L-Cysteine Desulfidase: An [4Fe-4S] Enzyme Isolated from *Methanocaldococcus jannaschii* That Catalyzes the Breakdown of L-Cysteine into Pyruvate, Ammonia, and Sulfide†

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ABSTRACT: A [4Fe-4S] enzyme that decomposes L-cysteine to hydrogen sulfide, ammonia, and pyruvate has been isolated and characterized from *Methanocaldococcus jannaschii*. The sequence of the isolated enzyme demonstrated that the protein was the product of the *M. jannaschii* MJ1025 gene. The protein product of this gene was recombinantly produced in *Escherichia coli* and purified to homogeneity. Both the isolated and recombinant enzymes are devoid of pyridoxal phosphate (PLP) and are rapidly inactivated upon exposure to air. The air-inactivated enzyme is activated by reaction with Fe^{2+} and dithiothreitol in the absence of air. The air-inactivated enzyme contains 3 mol of iron per subunit (43 kDa, SDS gel electrophoresis), and the native enzyme has a measured molecular mass of 135 kDa (gel filtration), indicating it is a trimer. The enzyme is very specific for L-cysteine, with no activity being detected with D-cysteine, L-homocysteine, 3-mercaptopropionic acid (cysteine without the amino group), cysteamine (cysteine without the carboxylic acid), or mercaptolactate (the hydroxyl analogue of cysteine). The activity of the enzyme was stimulated by 40% when the enzyme was assayed in the presence of methyl viologen (4 mM) and inhibited by 70% when the enzyme was assayed in the presence of EDTA (7.1 mM). Preincubation of the enzyme with iodoacetamide (17 mM) completely abolishes activity. The enzymatic activity has a half-life of 8 or 12 min when the enzyme is treated at room temperature with 0.42 mM *N*-ethylmaleimide (NEM) or 0.42 mM iodoacetamide, respectively. MALDI analysis of the NEM-inactivated enzyme showed Cys25 as the site of alkylation. Site-directed mutagenesis of each of four of the cysteines conserved in the orthologues of the enzyme reduced the catalytic efficiency and thermal stability of the enzyme. The enzyme was found to catalyze exchange of the C-2 hydrogen of the L-cysteine with solvent. These results are consistent with three of the conserved cysteines being involved in the formation of the [4Fe-4S] center and the thiolate of Cys25 serving as a base to abstract the α-hydrogen in the first step of the elimination. Although the enzyme has no sequence homology to any known enzymes, including the non-PLP-dependent serine/threonine dehydratases or aconitases, the mechanisms of action of all of these enzymes are similar, in that each catalyzes an α,β-elimination reaction adjacent to a carboxylate group. It is proposed that the enzyme may be responsible for the production of sulfide required for the biosynthesis of iron−sulfur centers in this archaea. A mechanism of action of the enzyme is proposed.

Several enzymes that catalyze the removal of sulfur from cysteine and its analogues have been described. Among these are L-cysteine desulfurase (EC 2.8.1.7), which converts cysteine to alanine and sulfane sulfur (*1*−*3*), D-cysteine desulfhydrase (EC 4.4.1.15) (*4*), L-cyst(e)ine *C*−*S*-lyase (C-DES) (EC 4.4.1.10) (*5*, *6*), L-cysteine *S*-conjugate β-lyase (EC 4.4.1.13) (*7*), selenocysteine lyase (EC 4.4.1.16) (*8*−*10*), alliinase (EC 4.4.1.4) (*11*), *S*-alkylcysteine lyase (EC 4.4.1.6) (*12*), and cystathionine β-lyase (EC 4.4.1.8) (*13*). Although several of these enzymes use slightly different strategies to carry out their specific reactions, each mediates its respective enzymatic mechanism using pyridoxal phosphate (PLP)[1] based chemistry. Here we report a previously uncharacterized enzyme, cysteine desulfidase, that uses a [4Fe-4S] center instead of PLP to catalyze the decomposition of cysteine to sulfide, ammonia, and pyruvate. The name of this enzyme was specifically selected to indicate its fundamental difference from the other enzymes that remove sulfur from cysteine.

The activity of this enzyme was first observed in cell extracts of *Methanocaldococcus jannaschii* (formally *Methanococcus jannaschii*) during experiments conducted to

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[1] Abbreviations: PLP, pyridoxal phosphate; MBB, 9, 10-dioxa-*syn*-(bromomethyl,methyl)-(methyl,methyl)bimane; TMS, trimethylsilyl; DTT, dithiothreitol; coenzyme M, 2-mercaptoethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); DTPA, diethylenetriaminepentaacetic acid; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid: TES, 2-[(2-hydroxy-1−1-bis[hydroxymethyl]-ethyl)amino]ethanesulfonic acid; NEM, *N*-methylmaleimide; IAA, iodoacetamide.

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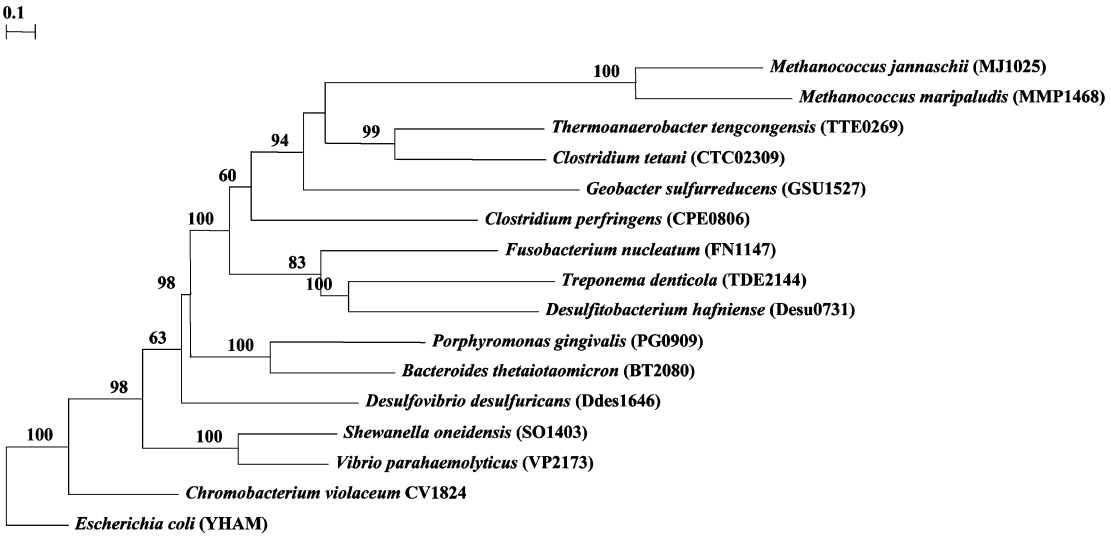

FIGURE 1: Phylogenetic tree based on MJ1025 gene sequence, showing the relationships between *M. jannaschii* and its other relatives. The tree was constructed using the neighbor-joining method and the Kimura two-parameter calculation model. The numbers (expressed as percentages) represent the confidence levels from 1000-replicate bootstrap samplings. Bar, $0.1K_{nuc}$. The numbers in parentheses are the gene numbers for the respective organisms.

establish the routes for the biosynthesis of cysteine and homocysteine in this organism (*14*). On the basis of sequence comparisons, the protein is found to be widely distributed among anaerobic bacteria (Figure 1). The mechanism of the enzyme may proceed like that observed in aconitase (*15*), the serine and threonine dehydratases (*16*), and serine deaminase (*17*), with a [4Fe-4S] center serving as the Lewis acid in the reaction. The enzyme may be identical to a "cysteine desulfhydrase" present in *Methanosarcina barkeri* that was described, but not isolated, by Mazumder (*18*).

MATERIALS AND METHODS

Materials. All reagents and enzymes were purchased from Sigma-Aldrich unless otherwise specified. *S*-(2-Sulfoethyl)-L-cysteine was prepared as previously described (*19*). DL-3-Mercaptolactate was prepared by sodium borohydride reduction of 3-mercaptopyruvate (*20*). D-Cysteine and cysteamine were produced by reduction of D-cystine and cystamine with excess (2×) dithiothreitol (DTT). The D-cystine used had an $[\alpha]^{20}_D$ +212° (c = 1, 1 N HCl). Since pure D-cystine has $[\alpha]^{20}_D$ +223° (c = 1, 1 N HCl), this indicates an anomeric purity of 94.9%.

Preparation of Cell Extracts. *M. jannaschii* were grown as previously described (*21*), and frozen cells were purchased from the University of Illinois Fermentation Facility (Urbana). A cell extract of *M. jannaschii* was prepared by sonication of 4.67 g of frozen cells suspended in 10 mL of TES buffer (50 mM TES/NaOH, 10 mM $MgCl_2$, pH 7.5) under Ar for 5 min at 3 °C. The resulting mixture was centrifuged under Ar (10 min, 27000*g*) and stored frozen at −20 °C until use. The protein concentration of the *M. jannaschii* extract used was 38 mg/mL. Protein concentrations were measured using the BCA total protein assay (Pierce) with bovine serum albumin as a standard.

Isolation and Identification of the Native Enzyme from M. jannaschii. *M. jannaschii* cell extract (1.0 mL) was applied to a Mono Q HR anion-exchange column (1 cm × 8 cm; Amersham Biosciences) equilibrated with buffer A (20 mM Tris/HCl, pH 7.5). Pumps attached to the column were controlled by a Biologic HR workstation (Bio-Rad). Bound protein was eluted with a 45-mL linear gradient from 0 to 1 M NaCl in buffer A at a flow rate of 1.0 mL/min. Both buffers were continuously degassed with argon, and each fraction was manually collected in a septated, sealed vial flushed with argon. Using the measurement of the sulfide released from cysteine as the activity assay, a single peak of activity was observed when elution was done at ~0.3 M NaCl. The most active fraction was assayed for PLP as previously described (*22*). Fractions containing activity were pooled and concentrated in a N_2-pressurized stirred cell with a YM10 ultrafiltration membrane (Millipore). Size exclusion chromatography of the concentrated sample (160 μL) was performed on a Superose 12HR column (1 cm × 30 cm; Amersham Biosciences) equilibrated with a buffer containing 50 mM HEPES/NaOH, 0.15 M NaCl, 2 mM DTT, pH 7.2, degassed with argon, and eluted at a flow rate of 0.4 mL/min. Each fraction was manually collected in a septated, sealed vial flushed with argon and assayed for activity under anaerobic conditions. After analysis, each fraction was concentrated to 10 μL using a Microcon YM-3 centrifugal filter device. The proteins contained in each concentrated fraction were solublized in the filter device with 20 μL of 2× SDS loading buffer (0.125 M Tris, pH 6.8, containing 20% glycerol, 4% SDS, 10% mercaptoethanol, and 0.0025% bromophenyl blue), and the entire sample from each fraction was separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The resulting gel was stained with Coomassie blue, and after destaining, the proteins contained in a single selected stained band were sequenced by mass spectrometry at the W. M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia Health System. Briefly, the procedure involved removal of the band from the gel and reductive alkylation of the proteins contained therein with iodoacetamide (IAA), followed by in-gel tryptic digestion. The resulting peptides were then analyzed by LC−MS and MS/MS analysis using a Finnigan LCQ ion trap mass spectrometer with a Protana nanospray ion source interfaced to a self-packed 8-cm × 75-

μm-i.d. Phenomenex Jupiter 10-μm C18 reverse-phase capillary column.

Cloning and Expression of the M. jannaschii MJ1025-Derived Protein. The *M. jannaschii* gene at locus MJ1025 (Swiss-Prot accession numbers Q58431) was amplified from genomic DNA by polymerase chain reaction (PCR). The primers used (Invitrogen) were MJ1025-Fwd (5′-GGTGGATCCATGAACAAAAATG-3′) and MJ1025-Rev (5′-GCTCTGCAGTTATTTCTTGTTTTTC-3′). PCR amplification was performed as described (*23*) using 50 °C as the annealing temperature. The PCR product was purified by using a QIAquick spin column (Qiagen, Valencia, CA), digested with *Bam*HI and *pst*1 restriction enzymes, and then ligated into compatible sites in plasmid pT7-7 to make the recombinant plasmid pMJ1025. DNA sequence was verified by dye-terminator sequencing at DAVIS Sequencing, LLC (Davis, CA). The resulting plasmid was transformed into *Escherichia coli* strain BL21-Codon Plus (DE3)-PIL (Stratagene). The recombinant protein production was induced by addition of lactose to a final concentration of 28 mM. After an additional culture of 2 h, the cells were harvested by centrifugation (4000*g*, 5 min) and frozen at −20 °C. Induction of the desired protein was confirmed by SDS−PAGE (12% T, 4% C acrylamide, using a Tris/glycine buffer system) analysis of total cellular proteins.

Purification of the Recombinant Enzyme. Soluble cell-free extracts of *E. coli* containing the recombinant enzyme were prepared by sonication and centrifugation as described previously (*24*). The apparent masses of the expressed, denatured proteins were determined by comparing protein migration to the migration of low-molecular-weight protein standards (Bio-Rad) separated by SDS−PAGE. Purification of the MJ1025 protein was performed by standard methods, which involved extraction of the protein from the cells by sonication and a heat treatment of the extract at 70 °C for 10 min to precipitate the *E. coli* proteins, followed by purification of the soluble protein by chromatography on a Mono Q column (*23*, *25*). Since the air-inactivated enzyme can be readily activated, this purification was undertaken in air since this was a simpler procedure.

Protein purity was evaluated by silver staining (Bio-Rad) of the SDS−PAGE separated proteins. The size of the denatured proteins was determined by comparison to low-molecular-weight protein standards (Bio-Rad). Protein concentrations were determined using the Bio-Rad Protein Assay with bovine serum albumin as the standard.

Site-Directed Mutagenesis. To test the possible function of conserved Cys25, Cys282, Cys322, and Cys329 residues of MJ1025, Cys25 was replaced by Ala (C25A), Cys282 was replaced by Ala (C282A), Cys322 and Cys329 were replaced simultaneously by Ala (C322A−C329A), Cys329 was replaced by Ser (C329S), and Cys329 was replaced by Ser (C329S). In addition to these residues, the following changes were made in other selected conserved residues: H139N and D323N. The Quick-Change TM site-directed mutagenesis kit (Stratagene) was used according to the manufacturer's instructions with template pMJ1025 (pT7-7 as the vector). The oligonucleotide primers (Invitrogen) used were C25A-Fwd (5′-GGCGTTAGGTGCCACAGAAGTTGG-3′), C25A-Rev (5′-CCAACTTCTGTGGCACCTAACGCC-3′), C282A-Fwd (5′-CATCTCAGCAATGGCTGGATGTGTAAATAG-3′), C282A-Rev (5′-CTATTTACACATCCAGCCATTGCTGAGATG-3′), C322A−C329A-Fwd (5′-CCAGGAATCGTTGCTGACGGAGGAAAAATTGGCGCTGCTTTAAAGATAG-3′), C322A−C329A-Rev (5′CTATCTTTAAAGCAGCGCCAATTTTTCCTCCGTCAGCAACGATTCCTGG-3′); C322S-Fwd (5′ CTTCCAGGAATCGTTAGTGACGGAGGAAAAATTG-3′), C322S-Rev (5′-CAATTTTTCCTCCGTCACTAACGATTCCTGGAAG-3′), C329S-Fwd (5-′GAGGAAAAATTGGCAGTGCTTTAAAGATAG-3′), C329S-Rev (5′-CTATCTTTAAAGCACTGCCAATTTTTCCTC-3′), H139N-Fwd (5′-GAAACAAAAGGGAGTAATTCTGGAAAATCTC-3′), H139N-Rev (5′-GAGATTTTCCAGAATTACTCCCTTTTGTTTC-3′), D323N-Fwd (5′-CCAGGAATCGTTTGTAACGGAGGAAAAATTGG-3′), and D323N-Rev (5′-CCAATTTTTCCTCCGTTACAAACGATTCCTGG-3′). DNA sequences of the mutated genes were confirmed by dye-terminator sequencing at DAVIS Sequencing, LLC.

Assay of Enzymatic Activity with L-Cysteine as Substrate. Two different methods were used to follow the decomposition of L-cysteine. The first involved a spectroscopic method measuring the loss of cysteine using a ninhydrin color reaction which is specific for cysteine (*26*). The second involved the measurement of sulfide produced using a micro gas diffusion cell similar to that previously described but at a smaller scale (*27*). This diffusion chamber consisted of a 1.5-mL screw-top glass vial containing a 4- × 10-mm glass collection test tube and fitted with a Teflon rubber-backed septum. Before incubation, the vial was flushed with argon, and 10 μL of anaerobic 1 M NaOH was added through the septum into the collection test tube, in such a manner that the inside wall of the collection tube was coated with the NaOH solution. Samples were added under anaerobic conditions with a syringe through the septum to the bottom of the glass vial. Vials were always maintained in the upright position so the contents did not mix. The standard assay consisted of adding 50 μL of anaerobic 25 mM TES/K^+, 10 mM $MgCl_2$, 20 mM DTT buffer, pH 7.2, to the sealed vial, followed by 5 μL of 0.1 M anaerobic cysteine, 5 μL of 1 M anaerobic DTT, 1 μL of 0.1 M anaerobic ferrous ammonium sulfate, and the desired amount of enzyme contained in 10 μL of solution. The final concentrations of cysteine, DTT, and ferrous ion in the standard assay were 7.0, 70, and 1.4 mM, respectively. The measured pH of the solution was 6.60 at 65 °C and 6.56 at 70 °C. The pH's of the other buffers reported were measured at room temperature. The samples were then incubated in an oven at 65 °C for 10−60 min, at which time 25 μL of anaerobic 1 M HCl was added through the septum to the incubation mixture and the samples were incubated an additional 30 min at 65 °C to allow the hydrogen sulfide to diffuse into the center tube. A syringe containing 80 μL of anaerobic water was then used to flush out and remove the NaOH absorption solution containing the sulfide, and the resulting solution was mixed with 420 μL of anaerobic water. The resulting sulfide-containing solution was assayed for sulfide by the addition of 100 μL of 0.5% dimethylphenylenediamine in 6 M HCl, followed by 200 μL of 0.023 M ferric chloride in 6 M HCl. After 1 h at room temperature, the sulfide was quantitated by measuring the absorbance at 560 nm from the produced methylene blue. The extinction coefficient for sulfide was 2.4×10^4 M^{-1} cm^{-1}. For determination of K_m and product formation with time, the assay bottles were contained in

a water bath at 65 ± 0.2 °C during the enzymatic reaction.

Substrate Specificity. Substrate specificity of the enzyme was investigated using analogues of cysteine at concentrations of 7−8 mM and testing for the production of sulfide in the standard diffusion assay.

For substrates not producing sulfide, products were assayed using a thin-layer chromatography (TLC) analysis of the monobromobimane (MBB) adducts of the products (*28*). Incubations were conducted in 50-μL volumes using the substrate concentrations and conditions listed in Table 2 (below). At the end of the incubation, 50 μL of a buffer consisting of 50 mM HEPPS and 5 mM DTPA, pH 8.0, was added followed by 10 μL of 0.083 M MBB in acetonitrile. After 15 min at room temperature, the MBB adducts were assayed by TLC separation using the TLC solvent consisting of acetonitrile−water−formic acid (88%), 19:2:1 v/v/v. In this solvent system, the MBB adducts of the following compounds had these R_f's: cysteine, 0.084; homocysteine, 0.12; coenzyme M, 0.34; DTT, 0.48. MBB had an R_f of 0.69. The MBB derivatives were detected as fluorescent spots upon exposure of the TLC plates to UV light.

Analysis of Pyruvate, Serine, and Alanine. To 50 μL of *M. jannaschii* extracts were added 5 μL of 0.1 M cysteine and 5 μL of 0.2 M DTT, and the mixture was incubated under argon for 30 min at 70 °C. For the analysis of serine and alanine, the sample was acidified by the addition of 5 μL of 6 M HCl, and the precipitated proteins were removed by centrifugation. The amino acids were isolated from the sample, converted into their trifluoroacetyl methyl ester derivatives, and analyzed by gas chromatography−mass spectrometry (GC−MS) as previously described (*2*). Pyruvate was analyzed in the incubation mixtures using lactate dehydrogenase-catalyzed reduction with NADH (*29*) and by GC−MS of the $(TMS)_2$ hydroxylamine derivative. For the GC−MS analysis, L-cysteine was incubated with the enzyme as stated in Table 2, and after the incubation, 50 μL of 0.1 M hydroxylamine was added and the reaction mixture was heated at 60 °C for 30 min to form the hydroxylamine derivative. The sample was acidified by the addition of 5 μL of 6 M HCl, and the precipitated proteins were removed by centrifugation (14000*g*, 10 min). The separated clear liquid was saturated with sodium chloride and extracted three times with 100 μL of ethyl acetate. After evaporation of the ethyl acetate, the sample was converted into the $(TMS)_2$ derivative by reaction with 20 μL of a mixture of pyridine, hexamethyldisilazane, and chlorotrimethylsilane (9:3:1 v/v/v) for 2 min at 100 °C and then analyzed by GC−MS. The control was treated in the same manner without the addition of cysteine.

Iron Analysis. The standard assay consisted of the addition of 100 μL of 5% HCl, 100 μL of 10% hydroxylamine, prepared by neutralization of the hydrochloride with 5 M sodium hydroxide, and 125 μL of 20 mM 2,2′-dipyridyl solution to the sample contained in a total volume of 300 μL. Standards consisted of solutions of ferrous ammonium sulfate of known concentration. For the analysis of the protein sample, the Mono-Q-purified recombinant protein, contained in 150 μL of elution buffer, was incubated at 80 °C for 10 min after the addition of 100 μL of 5% HCl. The precipitated proteins were removed by centrifugation, the pellet was washed with 150 μL of water, and the combined soluble material was mixed with 100 μL of 10% hydroxylamine solution and 125 μL of 20 mM 2,2′-dipyridyl solution. Samples were incubated at room temperature for 30 min, and absorbances were measured at 520 nm. Forty nanomoles of ferrous ammonium sulfate had an absorbance of 0.556 in this assay. The assay was patterned after that described by Fortune and Mellon but with the substitution of dipyridyl for *o*-phenanthroline (*30*).

Measurement of the Native Molecular Weight of MJ1025. Size exclusion chromatography was performed on a Superose 12HR column (1 cm × 30 cm; Amersham Biosciences) equilibrated with a buffer containing 50 mM HEPES/NaOH, 0.15 M NaCl, 2 mM DTT, pH 7.2. The Mono-Q-purified native enzyme or recombinant enzyme in a volume of 160 μL was applied to the column and eluted at a flow rate of 0.4 mL/min. Protein standards were used to calibrate the sizing column as described previously (*25*). Elution was monitored at 280 nm.

Heat Stability and pH Optimum of the Enzymes. To test the heat stability of the air-inactivated enzymes, 20-μL solutions of the purified enzymes (3−9 mg/mL) were heated in air at temperatures from 70 to 100 °C for 10 min, and the activity remaining after the heating was assayed at 70 °C after activation of the enzymes with ferrous iron and DTT. To test the heat stability of the reactivated purified enzymes, the enzymes were mixed with ferrous iron in the assay buffer and heated for 10 min at the indicated temperatures under argon, and the remaining activity was assayed at 70 °C. The remaining enzymatic activity was established by measuring L-cysteine loss using the ninhydrin color assay. The effect of pH on activity was studied by using a three-component buffer system in the pH range 6.0−9.0. Buffer mixtures contained 120 mM Bis-Tris, 61 mM HEPPS, and 60 mM CHES adjusted to pH 5.0−9.0 with sodium hydroxide or HCl.

Hydrogen Exchange at C-2 of Cysteine. To 50 μL of anaerobic 25 mM TES/K^+ and 10 mM $MgCl_2$ buffer, pH 7.2, in a sealed vial with an argon atmosphere was added 80 μL of anaerobic ^2H_2O (98% ^2H_2O), followed by 1 μL of 0.1 M anaerobic ferrous ammonium sulfate, 5 μL of 0.1 M anaerobic cysteine, 5 μL of 1 M anaerobic DTT, and 5 μL of a solution of the Mono-Q-purified enzyme. The calculated concentration of deuterium in the incubation mixture was 53.4%. The samples were then incubated in an oven at 67 °C. Two separate experiments were conducted, one with 0.28 μg of enzyme incubated for 20 min and the other with 0.56 μg of enzyme incubated for 30 min. Analysis of a sample incubated for 20 min showed that 50% of the cysteine had decomposed. After the incubation, 100 μL of methanol, 5 μL of 6 M ammonia, and 5 μL of methyl iodide were added to the incubation mixture, and the sample was briefly shaken to dissolve the methyl iodide. After 30 min at room temperature, the solvents were evaporated with a stream of nitrogen. The resulting *S*-methylated amino acids were converted into their methyl ester derivatives by reaction overnight at room temperature with 1 M HCl in methanol (0.5 mL). Following evaporation of the methanol/HCl, the *S*-methylated amino acid hydrochlorides were converted into their *N*-trifluoroacetyl derivatives by reaction for 4 h at room temperature with 0.2 mL of 50% trifluoroacetic anhydride in methylene chloride. After evaporation of the trifluoroacetic anhydride and methylene chloride, the sample was dissolved

in methylene chloride and assayed by GC−MS. The mass spectra of the cysteine derivatives were used to measure both the position and the extent of the deuterium incorporation. The fragment ions at *m*/*z* 186 ($M^+ - COOCH_3$), *m*/*z* 132 ($M^+ - CF_3CONH_2$), and *m*/*z* 61 ($CH_3SCH_2^+$) were used to measure the extent of incorporation of deuterium into the different parts of the molecule. The *m*/*z* 186 ions measured the total carbon-bonded deuterium at both C-2 and C-3, whereas the *m*/*z* 61 ($CH_3SCH_2^+$) fragment ion measured the deuterium incorporated at C-3. The *m*/*z* 132 ion ($M^+ - CF_3CONH_2$), resulting from the elimination of CF_3CONH_2 from C-2 and C-3, removed one of the hydrogens from C-3. Since the *m*/*z* 132 and the *m*/*z* 186 ions both showed the same amount of deuterium and the *m*/*z* 61 showed no deuterium, this indicated that deuterium was incorporated exclusively at C-2 of the cysteine.

Gas Chromatography−Mass Spectrometric Analyses. GC−MS analysis of the samples was obtained using a VG-70-70EHF gas chromatography−mass spectrometer operating at 70 eV and equipped with an HP-5 column (0.32 mm × 30 m), programmed from 95 to 280 °C at 10 °C/min. Under these conditions, the derivatives of the following compounds had the following retention times (min:sec) and mass spectral data [molecular weight, *base peak*, the most abundant ions listed in order of decreasing intensities]: ditrifluoroacetyl methyl ester of serine (1:58) [311, *139*, 140, 252]; trifluoroacetyl methyl ester of alanine (3:10) [199, *140*, 69, 92, 102]; trifluoroacetyl methyl ester of *S*-methylcysteine (5:58) [245, *61*, 132, 186, 117]; $(TMS)_2$ derivative of oxime derivative of pyruvate (6:10) [247, *73*, 147, 232, 130, 204, 247].

RESULTS AND DISCUSSION

Isolation and Identification of the Enzyme. We have previously shown (*14*) that cell extracts of *M. jannaschii* readily catalyze the decomposition of cysteine. GC−MS analysis showed that the product of the reaction was pyruvate, not alanine. Separation of a *M. jannaschii* cell extract on a Mono-Q column under anaerobic conditions in the presence of DTT produced one peak of activity decomposing cysteine to sulfide when the diffusion assay was used to monitor the production of sulfide. This activity eluted at 0.3 M NaCl and corresponded with a peak in absorbance at 405 nm. An identical separation under aerobic conditions in the absence of DTT produced no fractions with activity. The enzyme contained in the active fractions produced sulfide from cysteine (10 mM) in a linear fashion for up to 2 h at 70 °C, at which time more that 50% of the cysteine was decomposed. This activity was completely lost by incubating the native protein solution in air for 15 min prior to the incubation. Including 7.1 mM EDTA in the incubation mixture inhibited the enzymatic activity by 70%, and 4 mM oxidized methyl viologen stimulated the activity by 40%, whereas 34.5 mM hydroxylamine inhibited the reaction by only 12% (Table 1). These observations are similar to those reported Mazumder for an activity observed in crude extracts of *Methanosarcina barkeri* Fusaro (DSM 804) (*18*). We have no explanation for the stimulation of the reaction by methyl viologen or the weak inhibition by the hydroxylamine. Inhibition by IAA was a result of the alkylation of C25 (see below).

Table 1: Effect of Various Substances and Inhibitors on the Activity of Cysteine Desulfidase Isolated from *M. jannaschii*

incubation	relative activity[a]
control	100
control, no enzyme	0.00
methyl viologen (4 mM)	140
EDTA (7.1 mM)	31
hydroxylamine (34.5 mM)	88
iodoacetamide (17 mM)[b]	0.00
iodoacetamide (0.42 mM)[c]	25
N-ethylmaleimide (0.42 mM)[d]	20
L-serine (0.031 M)	120
L-serine (0.28 M)	60
oxygen[e]	5.1
oxygen, then Fe^{2+} and DTT[f]	100

[a] The incubations were conducted under anaerobic conditions at 65 °C for 10 min using 36 mM TES/K^+ buffer, pH 7.2, 14.2 mM $MgCl_2$, 7.1 mM cysteine, 143 mM DTT, and 0.28 μg of Mono-Q-purified native enzyme. In each case, the hydrogen sulfide was measured using the microdiffusion cell. The hydrogen sulfide produced in the control represented the decomposition of 36% of the cysteine, corresponding to a specific activity of 55.8 μmol of sulfide min^{-1} mg^{-1} of protein. [b] The enzyme was incubated under anaerobic conditions for 10 min at room temperature in a buffer of 70 mM Tris/HCl, pH 7.28, and 17 mM iodoacetamide prior to activation and incubation with the cysteine. [c] The enzyme had a half-life of inactivation of 12 min when incubated at room temperature in 25 mM TES/K^+, 10 mM $MgCl_2$ buffer, pH 7.3, containing 0.42 mM iodoacetamide. The activity reported was that remaining after 260 min of incubation with iodoacetamide. [d] The enzyme had a half-life of inactivation of 8 min when incubated at room temperature in 25 mM TES/K^+, 10 mM $MgCl_2$ buffer, pH 7.3, containing 0.42 mM NEM. The activity reported was that remaining after 260 min of incubation with NEM. The inactivation studies with 0.42 mM NEM and iodoacetamide were done only with the recombinant enzyme. [e] The protein (0.28 μg) in a 10-μL volume in a 0.5-mL centrifuge tube was exposed to air for 25 min at room temperature prior to analysis. [f] After exposure of the sample to the air for 25 min, the sample was made anaerobic by the addition of $Fe(NH_4)SO_4$ and DTT, and the sample was assayed for activity using the diffusion assay.

The active fractions did not contain pyridoxal-P (PLP) (*22*). Separation of the native protein on a Superose 12 column under anaerobic conditions showed one peak of activity eluting with an apparent molecular mass of 130 kDa. Correlation of the observed enzymatic activity of the collected fractions with the proteins contained in the fractions by SDS−PAGE analysis showed only one Coomassie staining band, with a molecular weight of 43 000, whose intensity was proportional with the measured activities in the fractions. The enzyme is therefore probably trimeric. The proteins contained in this band were sequenced and 22 proteins were identified, each corresponding to a known gene in the genome of *M. jannaschii*. Of these 22 proteins, eight were identified from only one or two peptide sequences and as a result were eliminated from consideration because of their expected low abundances. The remaining proteins were identified from five or more tryptic peptides. Seven of these peptides were from proteins annotated with expected known functions and included MJ0113, acetyl-CoA decarboxylase; MJ0203, phosphoribosylformyl-glycinamide cyclo-ligase; MJ0399, phosphomannomutase; MJ0785, biotin synthase; MJ1596, isocitrate dehydrogenase; and MJ1662, methyl-coenzyme M reductase component A2, and were not considered further. The remaining peptides were derived from expression of the *M. jannaschii* genes MJ1025, MJ1678, MJ0785, MJ0981, MJ1313, and MJ1678 and were

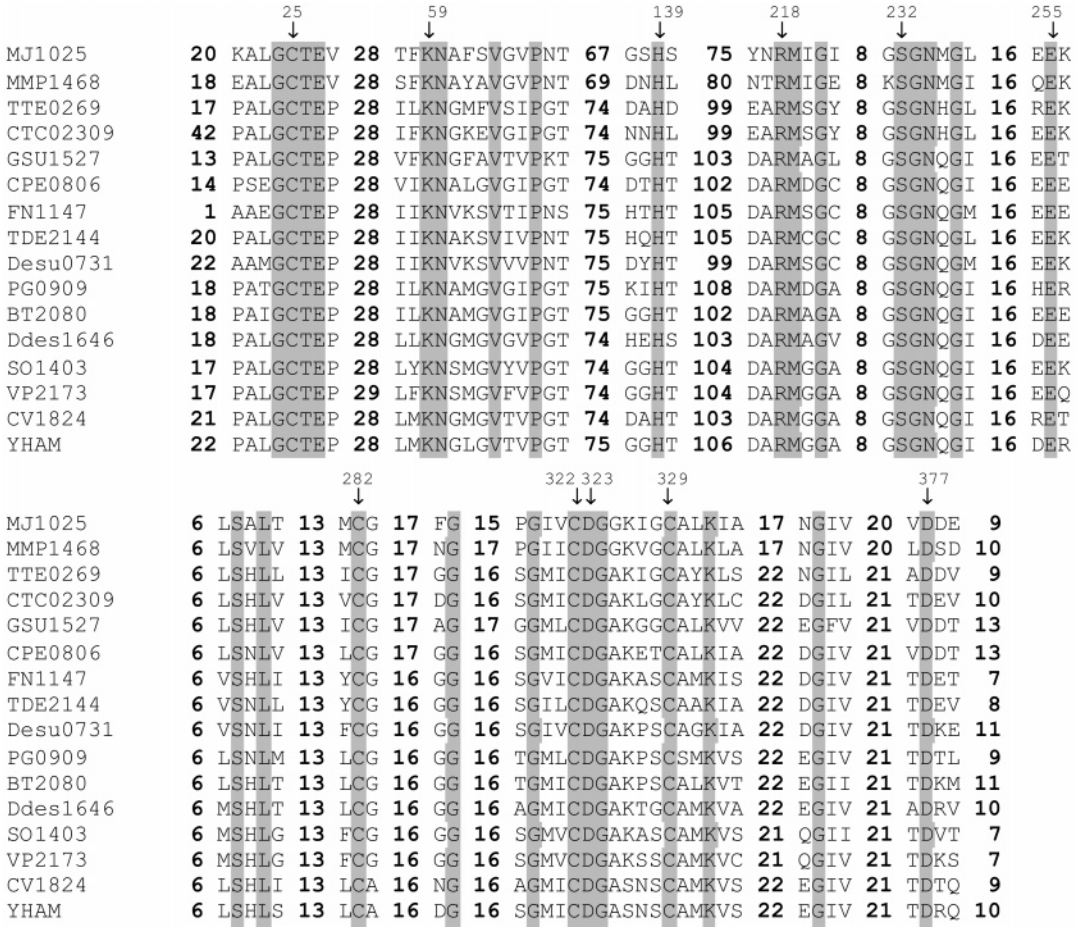

FIGURE 2: Conserved residues amount the orthologues of MJ1025. Proteins (and their gene numbers and database accession numbers) are *Methanocaldococcus jannaschii* (MJ1025, gb|AAB99029), *Methanococcus maripaludis* (MMP1468, gb|CAF31024), *Thermoanaerobacter tengecongensis* (TTE0269, gb|AAM23565), *Clostridium tetani* (CTC02309, gb|AAO36786), *Geobacter sulfurreducens* (GSU1527, gb|AAR34901), *Clostridium perfringens* (CPE0806, gb|BAB80512), *Fusobacterium nucleatum* (FN1147, gb|AAL95343), *Treponema denticola* (TDE2144, gb|AAS12664), *Desulfitobacterium hafniense* (Desu0731, gb|ZP_00097637), *Porphyromonas gingivalis* (PG0909, gb|AAQ66048), *Bacteroides thetaiotaomicron* (BT2080, gb|AAO77187), *Desulfovibrio desulfuricans* (Ddes1646, gb|ZP_00130024), *Shewanella oneidensis* (SO1403, gb|AAN54468), *Vibrio parahaemolyticus* (VP2173, gb|BAC60436), *Chromaobacterium violaceum* (CV1824, gb|AAQ59498), and *Escherichia coli* (YHAM, gb|BAB37413). The numbers within the alignment represent poorly conserved inserts that are not shown. A total of 29 conserved residues are indicated.

annotated with unknown or suspect function. Of these proteins, the MJ1025-derived protein was the strongest hit, with 13 peptides representing 90% coverage of the protein being identified. This protein, despite being apparently a major protein in the band, would not appear to be a major protein in the cell extracts since it was not detected in a "simple" proteome analysis of *M. jannaschii* (*31*). The genes for each these six proteins were cloned, and each of their respective proteins was heterologously produced in *E. coli* and tested for its ability to decompose cysteine. Only the MJ1025-derived protein was observed to decompose cysteine, so only the information for its expression is presented here. The recombinant protein was purified to homogeneity under aerobic conditions for subsequent analysis. Purification under aerobic conditions was preformed since this procedure is easier than anaerobic separation. SDS−PAGE analysis of the final enzyme showed a single protein band eluting with a molecular mass of 43 kDa. After activation with ferrous ions in the presence of DTT, the activity of the recombinant enzyme was found to be identical to that reported in Table 1 for the native enzyme isolated from the cell extract. The recombinant enzyme had a half-life of 8 and 12 min when treated at room temperature with 0.42 mM *N*-ethylmaleimide (NEM) and 0.42 mM IAA, respectively. The recombinant enzyme produced sulfide from cysteine in a linear manner (Figure 3) as long as the cysteine concentrate was 3-fold above the K_m, indicating that product inhibition of the reaction is not significant. The enzyme showed a sharp pH optimum with a maximum at pH 7.6. Activities at pH 6.5 and 8.5 were less than 20% of that observed at pH 7.6. The measured K_m of the purified recombinant enzyme for cysteine was 1.78 ± 0.14 mM. The specific activity of the pure recombinant enzyme was 55.8 μmol of sulfide min^{-1} mg^{-1} of protein at 65 °C. This corresponds to a turnover number of 39.6 s^{-1}. The k_{cat}/K_m for the enzyme was 22.2 mM^{-1} s^{-1}.

Analysis of Reaction Products. Incubation of cell extracts with cysteine readily led to its decomposition into sulfide and pyruvate. The sulfide was confirmed by the methylene blue assay, and the pyruvate was confirmed by GC−MS analysis and oxidation of NADH catalyzed by lactate dehydrogenase. GC−MS analysis showed that no alanine or serine was produced from the cysteine. An incubation of *M. jannaschii* cell extract with 10 mM L-cysteine for 30 min at 60 °C resulted in the production of 5 mM pyruvate in the incubation mixture, consistent with at least 50% of the cysteine being decomposed. Pyruvate was also detected when

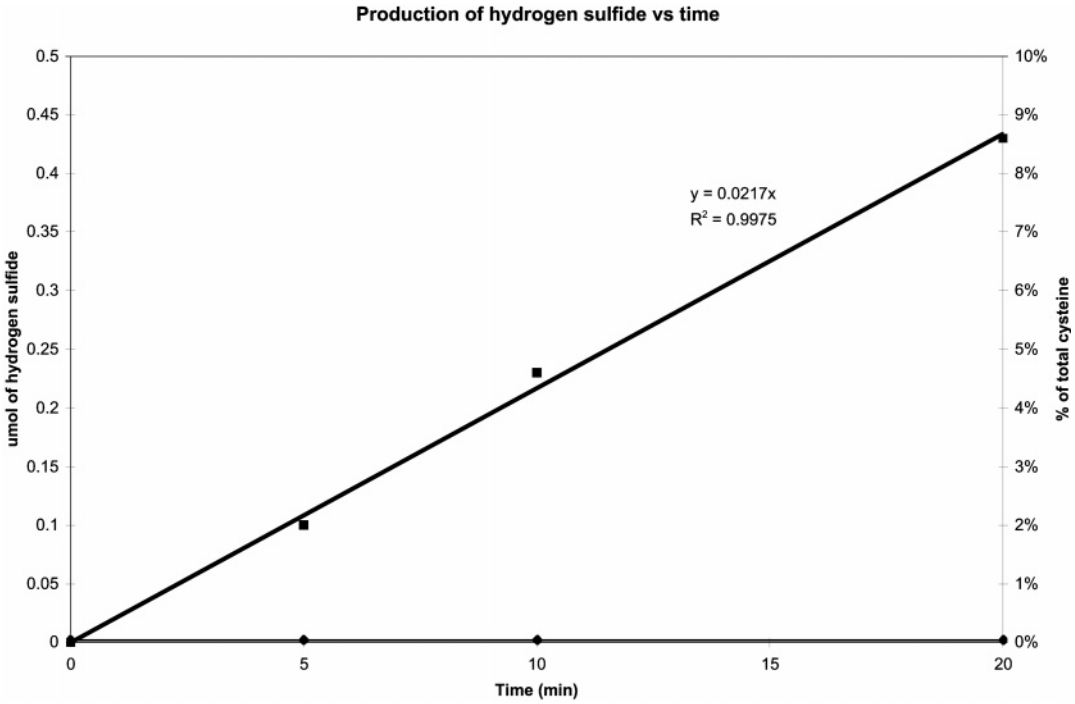

FIGURE 3: Production of sulfide (■) with a fixed amount of enzyme. The incubation was done with 0.367 μg of enzyme and assayed by the standard diffusion method. The initial cysteine concentration was 7 mM. No decomposition of the cysteine was observed unless the enzyme was added (level line).

Table 2: Testing Alternate Substrates for the Recombinant Cysteine Desulfidase from *M. jannaschii*

incubation	relative activity[a]
L-cysteine	100
D-cysteine[b]	6.7
L-homocysteine	<0.1
3-mercaptopropionic acid (7.1 mM)	<0.1
cysteamine (2-amino-1-ethanethiol) (7.1 mM)	<0.1
D/L-3-mercaptolactate (4.1 mM)	<0.1
D/L-selenocysteine (4.1 mM)	1.2[c]
S-methylcysteine (7.7 mM)	nd[d]
S-sulfoethyl-L-cysteine (3.1 mM)	nd[d]
L-cystathionine (3.1 mM)	nd[d]

[a] Enzymatic activities were established by the measurement of hydrogen sulfide production in a diffusion cell standard assay with 0.56 μg of Mono-Q-purified enzyme (specific activity 55.8 μmol of sulfide min^{-1} mg^{-1} of protein). [b] The D-cysteine contained about 5% of the L-isomer. [c] The cleavage was detected by pyruvate formation, and 5.6 μg of Mono-Q-purified activated enzyme was used. On the basis of the amount of pyruvate detected, only 12% of the D/L-selenocysteine was cleaved. [d] The last three samples were assayed for cleavage using the MBB adduct of their expected thiol product as described in the text. *S*-Methylcysteine was expected to produce methanethiol, *S*-sulfoethyl-L-cysteine was expected to produce 2-mercaptoethanesulfonic acid, and L-cystathionine was expected to produce homocysteine. The following concentrations of buffer and substrates were used in the incubations which were conducted at 70 °C for 30 min under Ar: 38 mM TES/K^+, pH 7.0; 7.7 mM Mg^{2+}; 0.7 mM *S*-methylcysteine; 7.7 mM *S*-sulfoethyl-L-cysteine; or 3.1 mM L-cystathionine. In each case, less than 0.02% of substrate was converted into product (nd = not detected).

L-cysteine was incubated with the purified activated recombinant enzyme.

Alternate Substrates. The data presented in Table 2, obtained using the pure activated recombinant enzyme, showed that the enzyme is very specific for L-cysteine, with only D-cysteine showing any measurable activity. Nearly all of this activity, however, could be accounted for by the presence of a small amount of L-cysteine in the sample of D-cysteine prepared from the commercial 95% pure D-cystine. No activity was observed with L-homocysteine, the homologue of L-cysteine; 3-mercaptopropionic acid, cysteine without the amino group; cysteamine, cysteine without the carboxylic acid; or D/L-mercaptolactate, the oxygen analogue of cysteine. The enzyme failed to cleave *S*-methylcysteine, *S*-sulfoethylcysteine, and L-cystathionine (Table 2). The latter two metabolites are involved in *M. jannaschii* metabolism (*14*, *19*). D/L-Selenocysteine was a poor substrate.

Evidence for a [4Fe-4S] Center. The aerobically purified recombinant enzyme had an absorbance spectrum with maxima at 365 and 425 nm, similar to that reported for the [3Fe-4S] center in L-serine dehydratase (*32*) but distinct from that of the [3Fe-4S] center in inactive aconitase (*33*). Analysis of the pure inactive enzyme showed 2.7 mol of Fe per subunit. Incubation of the enzyme with Fe^{2+} restored activity. Three of the four cysteines conserved among the orthologues of MJ1025 in other organisms, C25, C282, C322, and C329 (Figure 2), are likely the ligands for the [3Fe-4S] center. The loss of enzymatic activity by exposure to air is a common characteristic of several [4Fe-4S]-containing dehydratases, such as aconitase (*33*), L-serine dehydratase (*16*), and dihydroxy acid dehydratase (*34*), each of which contains a [4Fe-4S] center in the active enzyme which is converted into a [3Fe-4S] upon exposure to air (*35*, *36*). In each case, this inactivation is reversed after exposure of the enzymes, under anaerobic conditions, to ferrous ions, with re-formation of the [4Fe-4S] cluster (*37*).

Site-Directed Mutation. The MJ1025-derived enzyme contains seven cysteines, four of which are conserved among all orthologs of MJ1025 C25, C282, C322, and C329 (Figure 2). It is expected that three of these cysteines are ligands for the [4Fe-4S] center, with the other cysteine possibly serving as a base in the catalytic mechanism. Conversion of Cys329

Table 3: Relative Activities of Site-Directed Mutants of Cysteine Desulfidase

mutant	specific activity[a] (μmol of sulfide min^{-1} mg^{-1} of protein)
wild-type MJ1025	55.8
C329S	6.8
C322S	6.5
C282A	2.8
C25A	0.22
C322A and C329A	0.00
H139N	49
D323N	30

[a] The specific activities were measured under conditions where the wild-type enzyme has decomposed 50% of the cysteine using standard conditions of the sulfide assay.

or Cys322 into serine resulted in a protein still maintaining about 10% of the wild-type catalytic activity (Table 3). It is proposed that these are two of the three cysteines that are ligands for the [4Fe-4S] center. As expected, converting both of these cysteines to alanine completely eliminated enzymatic activity, presumably due to the inability of the protein to either form or maintain the [4Fe-4S] center. The loss of the [4Fe-4S] center in this double cysteine mutant protein was confirmed by the lack of a brown color associated with cell extracts containing this mutant enzyme.

There are several examples in the literature (*38*, *39*−*41*) where the cysteine ligand of an [Fe-S] center has been substituted with serine, producing active proteins. Many times this substitution has little effect on enzymatic activity but causes clear changes in the redox and spectroscopic properties of the [Fe-S] center. These include the high-potential [4Fe-4S] protein from *Chromatium vinosum*, where a C77S mutation had minimal effect of the function of the protein (*38*). Likewise, substitution of each of the cysteines for serine in the [4Fe-4S] of *Pyrococcus furiosus* ferredoxin (Fd) had little effect on the interaction and the electron transfer between Fd and FNOR (*39*). Cysteine-to-serine changes have also been observed in rubredoxin from *Clostridium pasteurianium* (*40*). Cysteine-to-serine mutants in the *Anabaena* [2Fe-2S] ferredoxin caused little change in the cluster, and the resulting ferredoxin was still competent in electron transfer with ferredoxins:$NADP^+$ reductase (*41*).

The C282A substitution of the enzyme produced an enzyme with only 5% of the activity of the native enzyme. Again, a few examples exist where the mutation of a cysteine for an alanine in an [Fe-S] protein still generated an active enzyme. Martin and Dean (*42*) showed that the mutation of Cys20 to alanine in *Azotobacter vinelandii* ferredoxin I still produced an active protein, likely a result of the enzyme rearranging so that Cys25 could serve as the fourth ligand. Ligand swapping has also been observed in the [2Fe-2S] ferredoxin from *Clostridium pasteurianum* (*43*). Since the *M. jannaschii* enzyme has a Cys284, it is possible that this cysteine could substitute in a similar manner to produce a partially active enzyme.

The C25A mutant enzyme was the least active enzyme of all the mutants generated and showed only 0.4% of the activity of the wild-type protein. We propose that this cysteine serves as the base responsible for the removal of the C-2 hydrogen of the cysteine substrate (Figure 3). This cysteine is the one alkylated by NEM, resulting in inactivation of the enzyme. This was confirmed by the identification of the modified tryptic peptide ALGC(NEM)TEVGLIGYTVAK (MH^+ calculated 1718.89, observed 1718.90 MALDI, observed 1718.85 electrospray) by both MALDI and electrospray mass spectrometry only in the NEM-inactivated enzyme. The reaction of this cysteine with NEM and IAA also indicates that Cys25 is not coordinated with an iron. If this cysteine is the base in the reaction mechanism, it may exist in the enzyme largely as the anion, which would explain its reactivity to both NEM and IAA.

In addition to these conserved cysteines, 29 additional amino acids are conserved among all the orthologues. Of these conserved amino acids, the following residues were considered to possibly be involved in the catalytic mechanism or binding of the substrate on the basis of their charge and/or side-chain structure: K59, H139, R218, S232, E255, S264, D323, K332, and D377 (Figure 2). Of these residues, H139 and D323 were considered most likely to be involved in the catalytic mechanisms, with the histidine serving as a general acid to protonate the sulfur leaving group and the aspartate, because of its closeness to C322, serving as the acid to protonate the cysteine carboxylate. Each of these residues was changed to an asparagine residue, and the H139N substitution reduced the enzymatic activity by 12% and D323N by 50% (Table 3). As a result of this low reduction in catalytic efficiency, we concluded that H139 and D323 were not directly involved in the reaction mechanism.

Exchange of C-2 of Cysteine. Incubation of L-cysteine with enzyme in the presence of deuterated water, under conditions where only partial decomposition of the cysteine occurred, resulted in the exchange of a portion of the cysteine α-hydrogen for solvent deuterium. This was established by GC−MS analysis of the recovered cysteine derivatized as its S-methyl derivative. After correction for the deuterium content of the water used in the incubations, samples incubated for 20 min with 0.28 μg of enzyme exchanged 5.2% of the α-hydrogen and samples incubated with 0.56 μg of enzyme for 30 min exchanged 17.6% of the α-hydrogen. These results demonstrate that the enzyme can catalyze the exchange of the α-hydrogen without elimination of sulfide.

Temperature Stability of the Native and Mutant Enzymes. In the wild-type enzyme as well as the site-directed mutants involving the conserved cysteines C278, C318, and C325 that we proposed to bind to the [4Fe-4S] center, the formation of the [4Fe-4S] cluster was found to significantly increase the thermal stability of each cluster and/or enzyme over the inactivated enzyme. Each of these activated enzymes with the [4Fe-4S] centers retained their activity after heating at 80 °C for 10 min but started to lose activity after 10 min at 90 °C. The extent of activity lost at 90 °C was 0% for the wt enzyme, 22% for C278A, 65% for C318S, and 80% for C325S. The activated wild-type enzyme did not start to lose activity until it was heated at 100 °C for 10 min, and this resulted in the loss of 80% of the activity. The extent of activity lost at 90 °C for the air-inactivated enzymes, with the [3Fe-4S] centers, was 50% for the wild-type enzyme, 55% for C278A, 100% for C318S, and 100% for C325S. These data are consistent with the idea that the enzymes with the catalytically active [4Fe-4S] centers are the most thermally stable and the mutant enzymes are each less stable that the wild-type enzyme. The D323Q mutation, both in the inactive form and in the active form, lost only about 20%

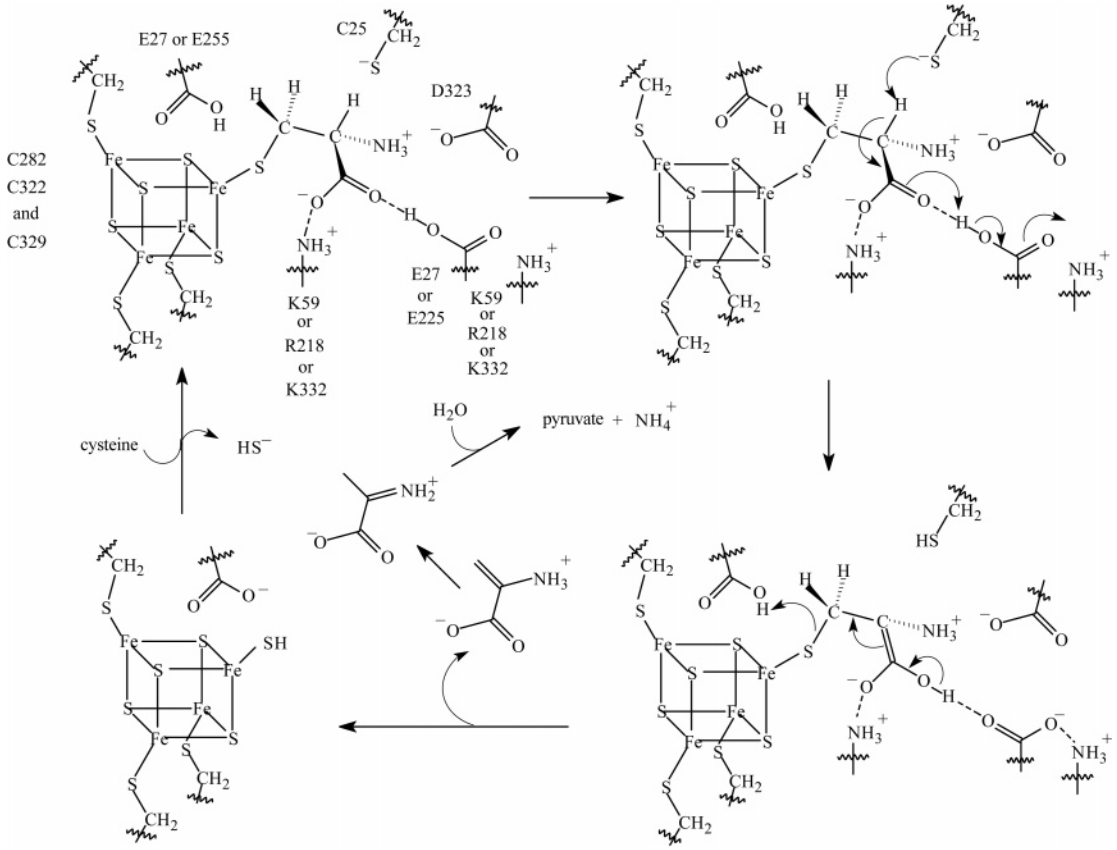

FIGURE 4: Proposed mechanism for cysteine desulfidase.

of its activity when it was heated for 10 min at 90 °C. This would indicate that this mutation simply reduces the protein stability and is unrelated to the [Fe-S] center. In contrast, the inactive oxidized H139Q mutant lost only 20% of its activity after 10 min at 90 °C, whereas the activated form of the enzyme lost 40% of its activity after 10 min at 80 °C and 90% of its activity after 10 min at 90 °C. This result may indicate that this histidine in the native enzyme may be a ligand for the fourth iron when no substrate is present. This ligation would help stabilize the active enzyme. Its substitution with asparagine blocks this ligation and thus greatly reduced its stability in the reduced state. The oxidized form of the enzyme does not have this iron, and thus the mutation has little effect. Having the final X-ray structure of the enzyme will provide insight into these observations.

Enzymatic Mechanism. Figure 4 shows a proposed mechanism for this enzyme that is consistent with the current information. The net outcome of the reaction is the same as that observed for serine/threonine dehydratase (*16*, *44*−*46*) and serine deaminase (*17*), where a [4Fe-4S] center catalyzed the elimination of water from L-serine or L-threonine to form pyruvate or α-ketobutyrate and ammonia. Upon binding to the enzyme, the thiol of the cysteine would coordinate to the non-cysteine-liganded iron, the carboxylate anion of the cysteine would interact directly with a conserved cationic amino acid, which could be either K59, R218 or K332, and the protonated amino group of the cysteine could interact with the conserved residue D323. The iron would serve as a Lewis acid, facilitating the elimination of sulfide. Concerted general acid−general base catalysis, with either of the conserved residues E27 or E225 functioning as the acid and the cysteine C25 anion acting as a general base, will allow formation of the enol/enolate intermediate (*47*, *48*). As depicted in Figure 4, the α-proton and the sulfur would be antiperiplanar, which is most favorable for the elimination. The other of the two conserved E27 or E255 residues could possibly function as the acid to protonate the bound thiol anion of the cysteine. If this should be the case, the active glutamate residues must be in a hydrophobic environment so that it would have a high enough pK_a to function as a hydrogen donor.

A group of enzymes containing a [4Fe-4S] cluster coordinated by three cysteinate ligands from the protein are the members of the *S*-adenosyl-L-methionine (SAM) radical superfamily. Recent spectroscopic evidence indicates that the non-cysteine-coordinated iron in these enzymes interacts with both the carboxylate and amino groups of the SAM substrate (*49*, *50*). Although this type of binding could be occurring here with the carboxylate and amino groups of the cysteine, this would not promote the elimination reaction with its antiperiplanar structure of the bound substrate and was not considered further.

The use of a thiolate anion to abstract an α-hydrogen from an amino acid is the norm in PLP-independent amino acid racemases, including proline racemases (*51*), glutamate racemase (*52*), aspartate racemases (*53*), and diaminopimelate epimerase (*54*). A histidine and thiol group pair would not, however, be like the thiolate−imidazolium ion pair, as seen in the cys/his/asn catalytic site motif (*55*) present in cysteine proteinases, since this would require the cysteine to have a pK_a of around 4 (*56*) and thus would not be a strong enough base to remove the α-hydrogen. Also mutation of the only conserved histidine, H139, had little effect on the enzymatic activity. Our mechanism is analogous to the elimination of

water from citrate by aconitase but with a cysteine serving as a base instead of serine (*57*−*59*). The immediate product of the reaction would be 2-aminoacrylate, which would decompose in the aqueous medium to pyruvate and ammonia (Figure 4).

Possible Function of This Enzyme. This enzyme generates sulfide from cysteine. Since this process does not serve a catabolic function in these autotrophic cells, it must have some other function. We propose this function is to generate sulfide for the biosynthesis of the abundant iron−sulfur centers (*60*) that occur in this organism to an extent 21 times more than found in *E. coli* (R. H. White, unpublished results). One of the lines of evidence for its possible involvement in iron−sulfur center biosynthesis is the absence of genes known to be involved in the biosynthesis of [Fe-S] proteins in this archaea as well most other archaea (*60*). These include the absence of ISC (*i*ron−sulfur *c*luster), NIF (*ni*trogen *f*ixation), and SUF (*suf*ur) machinery (*61*). Since this archaea contains only the SufC (MJ0035) and SufB (MJ0034) homologues (*61*), it is possible that the MJ1025 protein catalyzed the first reaction in yet another system for the formation of iron−sulfur centers.

As discussed previously (*14*, *62*), an important consideration involving sulfur metabolism and Fe-S cluster formation in particular is that the sulfur is transferred from protein to protein in a bound form in order that free sulfide not be present in the cell. In the case of NifS or IscU, the sulfur is bound as a persulfide to the NifS or IscU protein after its release from the cysteine and is then transferred to NifU or IscU, where the formation of the Fe-S cluster is proposed to first occur (*62*−*64*). For cysteine desulfidase we see the development of a similar story, where the sulfur is bound as a sulfide to the Fe-S center after its release from cysteine. On the basis of the observation of rapid exchange of thiol ligands in synthetic clusters (*65*) and the fact that the cysteine desulfidase produces sulfide, it is expected that the bound sulfide would be readily displaced by the cysteine substrate. Reaction of this sulfide with a disulfide in an anchoring and receiving protein would then generate a persulfide that could then be used to form Fe−S clusters. In this manner the sulfur can be effectively oxidized to sulfane sulfur and perhaps metabolized as with the NifU and IscU systems.

An important question related to [Fe-S] biosynthesis is why an organism, which grows in an environment rich in hydrogen sulfide, should require that sulfide be incorporated into cysteine before it is used for [Fe-S] protein biosynthesis. The explanation for this observation would appear to be that the cysteine here, as well as in other cells, serves as a specific sulfide carrier. By incorporating sulfide into cysteine, the cells put a handle on the sulfide so that the enzymes can recognize it, and toxic free sulfide is not present at high concentrations in the cells. An interesting question is how the sulfide is first sequestered by the cells, transported, and used for the biosynthesis of homocysteine, the precursor to the cysteine in these cells (*14*).

It is interesting to note that Flint established that *E. coli* extracts contained several different activities that mobilized the sulfur of cysteine for the synthesis of the [4Fe-4S] cluster of the *E. coli* dehydroxy acid dehydratase (*66*). Four peaks of activity were observed, and the enzymes responsible for three of the peaks were identified. It is possible that the enzyme responsible for the fourth peak of activity (YHAM in Figure 1) was a cysteine desulfidase.

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