal phosphate, 250 nM EDTA, 1 mM PMSF, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 350 mM NaCl, 1 % Tween 20, and 10 % glycerol), and frozen in small droplets in liquid nitrogen until use. Cells were broken by grinding the fozen droplets with a liquid nitrogen chilled mortar and pestle for 15 min (until a fine powder remained).

The cell homogenate was centrifuged twice at 13,000 × *g* for 10 min at 4°C, and the supernatant was stored in 0.05 ml aliquots at - 80°C.

Protein assay. Protein concentrations were determined by the method of Bradford [18], using Bio-Rad dye reagents and bovine serum albumin (Pierce) as a standard.

Enzyme assay. *O*-acetylserine sulfhydrylase activity was measured as described previously [19]. Cystathionine-γ-lyase activity assays were conducted as described previously [20] using the method of Droux [19] to detect the product cysteine. Cystathionine-β-synthase activity was measured as described previously [21]. All reactions were performed at 40°C with *M. thermophila* cell extract alone, and 30°C with *S. cerevisiae* cell extract alone or together with *M. thermophila* cell extract. Protein concentrations were 4 mg/ml per assay per organism (thus 8 mg/ml total protein concentration when using both cell extracts).

Gene isolation. *M. thermophila* DNA was a gift from Birgit Alber. Primers were designed using the *M. barkeri cysK* gene [11]. The *cysK* gene from *M. thermophila* was amplified through PCR (Ericomp DeltaCycler II™ System of the EasyCycler™ Series) and cloned into a pTAdvantage vector (Clontech). Using primers to the *M. thermophila cysK* gene, upstream DNA was obtained with the Universal GenomeWalker™ Kit (Clontech), and downstream DNA was obtained using *M. thermophila* DNA digested with Eco*RI* and ligated into a pUC 21 vector (a gift from Robert Barber). The resultant upstream and downstream DNA were cloned into pSTBlue-1 AccepTor Vector™ (Novagen). All transformants were obtained following the manufacturers instructions (Novagen). Plasmids were purified using QIAprep Spin Miniprep Columns (Qiagen), and were sequenced using a Perkin Elmer ABI377 (Penn State University Sequencing Facility).

The BLAST blastx program [22] was used to search the non-redundant sequence databases at the National Center for Biotechnology Information (Bethesda, MD) for genes with a similar sequence to the ORFs upstream (*nifS* and the hypothetical protein) and downstream (*cysE*) of *cysK*. The hypothetical protein from *M. thermophila*, *M. barkeri* [11], and AF0184 and AFO566 from *Archaeoglobus fulgidus* [8] were aligned by

using Pattern-Induced (local) Multiple Alignment 1.4 from the Baylor College of Medicine Search Launcher [23].

Results

Growth of *M. thermophila* with either cysteine, sulfide, or cysteine plus sulfide as the source of sulfur. Growth in minimal media, as determined by gas production, was similar in media that contained either cysteine plus sulfide, only sulfide, or only cysteine as the sole sulfur source (Figure 4-1). There was no significant difference in cell yield (lyophilized dry weight) for any of the cultures at the end of 10 days of growth (+Cys/+S^{2-}: 22 ± 4 mg; -Cys/+S^{2-}: 24.7 ± 0.9 mg; +Cys/-S^{2-}: 20 ± 5 mg). The results demonstrate that *M. thermophila* synthesizes cysteine de novo and can use either sulfide or cysteine as the sole sulfur source.

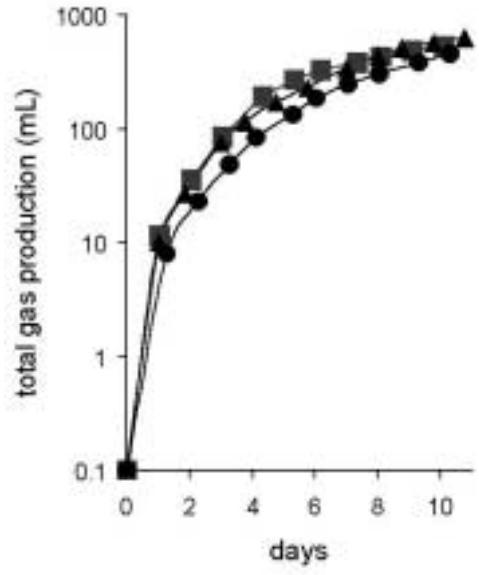


Figure 4-1: Growth of *M. thermophila* in minimal media with cysteine and sulfide (■), with sulfide only (▲), and with cysteine only (●) as sulfur source. Cultures (60 ml) were grown in 120-ml serum bottles

Activities in cell extracts of enzymes catalyzing steps in known cysteine biosynthetic pathways. When *M. thermophila* was grown in minimal media without cysteine and with sulfide, *O*-acetylserine sulfhydrylase activity in cell extract was elevated over 50-fold compared to growth in media containing both sulfide and cysteine (Table 4-1). When grown in minimal media with only cysteine as the sole sulfur source, *O*-acetylserine sulfhydrylase activity in extract was elevated over 150-fold compared to growth in media containing both sulfide and cysteine (Table 4-1). No other protein fractions with *O*-acetylserine sulfhydrylase activity were detected during the purification

Table 4-1: Specific activity of cysteine biosynthetic enzymes in *M. thermophila* grown in minimal media with different sulfur sources, and *S. cerevisiae* grown in standard media.

Organism and sulfur source	Cystathionine γ-lyase (mU/mg)[a]	Cystathionine β-synthase (mU/mg)[b]	*O*-acetylserine sulfhydrylase (mU/mg)[a]
M. thermophila cysteine and sulfide	< 0.04[c] (0.2 ± 0.06)[d]	< 0.3[c] (0.7 ± 0.3)	0.25 ± 0.05
M. thermophila sulfide	< 0.04[c] (0.2 ± 0.05)	< 0.3[c] (0.8 ± 0.4)	14 ± 1
M. thermophila cysteine	< 0.04[c] (0.2 ± 0.02)	< 0.3[c] (0.9 ± 0.1)	45 ± 4
S. cerevisiae yeast extract and peptone	0.3 ± 0.03	1.2 ± 0.4	N/A[e]

[a] mU = nmol cysteine/min

[b] mU = nmol cystathionine/min

[c] Limits due to sensitivity of assay

[d] Values in parentheses are with an equal amount of *S. cerevisiae* extract added, and are calculated based on *S. cerevisiae* extract alone.

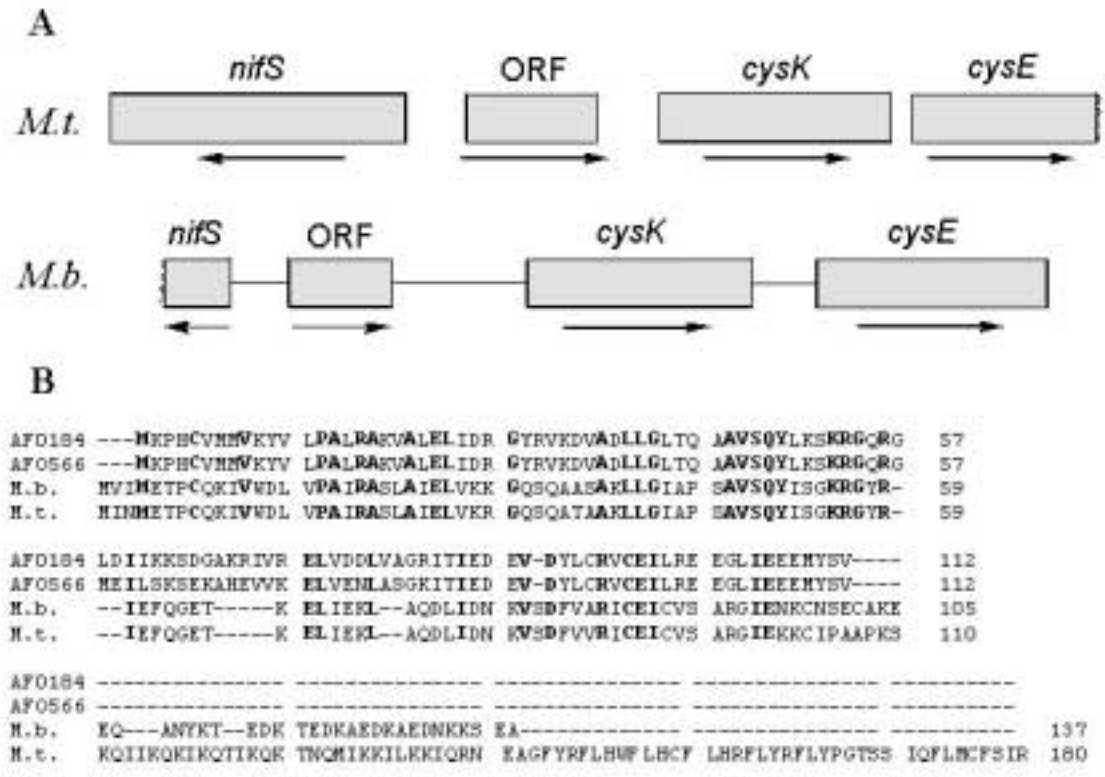
[e] N/A = not assayed

of the *O*-acetylserine sulfhydrylase from *M. thermophila* [13], indicating that the enzyme purified is responsible for the elevated activities.

Cystathionine γ-lyase and cystathionine β-synthase activities were not detected in cell extracts of *M. thermophila* grown under the conditions examined, including in the absence of cysteine (Table 4-1). Activities of these enzymes were detected in extracts of *S. cerevisiae* grown under conditions where both enzymes are expressed for the synthesis of cysteine [16,17]. Both enzyme activities from *S. cerevisiae* cell extract were nearly fully recovered when added to the individual *M. thermophila* cell extracts. These results indicate that no enzyme or other component in the cell extracts of *M. thermophila* significantly masks either cystathionine γ-lyase or cystathionine β-synthase activities.

Organization of genes that encode *O*-acetylserine sulfhydrylase and serine transacetylase. The *cysK* gene encoding the *O*-acetylserine sulfhydrylase from *M. thermophila* was obtained by PCR amplification of genomic DNA using primers derived from the published sequence for *cysK* [11]. The deduced sequence of the PCR-amplifed DNA was highly identical to *O*-acetylserine sulfhydrylase sequences from plants and procaryotes (53% identity with *Oryza sativa*, 63 % identity with *Mycobacterium tuberculosis*, 89% identity with *M. barkeri*). The first 20 N-terminal residues deduced from the sequence were 100% identical to those determined for the *O*-acetylserine sulfhydrylase isolated from *M. thermophila* [13]. The 1 kb sequence downstream, and 4 kb sequence upstream, of *cysK* were obtained by PCR amplification using primers derived from the *M. thermophila cysK* sequence. Analysis of the flanking sequences identified a partial ORF downstream of *cysK* (Figure 4-2A), the truncated deduced sequence (243 residues) of which was found to have high identity to *cysE* genes present in plants and procaryotes (39% identity with *Arabidopsis thaliana*, 55% identity with *Azotobacter vinelandii*, 87% identity with *M. barkeri*). Upstream of *cysK*, and transcribed in the opposite direction is a partial ORF designated *nifS* (Figure 4-2A). The deduced sequence of *nifS* has 50% identity to *nifS* from *Azotobacter vinelandii*. Situated between the *M. thermophila cysK* and *nifS* genes is an ORF encoding a hypothetical protein with 180 residues that is transcribed in the same direction as *cysK* and *cysE* (Figure 4-2A). *M. barkeri* has the same gene organization (Figure 4-2A). The deduced sequence of the *M. barkeri* ORF is 76% identical to the *M. thermophila* hypothetical protein (Figure 4-2B). The hypothetical proteins encoded by these ORFs have significant

identity to hypothetical proteins in the archaeon *Archaeoglobus fulgidus* [8] (AF0184 and AF0566 have 34% and 33% identity to the *M. thermophila* hypothetical protein respectively) (Figure 4-2B). Both *Archaeoglobus fulgidus* hypothetical proteins are clustered with *nifU* and *nifS* homologs that are transcribed in the same direction as the hypothetical proteins.




```
AF0184 ---MKPHCVMMVKYV LPALRASVALELIDR GYRVKDVADLLGLTQ AAVSQYLKSKRGQRG   57
AF0566 ---MKPHCVMMVKYV LPALRASVALELIDR GYRVKDVADLLGLTQ AAVSQYLKSKRGQRG   57
M.b.   MVIMETPCQKIVWDL VPAIRASLAIELVKK GQSQAASAKLLGIAP SAVSQYISGKRGYR-   59
M.t.   MINMETPCQKIVWDL VPAIRASLAIELVKR GQSQATAAKLLGIAP SAVSQYISGKRGYR-   59

AF0184 LDIIKKSDGAKRIVR ELVDDLVAGRITIED EV-DYLCRVCEILRE EGLIEEEMYSV----   112
AF0566 MEILSKSEKAHEVVK ELVENLASGKITIED EV-DYLCRVCEILRE EGLIEEEMYSV----   112
M.b.   --IEFQGET-----K ELIEKL--AQDLIDN KVSDFVARICEICVS ARGIENKCNSECAKE   105
M.t.   --IEFQGET-----K ELIEKL--AQDLIDN KVSDFVVRICEICVS ARGIEKKCIPAAPKS   110

AF0184 ---------------------------------------------------------------
AF0566 ---------------------------------------------------------------
M.b.   EQ---ANYKT--EDK TEDKAEDKAEDNKKS EA-------------------------------   137
M.t.   KQIIKQKIKQTIKQK TNQMIKKILKKIQRN EAGFYRFLHWFLHCF LHRFLYRFLYPGTSS IQFLHCFSIR  180
```

Figure 4-2: Gene arrangement and sequence alignment of the hypothetical protein from *M. thermophila* and *M. barkeri*. Panel A. Gene arrangement of *cysK*, *cysE*, ORF, and *nifS* in *M. thermophila* (*M.t.*) and *M. barkeri* (*M.b.*). Panel B. Sequence alignment of the hypothetical protein of *M. thermophila*, *M. barkeri* and *Archeoglobus fulgidus* (*AF0184*, *AF0566*). Identical residues are indicated in boldface. *M.t. cysE* and *M.b. nifS* sequences are truncated. The *M. thermophila* DNA sequence has been deposited at GenBank and the accession number is AF276772.

Discussion

The genomic sequence of five phylogenetically and metabolically diverse members of the Archaea provide little insight into mechanisms of cysteine biosynthesis in this domain. Although *cysK* was identified in *M. barkeri* [11], biochemical evidence for expression of *cysK* and involvement of CysK in cysteine biosynthesis was not reported. However, definitive biochemical evidence for expression of *O*-acetylserine sulfhydrylase and characterization of the enzyme was recently reported for *M. thermophila* [13]. Moreover, the enzyme from *M. thermophila* exhibits positive co-operativity indicative of involvement in a biosynthetic pathway [13]. Thus, the biochemical results reported for the *O*-acetylserine sulfhydrylase from *M. thermophila* suggests that pathway I operates in this archaeon for the synthesis of cysteine. Here we provide physiological and genetic evidence for involvement of this pathway. First, cystathionine γ-lyase and cystathionine β-synthase activities were not detected, indicating the only other pathway known for the synthesis of cysteine was not operable in *M. thermophila* under the conditions tested. Second, *O*-acetylserine sulfhydrylase activity in cells grown in media without cysteine and with sulfide was elevated 50-fold compared to the activity in cells grown in the presence of cysteine and sulfide where activity was fully repressed. Third, the *cysK* gene encoding *O*-acetylserine sulfhydrylase was found adjacent to an ORF with high identity to genes encoding serine transacetylase (*cysE*). Both *cysK* and *cysE* in *M. thermophila* are transcribed in the same direction and possibly form an operon.

Our previous results [13] describing the expression and allosteric properties of the *O*-acetylserine sulfhydrylase from *M. thermophila*, and the results reported here, suggest that the *cysK* gene identified in *M. barkeri* is also involved in cysteine biosynthesis and this pathway may operate in all *Methanosarcina* species. The apparent absence in the genomes of *M. jannaschii* and *M. thermoautotrophicum* of ORFs with identity to genes known to encode *O*-acetylserine sulfhydrylase and serine transacetylase suggests this pathway does not operate in all methanoarchaea; however, biochemical analyses are necessary to rule out convergently evolved enzymes with the requisite activities.

Likewise, biochemical approaches will be necessary to determine the pathways of cysteine biosynthesis in other members of the Archaea.

It was surprising to find that both cysteine and sulfide were necessary to repress fully the expression of *O*-acetylserine sulfhydrylase activity, assuming the enzyme functions only in the synthesis of cysteine. It was also unexpected to find a nearly 3-fold elevation of activity in cells grown with cysteine compared to cells grown with sulfide as the sole sulfur source. These results indicate another role for *O*-acetylserine sulfhydrylase other than cysteine biosynthesis. One possible alternative role is that *O*-acetylserine sulfhydrylase can mobilize sulfur from cysteine for other biosynthetic reactions. Additional observations are consistent with this hypothesis. First, growth with cysteine as the only sulfur source shows that cysteine can be imported, and, therefore, *O*-acetylserine sulfhydrylase is not needed for cysteine biosynthesis under these conditions. Second, the *O*-acetylserine sulfhydrylase purified from *M. thermophila* has a robust desulfurase activity of 240 nmols H_2S/min/mg (Figure 5-11). This activity compares favorably with the activity (89.4 nmols H_2S/min/mg) reported for *Azotobacter vinelandii* NifS [24] which catalyzes the desulfurization of cysteine for sulfide incorporation into iron-sulfur clusters. Third, during the purification of *O*-acetylserine sulfhydrylase from *M. thermophila* grown with cysteine as the sole sulfur source [13], only one other enzyme fraction with cysteine desulfurase activity was detected. This additional fraction was responsible for less than 10% of the total activity in extracts, indicating that the *O*-acetylserine sulfhydrylase is responsible for greater than 90% of the desulfurase activity in cells grown with cysteine as the only source of sulfur. When *M. barkeri* is grown with cysteine as the sole sulfur source, cysteine is rapidly taken up by the cells and desulfurated (as noted by the simultaneous evolution of sulfide into the media during cysteine uptake) [25]. If, as in *M. thermophila,* greater than 90% of the desulfurase activity is catalyzed by *O*-acetylserine sulfhydrylase then it is likely that this enzyme is largely responsible for the release of sulfide from cysteine in *M. barkeri*. Finally, the *E. coli O*-acetylserine sulfhydrylase also has desulfurase activity, and has been shown to aid in iron-sulfur cluster formation [26] similar to NifS.

A *nifS* gene is upstream of *cysK* and *cysE* in both *M. thermophila* and *M. barkeri*. NifS mobilizes sulfur from cysteine for the formation of Fe-S clusters [24]. Though it was originally discovered as one of a dozen enzymes required for nitrogen fixation in *Azotobacter vinelandii*, *nifS* has been found in non-nitrogen fixing organisms, suggesting a universal role for NifS in Fe-S cluster formation. It has been proposed that NifU may function in cluster formation by sequestering inorganic Fe, aiding in the release of sulfide from NifS [27], or donating Fe_2S_2 units for the synthesis of Fe-S clusters [28]. The hypothetical proteins encoded by the ORFs in *M. barkeri* and *M. thermophila* between *nifS* and *cysK* are similar to hypothetical proteins encoded by ORFs clustered with *nifU* and *nifS* homologs in the archaeon *A. fulgidus.* The location of these ORFs in archaeal species suggests the possiblity that the encoded hypothetical proteins may play a role in conjuction with CysK or NifS, possibly to assist in the mobilization of sulfur from cysteine or Fe-S cluster synthesis as proposed for NifU.

Acknowledgments

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Chapter 5

Experimental approaches towards defining an alternative role of *O*-acetylserine sulfhydrylase in addition to cysteine biosynthesis

Abstract

Two pathways for cysteine biosynthesis are known in nature; however, it is not known which, if either, the Archaea utilize. Recent genetic, biochemical, and physiological studies on *Methanosarcina thermophila* and *Methanosarcina barkeri* is suggestive that the *Methanosarcina* utilize *O*-acetylserine sulfhydrylase (CysK) and serine acetyltransferase (CysE) for cysteine biosynthesis. *O*-acetylserine sulfhydrylase has also been implicated to have additional roles in the cell apart from cysteine biosynthesis. Present here are further experimental evidence implicating *O*-acetylserine sulfhydrylase functions in the desulfuration of cysteine to supply sulfur for biosynthesis in *M. thermophila*. Over 90% of cysteine desulfurase activity in cell extracts of cells grown with cysteine as the sole sulfur source is due to *O*-acetylserine sulfhydrylase, suggesting that *O*-acetylserine sulfhydrylase provides the primary source of sulfide. Patterns of growth on trimethylamine, ammonia inhibition of the desulfurase activity, and changes in sulfide concentration of the media during growth support this interpretation. Experimental advances were made to obtain genetic knock-out mutants in *M. thermophila* deficient in *cysK* or *cysE*, to determine if an additional pathway exists for cysteine biosynthesis and to confirm the role of CysK in cysteine desulfuration. In addition a novel sensitive assay was developed for sulfide detection in cell extracts that is less prone to high background absorbances than published methods.

Introduction

Cysteine biosynthesis has been extensively studied in both the Eukarya and the Bacteria domains of life, yet little is known about cysteine biosynthesis in the Archaea. Both methanoarchaea genomes sequenced to date lack ORFs having a deduced sequence with significant identity to enzymes of either pathway of cysteine biosynthesis. However, recent data (as outlined in chapter 3 and chapter 4 of this thesis) suggest that at least the *Methanosarcina* utilize pathway I (consisting of CysK and CysE) for cysteine biosynthesis. However, these findings do not address how the other methanoarchaea (e.g., *M. janaschii* and *M. thermoautotrophicum*) synthesize cysteine. One possibility is that these organisms have the enzymes necessary for pathway I, but the genes could not be identified through sequence similarity searches because their sequences diverged too much from known pathway I genes. Another possibility is the methanoarchaea have a cysteine biosynthetic pathway unique to the Archaea. The question that was addressed here was if the *Methanosarcina* contain any unique alternative pathways for cysteine biosynthesis.

An additional role that has been proposed for *O*-acetylserine sulfhydrylase is mobilization of sulfur from cysteine for biosynthetic reactions, such as iron-sulfur cluster formation (5). The role of *O*-acetylserine sulfhydrylase to supply sulfide by cysteine desulfuration was investigated. This goal required a sensitive assay for sulfide in cell extracts. A common assay used in the literature detects sulfide by reaction with N,N-dimethyl-p-phenylene-diamine sulfate (DPD) in the presence of $FeCl_3$ to form methylene blue (20) (Figure 5-1). The assay is usually carried out by the addition of acidified solutions of DPD and $FeCl_3$ directly to the enzyme reaction mixture. Precipitated protein is pelleted by centrifugation, and the absorption at 650 nm is determined for the supernatant. Owing to the high concentration of cell extract required to detect desulfurase activity, the background resulting from nonspecific reactions was unacceptably high (up to 1.4 absorbance units). Presented here is the development of a more sensitive assay for sulfide formation by decreasing background absorbance.

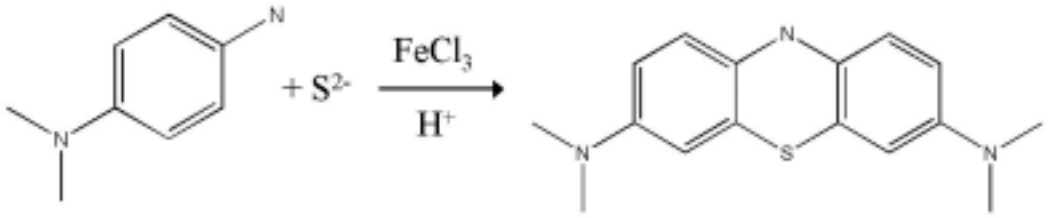

Figure 5-1: Reaction of sulfide with N,N-dimethyl-p-phenylene-diamine to form the indicator methylene blue.

An additional approach to investigate the physiological role of CysK in cysteine desulfuration, the role of CysK and CysE in cysteine biosynthesis and the possible existence of a yet unknown pathway for cysteine biosynthesis unique to the Methanoarchaea is through gene knock-out experiments. Presented here are experimental approaches for obtaining *M. thermophila* mutants deficient in *cysK* and *cysE*. Only recently have systems been developed for efficient transformation of Archaea (22). To my knowledge, there are only four published reports of successful archaeal gene disruptions, three in *Methanococcus voltae* (11) and one in *Halobacterium salinarum* (17). The method used in *Halobacterium salinarum* is not suitable for Methanoarchaea. One of the systems used in *Methanococcus voltae* was based on transposons, and is thus an uncontrolled form of mutagenesis. Homologous recombination of foreign DNA into the chromosome was used to obtain the other two gene disruptions in *M. voltae* (10, 18). Presented here are approaches for homologous recombination to obtain *M. thermophila* mutants deficient in *cysK* and *cysE*. Plasmids for eventual use in homologous recombination experiments to disrupt the genes were constructed. These plasmids contained *cysK* and *cysE* genes that were disrupted by insertion of a *pac* (puromycin acetyl transferase) gene from *Streptomyces alboniger* (15) flanked by the archaeal methyl CoM reductase promoter and terminator.

Materials and Methods

***M. thermophila* growth media and cell extract preparation.** General anaerobic methods for the growth of *M. thermophila* and preparation of extracts were as described (4, 16). Marine media (16) contained the following constituents in demineralized water at the final percent concentrations (wt/vol): NaCl, 2.3; $MgCl_2 \bullet 6H_2O$, 1.0; KCl, 0.076; $CaCl_2 \bullet 2H_2O$, 0.014; NH_4Cl, 0.05; $Na_2HPO_4 \bullet 7H_2O$, 0.11; Na_2CO_3, 0.3; trimethylamine, 1.0; resazurin, 0.0001; and 1% (vol/vol) each of vitamin and Wolfe's mineral solution as described (21, 25). Acetate or trimethylamine was added as substrate to a final concentration of 100 mM. The following were added individually or in combination to supply the sulfur source (final concentration in the media (wt/vol)): $Na_2S \bullet 9H_2O$, 0.025; cysteine-HCl $\bullet$ H_2O, 0.025. Transformed cells were grown in the presence of 1 μg/ml puromycin.

Cultures were grown at 37°C and transferred during late-log phase 10 successive times into fresh media containing either cysteine or sulfide or both (0.5 ml into 10 ml) before growth was recorded, to ensure no carryover of cysteine or sulfide from the original inoculum. At the end of the growth curves, a second batch was inoculated and the growth was recorded to ensure reproducibility. Growth was recorded by measuring absorbance at 550 nm (Spectronic 20, Bausch and Lomb).

Protein assay. Protein concentrations were determined by the method of Bradford (6), using Bio-Rad dye reagents and bovine serum albumin (Pierce) as a standard.

Sulfide mobilization assay. Sulfide was detected by conversion to methylene blue in an acidified solution of *N*,*N*-dimethyl-*p*-phenylenediamine and $FeCl_3$ (20). The enzyme catalyzed formation of sulfide was measured by incubating 1 mg of *M. thermophila* cell extract with 50 mM NaBicine (pH 7.8), 50 mM dithiothreitol, 10 mM L-cysteine, and the indicated salt concentrations for 1.5 hours at 40 ºC. The enzyme assay mix or media tested for free sulfide was placed in the base of a Warburg flask and acidified by addition of HCl (0.2 (vol/vol) final concentration). Free sulfide slowly transferred to a sidearm of the Warburg flask containing 0.8 ml of 1 mM NaOH and was

completed in 1.5 hours. After transfer, 100 µl of both 0.02 M *N*,*N*-dimethyl-*p*-phenylene-diamine sulfate (in 7.2 N HCl) and 0.03 M $FeCl_3$ (in 1.2 N HCl) were added in that order. Color was allowed to develop for 20 minutes before measuring absorbance at 650 nm.

Zymogram of cysteine desulfurase activity. An unboiled enzyme sample was applied to an SDS-PAGE gel. After electrophoresis SDS was removed from the gel by washing the gel twice for 10 minutes each in 50 ml of the following series of solutions: 2.5 % Triton in water, 2.5% Triton in Tris-HCl (50 mM, pH 7.4), and Tris-HCl (50 mM, pH 7.4). The gel was then incubated at 40 ^{o}C in developing solution for 1 – 12 hours (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM Cysteine, 0.5 M $PbNO_3$, 5 mM DTT, 5 mM pyridoxal phosphate). Bands of activity could be detected by precipitated PbS.

DNA isolation, purification and sequencing. Plasmids were purified from *E. coli* using QIAprep Spin Miniprep Columns (Qiagen). Digested DNA was purified on an agarose gel and extracted from the gel with the QIAEX II extraction kit (Qiagen). Genomic DNA was isolated from *Methanosarcina thermophila* with Puregene DNA isolation kit (PGC Scientifics Corporation). The manufacturers instructions were followed, except that a 1.5 ml aliquot of cells grown to a stationary phase in a 10 ml culture were pelleted and resuspended in 0.5 ml of TE buffer containing the following salts at the final percent concentrations (wt/vol): NaCl, 2.3 and $MgCl_2 \bullet 6H_2O$, 1.0; and cells were lysed by addition of 15 µL of 10 % SDS. DNA was sequenced using a Perkin Elmer ABI377 (Penn State University Sequencing Facility)

In vitro construction of *M. thermophila cysK*$^{-}$ and *cysE*$^{-}$ mutant alleles. The *M. thermophila cysK* and *cysE* genes were disrupted by insertion of a *pac* (puromycin acetyl transferase) gene from *Streptomyces alboniger* flanked by the archaeal methyl CoM reductase promoter and terminator from pJK3 (15). Plasmid pBB1 and pBB2 were constructed by ligating a 1753 bp PCR product containing *cysK* and *cysE* from *M. thermophila* into pSTBlue-1 and pETBlue-1 AccepTor Vector^{TM} (Novagen) respectively. Plasmid pBB3 was constructed by ligation of a BamHI-EcoRV *pac* gene fragment from pJK3 into pBB1 digested with BglII and EcoRV. The resulting construct was excised for *cysK* deletion from pBB3 by digestion with XhoI and BamHI (Figure 5-2a). Through site

directed mutagenesis, a PstI restriction site was engineered at the beginning of STA in pBB2. Plasmid pBB4 was constructed by inserting a PstI-EcoRV *pac* gene fragment from pJK3 into the mutant pBB2 digested with PstI and HincII. The resulting construct was excised for *cysE* deletion from pBB4 by digestion with NheI and AvrII (Figure 5-2b). Since both the construct and the remaining vector have the same size and thus not easily separated on a DNA gel, the vector/construct mix was digested with PvuI, which cuts the remaining vector in two locations. The two linear constructs containing a disrupted cysK and a disrupted cysE gene were sequenced to ensure proper insertion of the *pac* gene and were then used for transformation of *M. thermophila* cells.

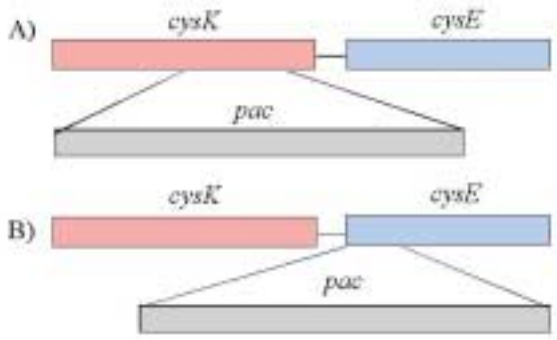

Figure 5-2: Diagram of insertion of the puromycin resistant gene into *M. thermophila* (A) *cysK* and (B) *cysE*.

Transformation. *E.coli* transformants were obtained following the manufacturers instructions (Novagen). *M. thermophila* cells were transformed by liposome mediated transformation as described in (2), except DNA was incubated with DOTAP (Boehringer Mannheim) for 1 hour before addition of cells. Plating of cells was essentially performed as described in (1), except the bottom agar contained puromycin (1 μg/ml).

Results

Development of a more sensitive sulfide assay than published methods. An additional function hypothesized for *O*-acetylserine sulfhydrylase is cysteine desulfuration. A major obstacle in analyzing desulfurase activity in cell extracts was the high background reading caused by reaction of the indicator DPD with cell extract components other than sulfide. Background readings were as high as 1.4 absorbance units. To alleviate this problem, it was attempted to separate the sulfide from the reaction mix before reacting it with DPD to form methylene blue.

Cell extract was assayed for cysteine desulfurase activity with Warburg flasks (Figure 5-3). The Warburg flasks were sealed from the atmosphere by glass or silicone rubber stoppers. Silicone was necessary because butyl rubber stoppers absorb sulfide. The reactions were quenched by addition of HCl which simultaneously volatilized the formed sulfide. The gaseous hydrogen sulfide migrated to the sidearm of the Warburg flask where DPD and $FeCl_3$ were added to detect the sulfide by methylene blue formation.

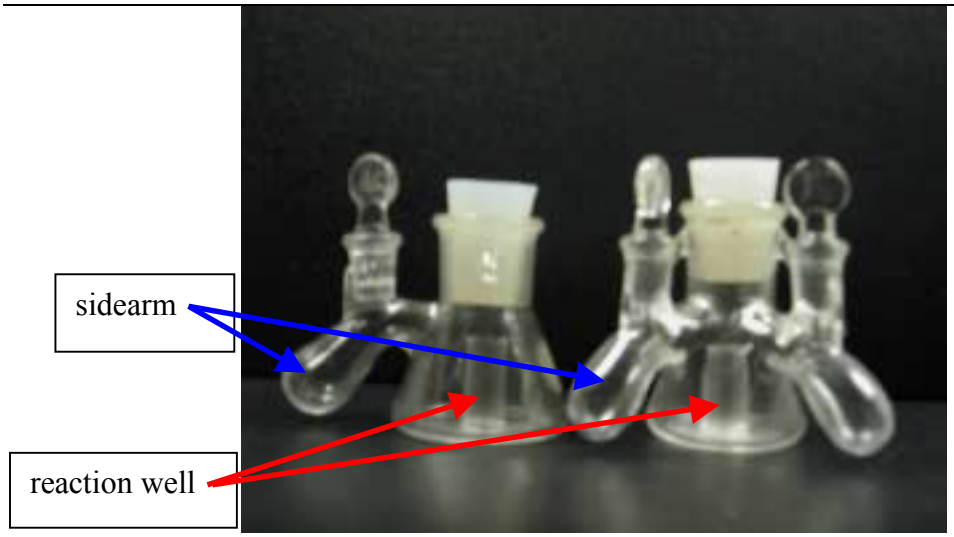

Figure 5-3: Warburg flasks.

Since the transfer efficiency of sulfide from the reaction well to the sidearm containing water proved poor (15%), a series of experiments were performed to determine the efficiency of various sulfide scavengers to increase sulfide transfer. First,

it was attempted to use a solution of DPD and $FeCl_3$ as scavenger, anticipating that the sulfide would react with the DPD and $FeCl_3$ to form methylene blue, as soon as the gaseous sulfide came in contact with the solution. However, while investigating the formation of methylene blue with respect to the order of addition of these components in a cuvette, it was discovered that more methylene blue was formed when the sulfide was already in solution, before addition of the acidic DPD and $FeCl_3$ solutions (Figure 5-4). It was concluded that acidic DPD and $FeCl_3$ solutions are not good candidates to scavenge sulfide, and that for maximal detection of sulfide, sulfide should first be transferred from the reaction well to the sidearm before adding the acidic DPD and $FeCl_3$ solutions. The second attempt involved buffering the pH of the solution in the sidearm containing DPD and $FeCl_3$ to a pH greater than 0. It was reasoned that the higher pH would allow sulfide to dissolve more readily in the solution, and the DPD and $FeCl_3$ would have more time to react with the sulfide to form methylene blue. However, while investigating the reaction of sulfide with DPD and $FeCl_3$ under various pH conditions in cuvettes, it was discovered that no methylene blue formed at pH 5 or higher (Figure 5-5). Finally 1 mM NaOH was used in the sidearm to keep the pH high enough for gaseous sulfide from the reaction well to dissolve in the sidearm solution, yet low enough so that upon addition of DPD and $FeCl_3$ the pH would decrease to 0, and methylene blue could easily form. Transfer efficiency from the reaction well to the sidearm under these conditions was a consistent 60% (Figure 5-6) over the range from 20 to 100 nmoles. Transfer of sulfide was at the maximum after 90 minutes (Figure 5-7), and there was a linear relationship between amount of sulfide added to the reaction well and the amount recovered in the sidearm over the range of 10 to 100 nmol (Figure 5-8).

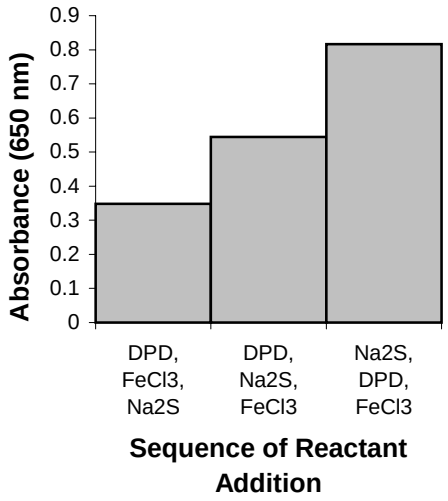

Figure 5-4: Detection of sulfide varying the sequence of addition of DPD, $FeCl_3$ and Na_2S.

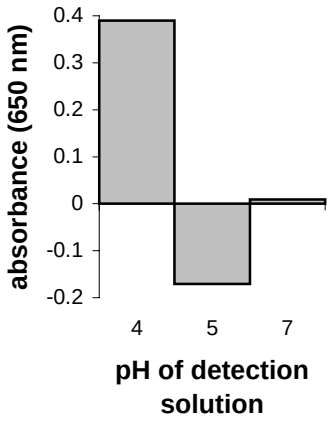

Figure 5-5: Detection of sulfide with DPD and $FeCl_3$ at various pH.

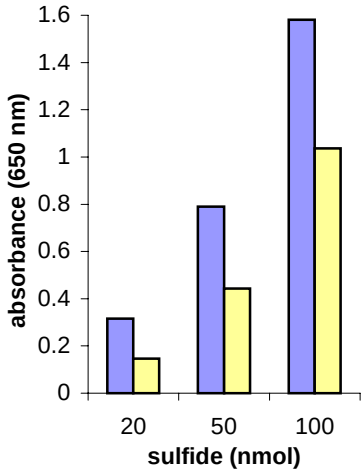

Figure 5-6: Transfer efficiency from Warburg reaction well to sidearm containing 1 mM NaOH. Symbols: amount of sulfide added (blue), amount of sulfide transferred (yellow).

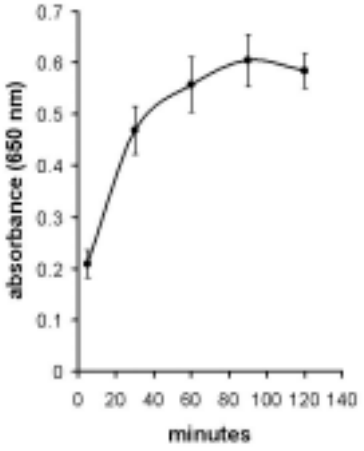

Figure 5-7: Time course for maximal transfer of sulfide from reaction well to sidearm of Warburg flask after addition of HCl to reaction well. The reaction well contained 75 nmol of sulfide before addition of HCl. The line was drawn to guide the eye.

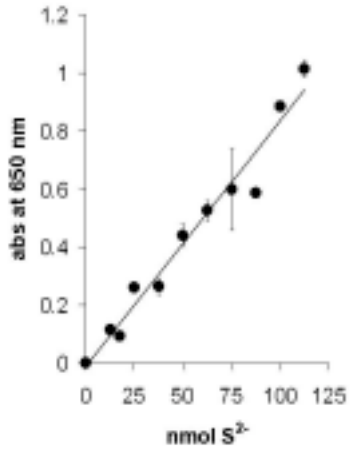

Figure 5-8: Standard curve relating the amount of sulfide added to the Warburg reaction well to absorbance at 650 nm of the sidearm solution after conversion of sulfide and DPD to methylene blue. The sidearm contained 1 mM NaOH.

Growth of *M. thermophila* with either cysteine, sulfide or cysteine plus sulfide as the source of sulfur. Several functions for *O*-acetylserine sulfhydrylase in cells have been hypothesized including formation of cysteine and desulfuration of cysteine for incorporation of sulfide into cofactors. The first question to be addressed was if *M. thermophila* can synthesize cysteine. The second question was if *M. thermophila* can desulfurate cysteine. *M. thermophila* was grown with only sulfide as the sulfur source to determine if the organism can synthesize cysteine, and *M. thermophila* was grown with only cysteine as sulfur source to determine if the organism can import cysteine and desulfurate it.

Growth with trimethylamine, as determined by optical density at 550 nm, was similar in media containing either cysteine plus sulfide or only sulfide as sulfur source (Figure 5-9). Thus *M. thermophila* is able to synthesize cysteine *de novo*. However, with only cysteine as the sulfur source, cells did not reach the same density as when sulfide only or cysteine plus sulfide were present as sulfur sources. Cells lysed easily and cell debris and intact cells accumulated at the bottom of the tube, explaining the apparent decrease in cell density at the end of log phase. Growth curves shown in Figure 5-9 are representative of a minimum of six separate growth curves measured.

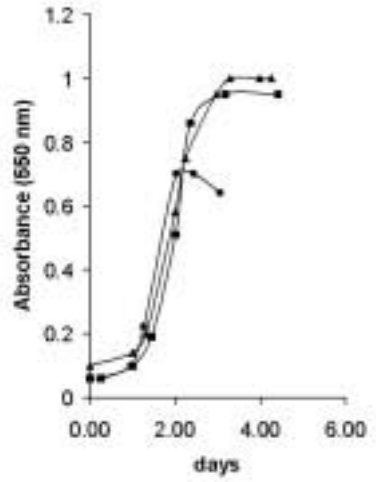

Figure 5-9: Growth of *M. thermophila* on trimethylamine in marine media. Symbols: with cysteine and sulfide (■), with sulfide only (▲), and with cysteine only (●) as sulfur source. Cultures (10 ml) were grown in 25 ml Hungate tubes at 37 ºC.

The low growth observed for *M. thermophila* with trimethylamine as substrate and cysteine as the sole sulfur source is not due to sulfide limitation. *M. thermophila* grown with acetate as substrate and cysteine as the sole sulfur source grew to the same density as acetate grown cells with sulfide or cysteine plus sulfide as sulfur sources (5). Instead, the low growth observed seems to be due to the switching of the growth substrate.

Inhibition of cysteine desulfurase activity by ammonia. During methanogenesis from trimethylamine, ammonia is synthesized, whereas no ammonia is synthesized during methanogenesis from acetate. Since the ammonia synthesized during methanogenesis from trimethylamine might provide insight into the cause for the low growth of *M. thermophila* with cysteine as the sole sulfur source, the inhibitory affect of ammonia on the desulfuration of cysteine by *M. thermophila* cell extract was investigated. The cysteine desulfurase activity in extracts of cells of *M. thermophila* is inhibited by millimolar concentrations of ammonia (Figure 5-10). Specific activity decreased to 60 % in the presence of 10 mM NH_4Cl and 40 % in the presence of 20 mM NH_4Cl. The cysteine desulfurase activity was not further inhibited by additional increase of NH_4Cl up to 100 mM. A similar inhibition curve was determined with $(NH_4)_2SO_4$

indicating the inhibition was due to ammonia and not chloride. Extract of *M. thermophila* cells grown with cysteine as the sole sulfur source were used for the assay. Under these growth conditions *O*-acetylserine sulfhydrylase is responsible for 90% of the desulfurase activity in extracts (data presented below), suggesting the ammonia inhibition of cell extract desulfurase activity is due largely to inhibition of *O*-acetylserine sulfhydrylase.

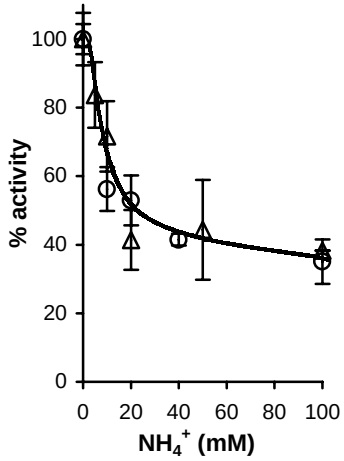

Figure 5-10: Inhibition of cysteine desulfurase activity of *M. thermophila O*-acetylserine sulfhydrylase. Symbols: NH_4Cl (Δ) and $(NH_4)_2SO_4$ (O).

The concentration of ammonia in the cells grown with trimethylamine can potentially reach 20 mM. The concentration of trimethylamine in the media at the start of the growth curves was 100 mM. During growth, the ammonia produced in the cells is probably exported into the media; however, as the concentrations of ammonia in the medium increases it probably becomes more difficult to export the ammonia. In this case the increased concentrations of ammonia in the cell could then inhibit cysteine desulfuration.

Another source of sulfide for *M. thermophila* cells grown with trimethylamine and cysteine as the sole sulfur source is trace amounts of sulfide contamination in cysteine. Media with cysteine as sole sulfur source contained 9.2 ± 0.6 μM sulfide due to these trace contaminations. The sulfide concentration decreased slightly when grown on trimethylamine (6.8 ± 1.1 μM), but remained the same when grown on acetate (9.9 ±

0.9 μM). The sulfide concentration was measured before inoculation and at the end of log phase. *M. thermophila* thus seems to import sulfide when grown with trimethylamine, but not to import sulfide when grown with acetate. These results are consistent with inhibition of *O*-acetylserine sulfhydrylase by ammonia.

Resolution of enzymes with cysteine desulfurase activity from *M. thermophila* cell extracts. An additional role for *O*-acetylserine sulfhydrylase has been implicated to be cysteine desulfuration (4). The question to be addressed was if *O*-acetylserine sulfhydrylases cysteine desulfuration activity is of physiological importance, or if another enzyme can provide adequate amounts of free sulfide for cell growth. Therefore resolution of all enzymes with cysteine desulfurase activity from extracts of *M. thermophila* cells grown with cysteine as the sole sulfur source was attempted. Specific cysteine desulfurase activity of the cell extract was 0.65 nmol S^{2-}/min/mg. Elution of *M. thermophila* cell extract from a Q-Sepharose column produced two distinct peaks containing cysteine desulfurase activity (Figure 5-11). The first peak contained 90% of all cysteine desulfurase activity. A zymogram of cysteine desulfurase activity of this peak exhibited one activity band (Figure 5-12, Lane 2), suggesting that only one enzyme is responsible for the cysteine desulfuration. The enzyme catalyzing cysteine desulfurase activity of this peak has been purified to homogeneity and shown to be *O*-acetylserine sulfhydrylase (4). Purified *O*-acetylserine sulfhydrylase from peak I has a robust desulfurase activity of 240 nmol S^{2-}/min/mg. Cysteine desulfurase activity was also dependent on DTT in the reaction mix.

Table 5-1: Effect of DTT on cysteine desulfuration activity of *O*-acetylserine sulfhydrylase. All experiments were performed in triplicate.

[DTT] (mM)	k_{cat} x 10^3 (s^{-1})
0	0.16 ± 0.04
28	1.1 ± 0.3
55	1.4 ± 0.2

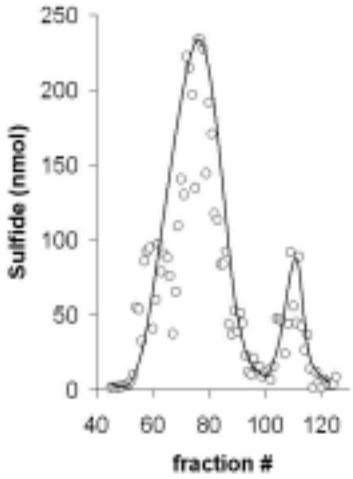

Figure 5-11: Q-Sepharose elution profile of cysteine desulfurase activity in *M. thermophila* cell extract. The line was drawn to guide the eye.

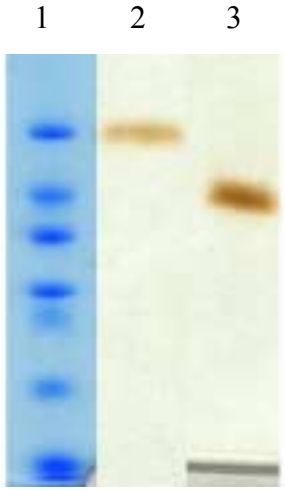

Figure 5-12: Zymogram detecting cysteine desulfurase activity of *M. thermophila* proteins. Lane 1: molecular mass markers: 66, 45, 36, 29, 24, 20, 14.2 kDa. Lane 2: 2 μg of enzyme from peak I enriched for cysteine desulfurase activity over 4 columns. Lane 3: 5 μg of enzyme from peak II enriched for cysteine desulfurase activity over 4 columns. (Enrichment as outlined in materials and methods of Chapter 3)

The cysteine desulfurase activity of peak II also seems due to only one enzyme since a zymogram of that fraction revealed only one band of activity (Figure 5-12, Lane 3). The protein responsible for cysteine desulfurase activity is distinct from *O*-acetylserine sulfhydrylase, since the zymogram of peak II revealed a cysteine desulfurase activity band at 45 kDa while peak I revealed a band at 66 kDa. Enzyme of peak II also did not have *O*-acetylserine sulfhydrylase activity.

Attempts to purify and identify the enzyme responsible for the cysteine desulfurase activity of peak II was unsuccessful because of the low abundance of the protein. Desulfurase activity of peak II of 60 grams of *M. thermophila* cells was enriched over four column steps. All of the proteins recovered from the last column were subjected to N-terminal analysis. An N-terminal analysis was obtained from the most abundant proteins and identified as methyl CoM reductase and 3-isoproplymalate dehydrogenase, neither of which contain cysteine desulfurase activity. None of the other proteins were of high enough abundance to obtain N-terminal analysis. Thus the identity of the enzyme or enzymes from peak II containing cysteine desulfurase activity was not obtained.

Experimental approaches for recombination of *in vitro* constructed *cysK*$^{-}$ and *cysE*$^{-}$ mutations onto the *M. thermophila* chromosome. Further understanding of the role of CysK in cysteine desulfuration and CysK and CysE in cysteine biosynthesis in *M. thermophila* can be obtained by studying mutants defective in *cysK* and *cysE*. In addition we wanted to address the question if *M. thermophila* contains a second cysteine biosynthetic pathway distinct from CysK and CysE. Since homologous recombination of mutant DNA into chromosomal DNA is the most efficient direct method for gene knock-out experiments in the Methanoarchaea, plasmids were constructed that contain disrupted *cysK* and *cysE* genes from *M. thermophila*. Plasmid pBB3 contained *cysK* and *cysE* with the *cysK* being disrupted by insertion of the *pac* gene which confers resistance to puromycin to the Archaea (Figure 5-2a). Plasmid pBB4 contained *cysK* and *cysE* with the *cysE* being disrupted by insertion of the *pac* gene (Figure 5-2b). Homologous sequences between plasmid DNA and host chromosome DNA were 488 and 942 bp for the *cysK*$^{-}$ and 1025 and 547 bp for *cysE*$^{-}$.

The *cysK*$^{-}$ and *cysE*$^{-}$ alleles were excised from pBB3 and pBB4, respectively. Recombination of the alleles (Figure 5-2) into the *M. thermophila* chromosome was attempted by double homologous recombination after transformation with the excised DNA. Recombination between homologous sequences of the excised plasmid DNA and the host chromosome should replace the wild-type allele with the mutant allele. Organisms in which the transformation was successful will be resistant to puromycin owing to the newly acquired *pac* gene. Recombinants were selected through their puromycin resistance (Pur^{R}).

Transformed *M. thermophila* were grown in the presence of sulfide and cysteine as sulfur source because under these conditions CysK was expressed at the lowest level (5), thus giving the knock-out mutants the greatest chance of survival. Half of all transformations of *M. thermophila* with approximately 2 μg of excised plasmid DNA grown in the presence of puromycin yielded no colonies at all. The other half of the transformations yielded 2 (±2) colonies within three weeks, an additional 2 (±3) colonies within one month, and an additional 12 (±13) colonies after two months of incubation. All colonies were restreaked once and then grown in liquid culture. Genomic DNA was isolated from the liquid cultures, the *cysK* and *cysE* genes were amplified via PCR, and the size of the amplified DNA was determined. Mutant *cysK* and *cysE* genes containing *pac* are 1 kb longer than wild type *cysK* and *cysE*.

Nine out of ten colonies picked were not transformants, and one colony was a mixture of transformed and non-transformed cells, as determined by the size of amplified *cysK* and *cysE*. Transfer of an aliquot of a growing culture of the mixed cells into fresh media resulted in complete loss of the transformants. Growth rate and cell density at the end of log phase of wild type *M. thermophila* in the presence and absence of puromycin were similar, showing they are resistant to puromycin. However, it was recently discovered that the wild-type cells were contaminated with cells already containing the puromycin resistant gene.

Discussion

An additional role for *O*-acetylserine sulfhydrylases has been hypothesized to be cysteine desulfuration (5). Desulfuration of cysteine in the absence of free sulfide in the media is essential to supply sulfur for biosynthesis. *M. thermophila* contains many enzymes with iron-sulfur clusters, and *O*-acetylserine sulfhydrylase might provide the necessary sulfide to form the clusters. The *O*-acetylserine sulfhydrylase from *E. coli* has been shown to aid in iron-sulfur cluster formation *in vitro*, but it is unknown if the enzyme also does so *in vivo*; thus additional experiments are necessary to establish a role for O-acetyserine sulfhydrylase in iron-sulfur cluster formation *in vivo*.

During growth of *M. barkeri* with methanol with cysteine as the sole sulfur source, the concentration of sulfide in the media increased rapidly from 0 mM to 0.8 mM after 35 hours of growth, and then slowly decreased to 0.5 mM after 95 hours of growth (14). The increase in sulfide was attributed to the activity of a cysteine desulfurase. Although the enzyme was not purified and characterized, it is most likely not a NifS or IscS-like enzyme, because the additional product of cysteine desulfuration by *M. barkeri* cell extract was pyruvate (14), whereas in NifS and IscS the additional product is alanine. This raises the possibility, that the enzyme responsible for the cysteine desulfuration activity in *M. barkeri* is an *O*-acetylserine sulfhydrylase, because under similar growth conditions using acetate as substrate *O*-acetylserine sulfhydrylase with robust desulfurase activity was expressed in *M. thermophila* (5), and accounted for 90% of the desulfuration activity in the cell extract (Figure 5-11). However, it is unknown if the additional product of cysteine desulfuration by *O*-acetylserine sulfhydrylase is pyruvate, and therefore is similar in mechanism to the cysteine desulfurase of *M. barkeri*.

If *O*-acetylserine sulfhydrylase is responsible for the increase in sulfide in the media in *M. barkeri* when grown with cysteine as the sole sulfur source, then a similar increase in sulfide in the media should be observed for *M. thermophila* when this organism is grown with cysteine as the sole sulfur source. However, during growth of *M. thermophila* with acetate with cysteine as the sole sulfur source, the concentration of sulfide in the media remained constant (9.2 ± 0.6 μM at beginning, 9.9 ± 0.9 μM at end).

It is possible that the concentration of sulfide in the media increased and decreased during growth as was observed with *M. barkeri*, however, at the end of growth the concentration of sulfide in the media in the *M. barkeri* culture was still higher (0.5 mM) than the sulfide concentration in the media from *M. thermophila*. An alternative explanation why the concentration of sulfide increased in the media of the *M. barkeri* culture, but not in the *M. thermophila* culture is that the growth substrates used were different. *M. barkeri* was provided with methanol and *M. thermophila* was provided with acetate. Acetate is a source of fixed sulfur and methanol is not. *M. barkeri* might have desulfurated cysteine not only to use the sulfide as a sulfur source, but also to use the pyruvate derived from cysteine desulfuration as a fixed carbon source. The cell yield from *M. barkeri* cells grown with methanol and cysteine as sole sulfur source was higher with respect to the amount of methanol consumed, than when sulfide was the sole sulfur source (14). Thus *M. barkeri* desulfurates more cysteine than is necessary if only the sulfide was needed. In comparison, *M. thermophila* grown with acetate as substrate could use acetate as a fixed carbon source, instead of pyruvate from desulfurated cysteine, thus negating the necessity to desulfurate cysteine at such high rates.

However, when *M. thermophila* was grown with cysteine as the sole sulfur source using trimethylamine as substrate, the organism grew very poorly, lysing easily, and the concentration of sulfide in the media decreased from 9.2 ± 0.6 μM to 6.8 ± 1.1 μM. Instead of desulfurating cysteine as a sulfur source, *M. thermophila* seems to import sulfide from the media under these growth conditions. During growth on trimethylamine, high amounts of ammonia are produced in the cell as an additional product of methanogenesis. Ammonia inhibits desulfuration activity of *O*-acetylserine sulfhydrylase from *M. thermophila* up to 60% at ammonia concentrations of 20 mM.

Inhibition of *O*-acetylserine sulfhydrylases desulfuration activity by ammonia could partly account for the minimal growth with trimethylamine in the presence of cysteine and absence of sulfide. As *M. thermophila* demethylates the amine to form ammonia during methanogenesis, the concentration of ammonia in the cell could potentially reach concentrations that inhibit *O*-acetylserine sulfhydrylase, which could account for the early termination of growth due to sulfur starvation. The decrease of

sulfide in the media during growth on trimethylamine is consistent with this interpretation.

The mechanism of *M. thermophila* OASS cysteine desulfuration is currently unknown; however, it is most likely distinct from the mechanism of NifS and IscS cysteine desulfuration. In NifS and IscS a reactive cysteinyl in the active site is crucial for activity (Figure 2-3). Although *S. typhimurium* and *E. coli* OASS contain a cysteine residue in their active sites (C42), the functional group of the amino acid is buried in the protein, away from the active site (7). Replacement of the cysteine residue of *S. typhimurium* OASS with serine resulted in an enzyme that could still synthesize cysteine, but that had a 50-fold lower stability in the α-aminoacrylate intermediate, suggesting that the cysteine residue is not essential for catalysis, but does play an important role in stabilizing the α-aminoacrylate intermediate (23). The OASS enzyme from *M. thermophila* does not contain a cysteine residue at the equivalent position of C42 in *S. typhimurium*; however, the enzyme does contain two cysteine residues along its peptide chain (C82 and C84). It does not appear that these cysteine residues are near the active site by comparing the primary sequence of *M. thermophila* OASS with the *S. typhimurium* OASS, which has been crystallized. Therefore, in the absence of a reactive cysteine in the active site, the cysteine desulfuration mechanism of OASS from *M. thermophila* must follow a different reaction mechanism than that proposed for NifS and IscS.

Recently a second type of cysteine desulfurase (C-DES) enzymes was isolated from *Synechocystis*. This enzyme also catalyzes the desulfuration of cysteine for incorporation of the sulfide in iron-sulfur clusters similar to NifS and IscS (13). Yet the mechanism of cysteine desulfuration in this enzyme is unique from other NifS and IscS enzymes, because C-DES does not contain a reactive cysteine residue in its active site and the enzyme is not inactivated by alkylating reagents. In addition, the products of cysteine desulfuration are not sulfur and alanine, but sulfide, pyruvate and ammonia (13). The enzyme therefore follows a different reaction mechanism from the one depicted in Figure 2-3.

Though the C-DES from *Synechocystis* does not contain a reactive cysteinyl in its active site, there is evidence that the product of the cysteine desulfuration reaction is a persulfide of a cysteine. C-DES prefers cystine over cysteine, and the cysteine forming the persulfide can thus be substrate derived (13). Evidence for a substrate derived persulfide was obtained by studying the reaction of desaminocystine with C-DES. The reaction products were pyruvate and 3-(disulfanyl)-propionic acid, which spontaneously cyclize to form 1,2-dithiolan-3-one, a stable derivative of the linear persulfide (12). The crystal structure of C-DES also identifies the product of cystine desulfuration as a cysteine persulfide. The reactive cysteine persulfide is stabilized through a hydrogen bond with a histidine residue in the active site of the enzyme (8). Since persulfides are not better leaving groups than sulfide, the existence of a persulfide as the products of catalysis by NifS, IscS, and C-DES might be indicative of the requirement of a persulfide by the enzymes accepting the sulfur from the cysteine desulfurating enzymes (e.g. NifU and IscU) (8). With the above information, the mechanism depicted in Figure 5-13 has been proposed for the C-DES enzyme (8).

OASS from *M. thermophila* could plausibly desulfurate cysteine following a similar mechanism, except that cysteine is the substrate instead of cystine, and no cysteine persulfide is formed as a product. When the α-aminoacrylate intermediate of OASS from *S. typhimurium* is preformed by reaction of *O*-acetylserine with OASS in the absence of a nucleophile, the α-aminoacrylate is slowly released from the enzyme and can undergo hydrolysis to ammonia and pyruvate (9). However the k_{cat} (0.07 s^{-1}) of this reaction for OASS from *S. typhimurium* is extremely low compared to the turnover rate found for *M.thermophila* OASS cysteine desulfuration activity (9 s^{-1}).

Inhibition of OASS cysteine desulfurase activity by ammonia was initially assumed to be due to product inhibition, which would lend support to an OASS cysteine desulfurase reaction mechanism similar to C-DES; however, similar inhibition was determined with NaCl, suggesting that the inhibition is not due to the identity of the salt, but rather due to an ionic strength effect. An additional problem with the mechanism depicted in Figure 5-13 is that it does not account for the apparent dependence of cysteine desulfurase activity on DTT (Table 5-1).

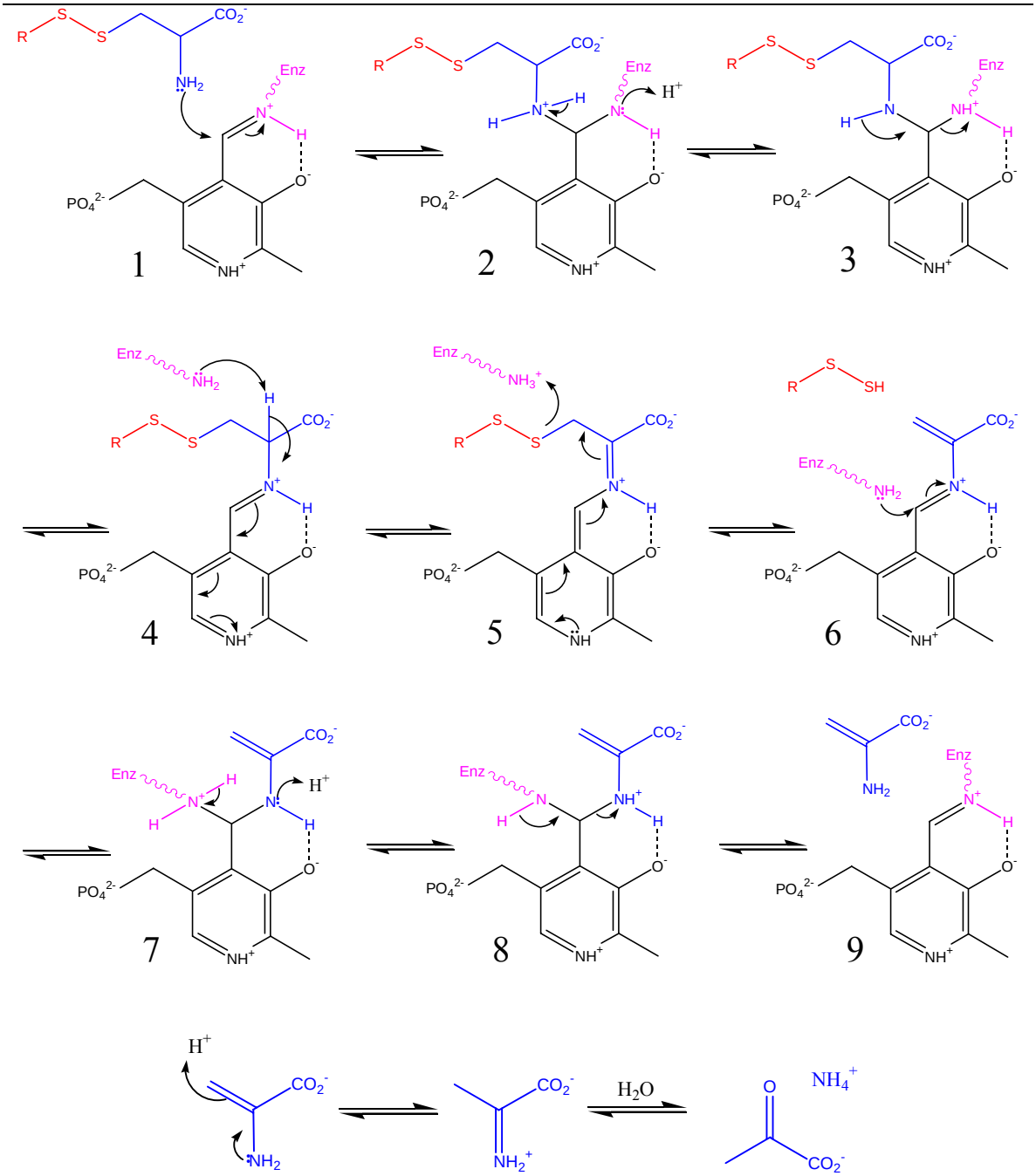

Figure 5-13. Proposed enzymatic mechanism of C-DES from *Synechocystis* (8). Intermediates: internal [1, 9] and external [4] aldimine; geminal diamine [2, 3, 7, 8]; quinonoid [5]; and α-aminoacrylate [6].

An alternative explanation for the low growth of *M. thermophila* with TMA as substrate and cysteine as the sole sulfur source is, that the strain used to obtain the growth

curves was defective in the cysteine transport chain. The strain used to obtain the growth curves was the mutant strain that exhibited resistance to puromycin and contained the *pac* gene. It is possible that the *pac* gene in this organism disrupts one of the genes necessary for cysteine transport. All acetate growth curves were recorded using the wild type strain.

An alternative mechanism for cysteine desulfuration that does account for the dependence of cysteine desulfuration by OASS on DTT is outlined in Figure 5-14, where R-S in this case would be DTT. Upon binding of cysteine to OASS from *S. typhimurium*, an external aldimine is formed (9). Data from spectral analysis suggest that cysteine does not undergo α-β elimination to form the α-aminoacrylate (19). However, when OASS from *S. typhimurium* was incubated with elevated concentrations of cysteine, L-lanthionine was formed (Figure 5-15, structure 1) (24). It seems under these conditions cysteine took the place of the competing nucleophile (R-S in Figure 5-14). Kinetic analysis of the formation of lanthionine resulted in a k_{cat} for α-aminoacrylate formation from cysteine of 100 s^{-1}. Under the conditions used for the spectral determination of cysteine binding to OASS, the absence of a competing nucleophile ensured that sulfide attacked the α-aminoacrylate once formed to regenerate cysteine. The equilibrium constant of this reaction must lie far towards synthesis of cysteine, so that spectral analysis only detected formation of the external aldimine containing the cysteine in Schiff base with the pyridoxal 5'-phosphate.

In the proposed mechanism for cysteine desulfuration, DTT could act as the competing nucleophile. In OASS from *S. typhimurium*, many substrate analogs able to react with the α-aminoacrylate in place of HS^- have been identified. Substrate specificity appeared independent from the size of the substrate, and more dependent on the pK_a of the nucleophile. Therefore in the presence of an excess of DTT in the reaction mix, it is possible that OASS could use this substrate in place of sulfide, to form 1-cysteinyl-4-thio-threitol (Figure 5-15, structure 2). Future research should address what the products are for cysteine desulfuration with OASS from *M. thermophila*.

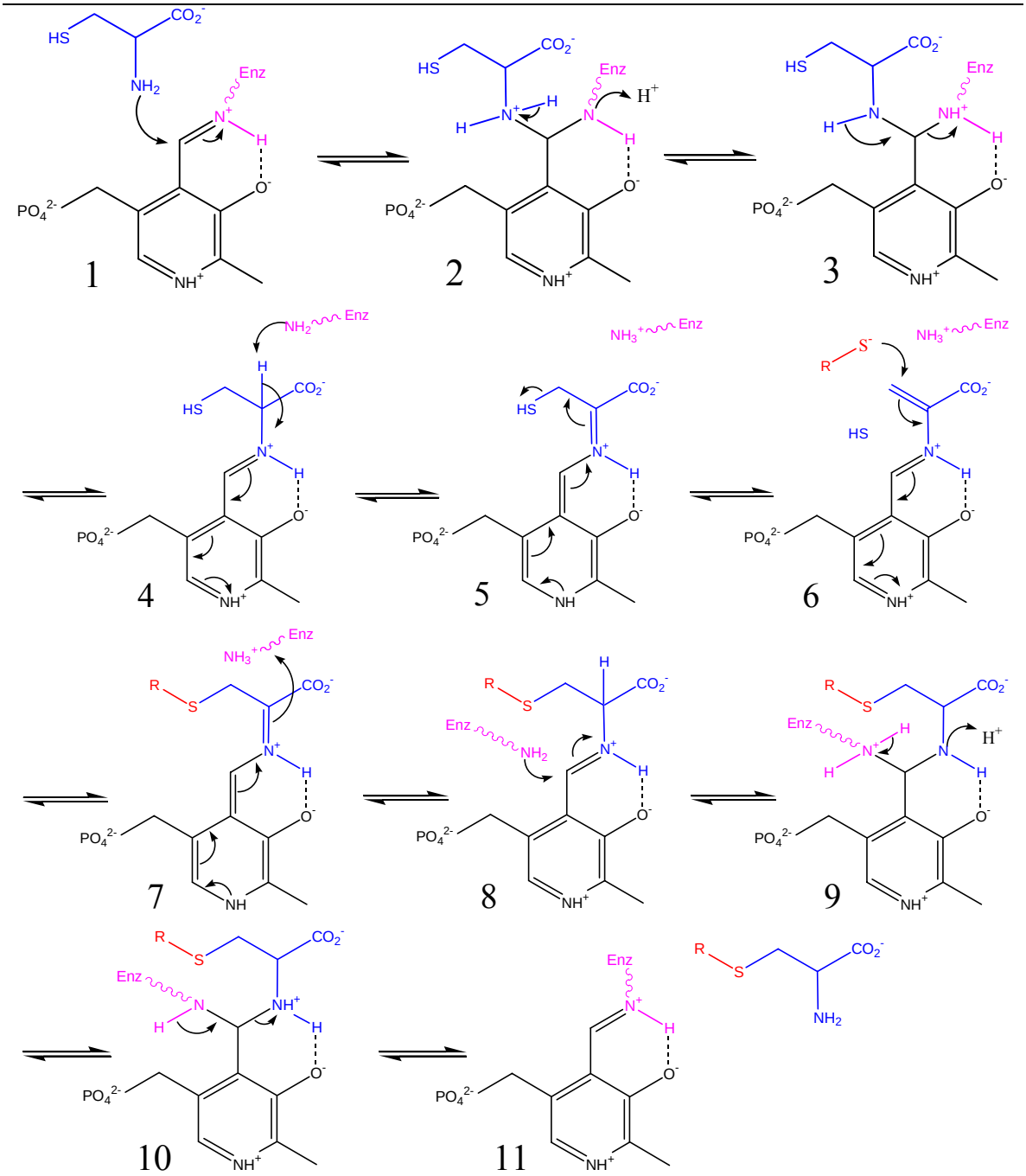

Figure 5-14. Proposed mechanism of cysteine desulfuration by OASS. Intermediates: internal [1, 11] and external [4, 8] aldimine; geminal diamine [2, 3, 9, 10]; substrate [5] and product [7] ketimine quinonoid; and α-aminoacrylate [6].

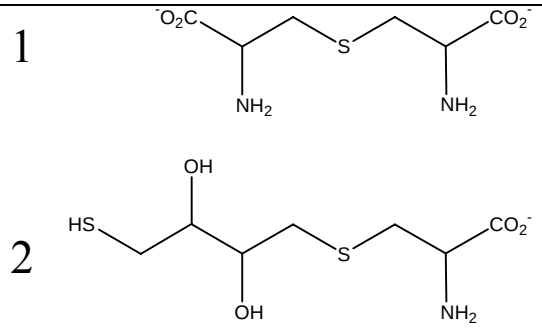

Figure 5-15. Structure of (1) lanthionine and (2) 1-cysteinyl-4-thio-threitol.

Assuming OASS from *M. thermophila* is involved in cysteine desulfuration *in vivo*, it would be interesting to identify the competing nucleophile that would replace the sulfide, once the α-aminoacrylate is formed from cysteine in the OASS active site. In addition, if OASS provides the sulfur for iron-sulfur cluster formation, it would be interesting to determine if OASS can interact with and donate the sulfide to a NifU or IscU protein. It is possible that OASS requires an additional protein for this transfer, especially if NifU or IscU can only accept a sulfane sulfur. The protein product of a conserved ORF upstream of *cysK* and *cysE* in *M. thermophila* and *M. barkeri* has been hypothesized to be involved in such a transfer (5).

However, it is possible that the high expression of OASS in cells grown with cysteine as the sole sulfur source is not because the enzyme is the major source of free sulfide to cells, and that a different enzyme performs this function. In the cell extract of *M. thermophila*, a second enzyme or enzymes was detected that accounted for 10% of the cysteine desulfuration activity in the cell extract. It is possible that this second enzyme or enzymes is inhibited at the concentrations of DTT and cysteine used for the assay, and that its/ their activity accounts for more than 10% of the desulfurase activity under *in vivo* conditions (3). In addition, two open reading frames were identified upstream of *cysK* and *cysE* that have sequence identity to *nifS* and *nifU*. These genes need to be expressed and the gene products characterized to determine if they are involved in cysteine desulfuration for incorporation of the sulfide in FeS clusters.

A novel assay was developed for the detection of sulfide in cell extracts. Conversion of sulfide to methylene blue with *N*,*N*-dimethyl-p-phenylene-diamine in the

presence of the cell extract resulted in high background absorbances of up to 1.4 absorbance units. In the developed assay, the sulfide is first separated from the cell extract before conversion to methylene blue, thus decreasing the background noise to 0.0002 absorbance units. Even though only 60 % of the sulfide could be recovered after transfer, the assay was sensitive to nmol quantities of sulfide.

Isolation of mutants defective in genes whose products are involved in cysteine biosynthesis are a powerful method used in studying cysteine biosynthesis. Homologous recombination of mutant alleles into the genome is one method used to obtain mutants in the Archaea. The homologous recombination event is dependent on the length of homologous DNA on both sides of the mutated genes. Currently the length of homologous DNA around the mutated *cysK* and *cysE* genes ranges from under 0.5 to over 1 kb, which could account for the small amount of transformants obtained. Ideally the length of homologues DNA flanking both sides of the mutated genes would be well over 1 kb.

An unforeseen challenge of the experiment was the unexpected high incidence of resistance of *M. thermophila* to puromycin. Almost 90% of all puromycin resistant colonies isolated were not transformants and did not contain the *pac* gene inserted into their *cysK* or *cysE* genes. Additionally, naturally resistant organisms tended to have a growth advantage over transformants, since transfer of a mixed culture containing wild type and knock-out mutants into new media containing puromycin resulted in complete loss of the transformants. Increasing the concentration of puromycin from 1 μg/ml to 2 μg/ml or higher might decrease the amount of naturally resistant organisms able to grow in the presence of the selection marker. The complete loss of $cysK^{-}$ knock-out mutants after transfer of the mixed culture into new media might also be indicative that the $cysK^{-}$ mutation is lethal to the cell. However, it was recently discovered that the *M. thermophila* culture used for the transformation experiments was a mixed culture, containing cells that already had the puromycin resistance gene which could account for the high rate of naturally resistant colonies isolated. A new culture of *M. thermophila* cells was obtained and analyzed to ensure it does not contain the *pac* gene. The strain is now being used to continue the genetic experiments in the laboratory.

Acknowledgements

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Chapter 6

Conclusion

The archaea seem to utilize two or more pathways for cysteine biosynthesis. The presence of open reading frames (ORFs) in the genomes of *A. pernix*, *Halobacterium* sp. NRC-1, *P. horikoshii* and *T. acidophilum* with deduced sequence having identity to cystathionine β-synthase and cystathionine γ-lyase sequences is consistent with pathway II for cysteine biosynthesis in these Archaea. Additionally, *Halobacterium* sp. NRC-1 has ORFs in the genome with deduced sequence having identity to *O*-acetylserine sulfhydrylase and serine acetyltransferase, which is consistent with pathway I for cysteine biosynthesis in the Archaea. However, the genomes of both methanoarchaea sequenced contain no ORFs with deduced sequences having identity to any known enzymes involved in cysteine biosynthesis suggesting an alternative pathway for cysteine biosynthesis in the methanoarchaea.

The work presented here outlines evidence supportive of pathway I for cysteine biosynthesis in the *Methanosarcina*. First, the genome of *M. thermophila* contains genes with deduced sequences having identity to *O*-acetylserine sulfhydrylase and serine acetyltransferase. The genes are transcribed in the same direction and possibly form an operon. Second, biochemical studies of *O*-acetylserine sulfhydrylase revealing the enzymes cooperativity, are consistent with its involvement in cysteine biosynthesis. Third, expression of the enzyme under cysteine limiting growth conditions and repression in the presence of cysteine and sulfide in the growth media are indicative of its involvement in cysteine biosynthesis. Fourth, activities of enzymes of pathway II, cystathionine γ-lyase and cystathionine β-synthase, were not detected under all growth conditions tested, suggesting that the other known cysteine biosynthetic pathway is not operative under the conditions tested.

The work presented here also outlines evidence supportive of an additional role for *O*-acetylserine sulfhydrylase apart from cysteine biosynthesis. First, *O*-acetylserine sulfhydrylase expression was elevated nearly 3-fold in cells grown with cysteine as the sole sulfur source compared to cells grown with sulfide as the sole sulfur source. Second, *O*-acetylserine sulfhydrylase desulfurates cysteine at a rate comparable to NifS – an enzyme which primarily functions in cysteine desulfuration for iron-sulfur cluster formation. Third, *O*-acetylserine sulfhydrylase accounts for over 90% of the desulfurase activity in cell extracts grown with cysteine as the sole sulfur source, and the concentration of sulfide in the media increases under these growth conditions, suggesting *O*-acetylserine sulfhydrylase catalyzes cysteine desulfuration *in vivo*, and provides the primary source of sulfide for biosynthesis. Patterns of growth on trimethylamine and inhibition of cysteine desulfurase activity by ammonia support this interpretation. During growth on trimethylamine, an excess of ammonia is produced during methanogenesis. Ammonia inhibits cysteine desulfuration by 60% at ammonia concentrations above 20 mM. Switching of growth substrate from acetate to trimethylamine with cysteine as the sole sulfur source resulted in slow growth with unhealthy cells and a marked decrease in free sulfide concentration in the media during growth.

Genetic knock-out work is in progress to determine if an additional pathway for cysteine biosynthesis exists in the *Methanosarcina*, and to confirm the role of *O*-acetylserine sulfhydrylase in the desulfuration of cysteine to supply sulfur for biosynthesis. Unforeseen challenges in this work have been the high rate of resistant to the selection marker puromycin and the low frequency of homologous recombination events. Increasing the amount of puromycin should decrease the amount of naturally resistant cells able to grow. The frequency of homologous recombination events is partly due to the length of homologous DNA available and partly due to the organisms physiology. Thus increasing the length of homologous DNA on both sides of the mutated genes or working with *Methanosarcina acetivorans*, an organism known to undergo homologous recombinations more easily (1), could increase the frequency of the recombination event. However the main obstacle towards isolation of *cysK*$^{-}$ and *cysE*$^{-}$ mutants has been that I only recently discovered that the original *M. thermophila* culture

used for the genetic knock-out work was contaminated with cells already containing the resistance marker.

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Appendix A

Protocols for assays used

Cysteine desulfuration activity assay:

liquid (use Warburg flask)

Place glass beads in bottom of Warburg flask to weigh down flask.

In one sidearm of Warburg flask, add 0.8 ml of 1 mM NaOH.

In center of Warburg flask mix the following assay solutions:

- 20 µl NaBicine (0.05 M, pH 7.8)
- 10 µl DTT (1 M)
- 20 µl L-cysteine (0.1 M)
- x µl enzyme

H_2O to make a total volume of 200 µl.

Place Warburg flask in water bath at 40 ^oC.

Let reaction proceed for 1 to 1 ½ hours.

Stop reaction by addition of 200 µl of dilute HCl (3 part HCl/ 2 part H_2O)

Let sulfide transfer to NaOH solution for 1½ hours at room temperature.

Then add to side-arm containing NaOH solution

- 100 µl DPD (0.02 M N,N-dimethyl-p-phenylene-diamine sulfate in 7.2 N HCl)
- 100 µl $FeCl_3$ (0.03 M $FeCl_3$ in 1.2 N HCl)

Let color develop for 20 min. at room temperature

Measure absorbance at 650 nm.

in gel (Zymogram)

Run SDS or non-denaturing protein gel on unboiled sample.

To remove SDS wash gel in following solutions 10 min. each:

2 times in 50 ml 2.5% Triton in H_2O

2 times in 50 ml 2.5% Triton in Tris (50 mM, pH 7.4)

2 times in 50 ml Tris (50 mM, pH 7.4)

Incubate gel in freshly made developing solution at 37^{o}C for 1 – 16 hours.

Developing solution contains: 50 mM Tris pH 7.4, 10 mM $MgCl_2$,

10 mM Cysteine, 0.5 mM $PbNO_3$, 5 mM DTT, 5 mM pyridoxal phosphate)

Cysteine biosynthetic enzymes:

Into eppendorf tube add all components (listed below) except enzyme.

Preincubate at reaction temperature (usually 40oC) for 5 min.

Start reaction by addition of enzyme.

Let reaction proceed for 2-5 min.

Stop reaction by adding 50 µl 20% TCA; vortex and set on ice for 10 min.

Centrifuge on high for 5 min.

***O*-acetylserine sulfhydrylase activity assay:**

10 µl Tris (500 mM, pH 7.3)

10 µl *O*-acetylserine (100 mM)

note: make fresh daily and leave on ice

10 µl Na_2S (10 mM)

1 µl pyridoxal phosphate (25 mM)

note: optional

x µl enzyme

H_20 to make a total volume of 100 µl

Analyze 130 µl of supernatant for cysteine.

cystathionine γ-lyase activity assay:

10 µl phosphate buffer (1 M, pH 7.3)

10 µl cystathionine (0.02 M in 0.05 N HCl)

note: cystathionine is very insoluble in H_2O

1 µl pyridoxal phosphate (25 mM)

note: optional

x µl enzyme

H_2O to make a total volume of 100 µl.

Analyze 130 µl of supernatant for cysteine.

cystathionine β-synthase activity assay:

10 µl Tris (500 mM, pH 7.3)

10 µl serine (100 mM)

10 µl homocysteine (100 mM)

0.5 µl $CuSO_4$ (10 µM)

note: $CuSO_4$ inhibits cystathionine lyase activities

1 µl pyridoxal phosphate (25 mM)

note: optional

x µl enzyme

H_20 to make a total volume of 100 µl

Analyze 130 µl of supernatant for cystathionine.

Cysteine Analysis:

Ninhydrin reagent: Dissolve 250 mg Ninhydrin in
6 ml glacial acetic acid/ 4 ml conc. HCl.
Incubate at room temperature for one day.
Hide from light.

Analysis setup: In a glass vial mix
130 µl of supernatant
100 µl glacial acetic acid
and 200 µl Ninhydrin reagent
Heat in boiling water 10 min.
Cool on ice 1 min., then add 600 µl ethanol
Cool 5 min. more on ice.
Let color develop at room temperature for 10 min.
Measure absorbance at 560 nm.

Cystathionine Analysis:

Ninhydrin reagent: Dissolve 1 g Ninhydrin in 100 ml glacial acetic acid.
Then add 33 ml glacial phosphoric acid.
Mix well and hide from light.

Analysis setup: In a glass vial mix
130 µl of supernatant
and 1 ml Ninhydrin reagent
Heat in boiling water 5 min.
Cool 2 min. on ice.
Let color develop at room temperature for 20 min.
Measure absorbance at 455 nm.

Appendix B

M. thermophila genomic sequence

The following is the entire DNA sequence that I sequenced around *cysK*. All open reading frames are color coded. The open reading frames were compared with genes of known function through the non-redundant BLAST search, and are identified as such below. Numbers in parenthesis indicate length of open reading frame (in nucleotides) and numbers between open reading frames indicate amount of nucleotides between each open reading frame. *nadA* (472) 108 *nifU* (390) 145 *nifS* (1217) 244 hypothetical (543) 269 *cysK* (927) 97 *cysE* (729).

Note: *nadA* and *cysE* are partial open reading frames

nadA is quinolinate synthase.

TCGTGAACATGGCAAAATCCAGGCCAGAGATGTATCTTCTTGTCGGTATGAAGAGCTAC
GAAAACACCCAGGTTTTTATCAGGAACAAAAAGAACCTCCTGGCTTTCAAGTGAATTTA
CCACGTTTATAGCATTTGCTGAAGTGCAGCAGATATCAGATTCGGCTTTAACTGCCGCC
GAACTGTTCACATAGCACACGACTGCAGCATCGGGATGTTTCTCTTTTGCATCCCGCAG
AGCCTCCGCAGTTATCATATCCGCCATAGGGCAGCCTGCATCGGGTACAGGTAAGAGCA
CCACTTTGTCAGGGGAAATTATTGATGCCGATTCTGCCATGAAGTGAACCCCTGCAAAG
ACTATAATATCGGCTTTTGAATCAACTGCGGCTTTGCAAAGGGAAAGAGAATCCCCGAC
AAAGTCCGCAACTTCCTGAACTTCACGCCTGGTGTAAAAGTGAGAAAGAATGACAGCAT
TCCTTTCGGCTTTAAGTTTTTTTATTTCTTCTATAAGTAACTCGTTTTCCATAACAATC
TTATTCCCTATATTGCAAGCACATTCCTGTACTGAAGGAACAACCGGAATCAGTCATCC
CACGGCTCAAGTCCCTTTTTCCTGAGATAATCGTTAATAGCCGCGTGGATCGCCTCTTC
TGCAAGCATTGAACAGTGCATCTTTATTGGAGGAAGTCCGTCAAGAGCCTCGGCAACAG
CTTTGTTTGAAAGTTTCCAGGCTTCCTCCAGCGTTTTTCCTTTAATAAGTTCGGTCGCC
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CCGGTTATCCTCAACTTTGAGATAAATTTTCATTATGTCTCCACACTTCGCGTTTCCGA
CCTCTCCTACTCCATCACTGTCTTCAATGATTCCCATGTTTCTCGGATTTGAGAAATGA
TCCATTACTTTAAGGCTGTAGTCCATTTGCTGTCCTCCAGTGTTATTTCAATTCTATTC
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TCTTTAAAATGGTCTTCCTTTTTCAATTGCCGACCTGCATTGCTGCTGTTGCCTCTCAA
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GGTTCAAGAGAAGAAGAATTACATGCGCTTCCCGTAGATGCATAGATTCCTTTCGCGTT
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AATATTGAGCTTTTTGACATCTATAGGGACATGACCTATTGCCTGCACCGCATCCGTAT
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CCGATTTCGTTGTTTGCAAACATTATGGAAATAAGGATGGTGTCATCCCGAATTGCATT
TCTGAGTTCGTCAGGAGACACCATTCCGTATTTGTCCACAGGCAGGTAGGTTACCTCAA
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CAACCAGCGGCAATACCGGCATCGCACTTGCATGCGTTTGTGCTGCCCGCGGCTACAAT
CTTATACTGGTTATGCCTGAAACTATGACTGTAGAACGAAGAAAACTGCTTAAAGCTCT
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CCGAACAGATGGCTGCCGACGATTCTGCACTTTATTTCATACCCCAGCAATTCCAGAAC
CCCGCAAACCCTGAGATTCACAGAAGGACAACAGCCGAAGAGATCTGGAGAGATACTGA
CGGCAAAGCCGATGTTATTGTAGCGGGCGTGGGAACTGGCGGAACTATTACAGGGATTG
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TCTCCTGTACTCAGCGGAGGAAGCCCTGGCCCGCACAGGATCCAGGGAATCGGTGCAGG
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GGTGCTGGCTCGGTTGTTATCCGACCAGTCCCTCCCGGAGCTACGGTTGTGGGAGTACC
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TTCCTGATCCTGGACTTATTGTTATAGGACAGATACTCGACCGGTTGAGCAGATTAGAA
GACCAGTTCAAAGCACATGAGAAGTCAATCTCCAGAGAGGAGAAAACTACACAGGAAGC
AGCACTTAAGGACGAGCAAATCTATTCCCTACTAAAAGAAGTGATTGACCCTGGAGTAG
GCATGGAT

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