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Protein Thiocarboxylate-Dependent Methionine Biosynthesis in *Wolinella succinogenes*

Kalyanaraman Krishnamoorthy and Tadhg P. Begley*

Department of Chemistry, Texas A&M University, College Station, Texax 77840, United States

Received August 17, 2010; E-mail: begley@chem.tamu.edu

Abstract: Thiocarboxylated proteins are important intermediates in a variety of biochemical sulfide transfer reactions. Here we identify a protein thiocarboxylate-dependent methionine biosynthetic pathway in *Wolinella succinogenes.* In this pathway, the carboxy terminal alanine of a novel sulfur transfer protein, HcyS-Ala, is removed in a reaction catalyzed by a metalloprotease, HcyD. HcyF, an ATP-utilizing enzyme, catalyzes the adenylation of HcyS. HcyS acyl-adenylate then undergoes nucleophilic substitution by bisulfide produced by Sir to give the HcyS thiocarboxylate. This adds to *O*-acetylhomoserine to give HcyS-homocysteine in a PLP-dependent reaction catalyzed by MetY. HcyD-mediated hydrolysis liberates homocysteine. A final methylation completes the biosynthesis. The biosynthetic gene cluster also encodes the enzymes involved in the conversion of sulfate to sulfide suggesting that sulfate is the sulfur source for protein thiocarboxylate formation in this system.

1. Introduction

Sulfur-containing natural products are widely distributed in nature, for example, in amino acids, carbohydrates, proteins, nucleic acids, antibiotics, cofactors, siderophores, alkaloids, etc.[1,2] Three major sulfur sources have been identified in bacterial metabolism: free sulfide used for example in cysteine biosynthesis, protein persulfides (R-S-SH) used for example in iron sulfur cluster biosynthesis, and protein thiocarboxylates (R-COSH). Protein thiocarboxylates are members of a growing family of biosynthetic sulfide donors and are involved in a variety of biosynthetic pathways, including vitamin B_1 (ThiS-COSH),[3] molybdopterin (MoaD-COSH),[4] cysteine (CysO−COSH),[5] thioquinolobactin (QbsE-COSH),[6] 2-thioribothymidine (TtuB-COSH),[7] and 5-methoxy-carbonyl-methyl-2-thiouridine (Urm1p-COSH).[8] A significant gap in our understanding of the biosynthetic role of protein thiocarboxylates is the identification of the sulfur source for thiocarboxylate formation. For ThiS-COSH,[9] MoaD-COSH,[4,10] and Urm1p,[8,11] a desulfurase persulfide is the sulfur donor; for the others shown in Figure 1, the sulfur source is unknown.

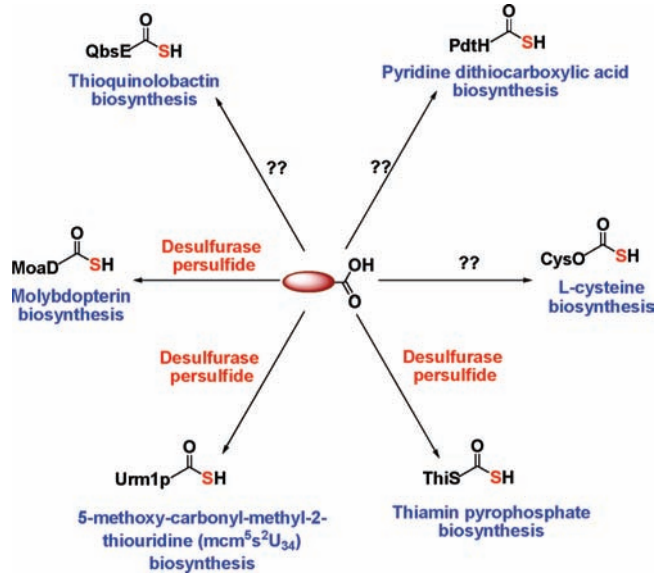

Figure 1. Protein thiocarboxylates function as sulfide donors in the biosynthesis of a variety of natural products. In many cases, the sulfur source for thiocarboxylate formation is unknown.

A search for ThiS-COOH orthologs in the SEED database (http://theseed.uchicago.edu/FIG/index.cgi), revealed in *Wolinella succinogenes* the gene for a putative thiocarboxylate-forming protein clustered with the sulfate assimilation proteins and putative methionine biosynthetic genes (Figure 2). This protein thiocarboxylate/sulfate assimilation clustering pattern, and variations of it, was seen in many other micro-organisms (e.g., *Clostridium kluyveri*, *Clostridium thermocellum*, *Desulfitobacterium hafniense*, *Carboxydothermus hydrogenoformans Z-2901*, *Caldicellulosiruptor saccharolyticus DSM 8903*, *Alkaliphilus metalliredigens QYMF*, *Geobacter metallireducens GS-15*,

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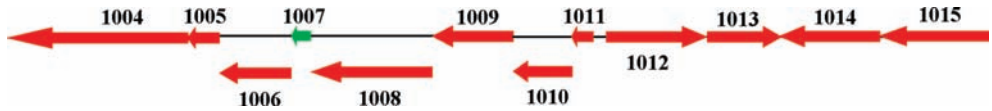

Figure 2. Putative thiocarboxylate forming protein, WS1007 (shown in green), clustered with sulfate assimilation proteins, WS1004, WS1008, WS1009, and WS1010, and homocysteine biosynthetic proteins, WS1012 and WS1015, in *Wolinella succinogenes*. Hypothetical functions for these genes are given in Table 1.

Table 1. Hypothetical Functions of Proteins Adjacent to the Putative Thiocarboxylate-Forming Protein, HcyS-Ala (WS1007)

protein IDs	putative function of the proteins
WS1004, NP_907206 *(sir)*	Ferredoxin sulfite reductase[a]
WS1005, NP_907207 *(hcyD)*	Metalloprotease involved in the removal of C-terminal alanine of HcyS-Ala prior to sulfur transfer and also in the release of homocysteine from HcyS-Homocysteine adduct[a]
WS1006, NP_907208 *(hcyF)*	ATP-utilizing enzyme involved in adenylating HcyS C-terminal prior to sulfur transfer[a]
WS1007, NP_907209 *(hcyS-ala)*	Sulfur transfer protein involved in methionine biosynthesis[a]
WS1008 and WS1009, NP_907210 and NP_907211 *(cysD* and *cysN)*	Sulfate adenylyltransferase, subunits 1 and 2
WS1010, NP_907212 *(cysH)*	Phosphoadenylyl-sulfate reductase/Adenylyl-sulfate reductase
WS1011, NP_907213	Conserved hypothetical protein probably involved in assimilatory sulfate reduction
WS1012, NP_907214 *(metY)*	*O*-acetylhomoserine sulfhydrylase[a]
WS1013, NP_907215	Methyltransferase (EC 2.1.1.-)
WS1014, NP_907216	Putative efflux protein
WS1015, NP_907217 *(metZ)*	*O*-acetylhomoserine or *O*-succinylhomoserine sulfhydrylase[a]

[a] Experimentally characterized in this study.

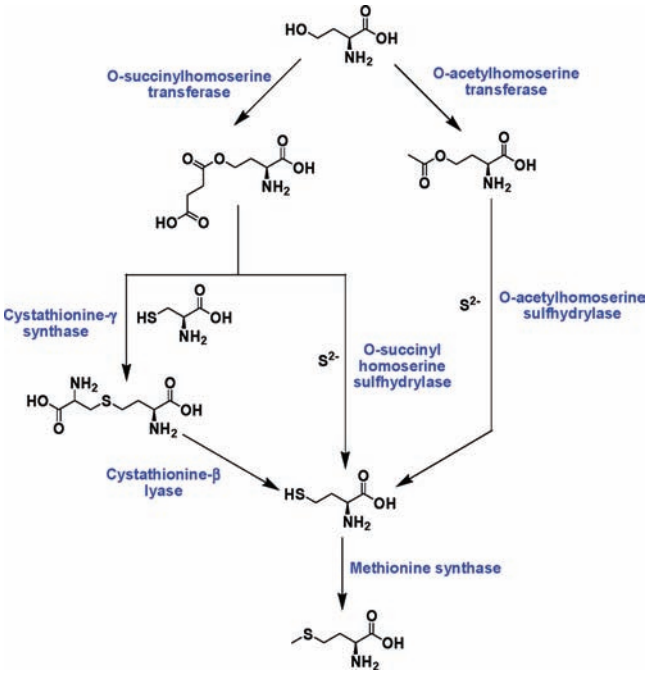

Figure 3. Three well-characterized pathways for L-methionine biosynthesis. The transsulfuration pathway that makes homocysteine from cysteine through cystathionine is absent in *W. succinogenes* (http://seed-viewer.theseed.org/seedviewer.cgi).

Geobacter uraniireducens Rf4, *Acidovorax sp. JS42* and *Pelodictyon luteolum DSM 273*), suggesting that the genes are functionally related and that sulfate may be used as the sulfur source for protein thiocarboxylate formation in these systems.

The well-characterized biosynthesis of methionine is outlined in Figure 3. In this pathway homocysteine formation occurs either by direct addition of sulfide to *O*-succinylhomoserine or *O*-acetylhomoserine, or by breakdown of cystathionine.[12] In this paper, we describe the identification of a new protein thiocarboxylate-dependent methionine biosynthetic pathway in *Wolinella succinogenes* and identify sulfate as the probable sulfur donor.

2. Materials and Methods

2.1. Materials. *W. succinogenes g*enomic DNA (ATCC 29543D-5) was purchased from ATCC (Manassas, VA). pTYB1, SapI, NdeI, XhoI and chitin beads were bought from New England Biolabs (Ipswich, MA). EMD biosciences (Gibbstown, NJ) supplied Luria−Bertani, glycerol, chloroform, methanol, acetonitrile, potassium phosphate and ammonium acetate. IPTG, amplicillin and kanamycin were procured from Lab Scientific (Livingston, NJ). L-Cysteine, arabinose, DL-methionine, D-glucose, M9 minimal salts, formic acid, EDTA, urea, D_2O, triton X-100, methyl viologen, TCEP, *O*-acetyl-L-serine, *O*-succinyl-L-homoserine, DL-homocysteine, 10% Pt on activated carbon, hydroxycobalamin hydrochloride, *S*-adenosylmethionine chloride, 3-mercaptopropionic acid and *o*-phthalaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). Ferrous ammonium sulfate, nickel sulfate, chloramphenicol, boric acid, Na_2SO_3 and Tris.HCl were acquired from Fisher Scientific (Fairlawn, NJ). $CaCl_2$, $ZnSO_4$, $MgCl_2$, $MgSO_4$ and NaCl were from Mallinckrodt. Microcon YM-10 (MWCO 10 kDa), YM-3 (MWCO 3 kDa) and amicon (MWCO 5 kDa) cellulose filters were obtained from Millipore Corporation (Billerica, MA). Aminolevulinic acid, ATP and sodium sulfide were from Acros. *O*-Acetyl-L-homoserine and 5-DL-methyltetrahydrofolic acid calcium salt trihydrate was from TRC Canada (North York, Ontario). Typhoon trio and chelating sepharose fast flow (used for Ni-NTA affinity purification) were products of GE healthcare biosciences (Piscataway, NJ). All bacterial cultures were grown in a New Brunswick Scientific (Edison, NJ) Excella E25 shaker incubator and lysed by sonication using Misonix sonicator 3000 (Misonix Inc., Farmingdale, NY). Absorbance data was obtained on a Cary 300 Bio UV−visible spectrophotometer (Varian, Palo Alto, CA). The glovebox was made by Coy Laboratory products (Grass lakes, MI). ESI-MS analysis was performed using an Esquire-LC_00146 instrument (Bruker, Billerica, MA) in the positive ion mode. MALDI-MS data were recorded in positive mode on an Applied Biosystems Voyager STR (matrix: sinapinic acid). LC-MS data

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were obtained on an Agilent 1200 capillary HPLC system interfaced to an API QSTAR Pulsar Hybrid QTOF mass spectrometer (Applied Biosystems/MDS Sciex, Framingham, MA) equipped with an electrospray ionization (ESI) source. Liquid chromatography (LC) separation was achieved using a Phenomenex Jupiter C4 microbore column (150 × 0.50 mm^2, 300 Å) at a flow rate of 10 μL/min. MALDI-MS and LC-MS data were provided by the Laboratory of Biological Mass spectrometry at Texas A&M University, College Station, Texas. All the protein concentrations were measured by the Bradford assay.[13] The protein stock samples are in 100 mM Tris, 150 mM NaCl, 2 mM TCEP, 30% glycerol, pH 8.0 unless otherwise mentioned. Methionine-auxotroph *E. coli* B834(DE3), harboring the iron sulfur cluster biosynthetic genes in the vector pDB1282, was a gift from Dr. Squire Booker.[14]

2.2. Methods. Cloning and Overexpression. The *sir*, *hcyD*, *hcyF*, *hcyS-ala*, *metY*, *metZ* and *metE* genes, from *Wolinella succinogenes* FDC 602W, were inserted between NdeI/XhoI restriction sites of the THT vector (Table 1s, Supporting Information). This vector is a pET-28 derived vector which allows attachment of a modified 6xHisTag followed by a TEV protease site onto the N-terminus of the expressed protein. *Salmonella typhimurium cysG* (siroheme synthase) was cloned into the pACYCDuet vector. All the genes, except *sir*, were overexpressed in *E. coli* BL21(DE3). Luria−Bertani cultures containing 40 mg kanamycin per liter were grown at 37 °C until an OD_{600} of 0.6, cooled to 15 °C and then induced with a final concentration of 500 μM IPTG before continuing growth at 15 °C for another 12−16 h. The *sir* gene was coexpressed with the *S. typhimurium cysG* and the *A. vinelandii* IscS cluster in *E. coli* B834(DE3). M9 minimal medium (1.5 L) was supplemented with 30 mL 20% glucose, 3 mL of 1 M $MgSO_4$, 150 μL of 1 M $CaCl_2$, 120 mg DL-methionine, 150 mg ampicillin and 60 mg each of kanamycin and chloramphenicol, inoculated with a starter culture and grown at 37 °C to an OD_{600} of 0.1. At this point, 3.75 g of L-arabinose, 88 mg of ferrous ammonium sulfate and 90 mg of L-cysteine were added and the culture was shaken at 100 rpm until the OD_{600} reached 0.6. The culture was then cooled for 4 h at 4 °C, 45 mg of aminolevulinic acid and IPTG (final concentration = 0.5 mM) were added and the culture was incubated with shaking at 15 °C for 12−16 h.

All the cultures were harvested by centrifugation and the cell-pellets were lysed by sonication on ice. The proteins were purified by Ni-NTA affinity chromatography at 4 °C. All buffers contained 1 mM TCEP. After purification, all proteins were buffer exchanged into 100 mM Tris, 150 mM NaCl, 2 mM TCEP, 30% glycerol, pH 8.0 and stored in frozen aliquots at −80 °C.

To make HcyS-COSH and HcyS-DL-homocysteine, *hcyS* (with the C-terminal alanine removed, see below) was inserted between the NdeI/SapI restriction sites in pTYB1, an intein encoding plasmid.[15] The HcyS-Intein fusion was overexpressed in *E. coli* BL21(DE3) as follows: Luria−Bertani cultures containing 100 mg of ampicillin per liter were grown at 37 °C until an OD_{600} of 0.6−0.8 when the temperature was reduced to 15 °C and the cultures were induced with IPTG (final concentration = 0.5 mM). Further growth was carried out at 15 °C for 12−16 h with constant shaking. The cells were harvested by centrifugation and lysed by sonication on ice in 20 mM Tris, 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, pH 7.8. The samples were then loaded onto a chitin column (20 mL) at a flow rate of 0.5 mL/min and washed with 300 mL of 20 mM Tris, 500 mM NaCl, 1 mM EDTA, pH 7.8 at a flow rate of 2 mL/min. Cleavage of the HcyS-Intein fusion was carried out at 4 °C for 12−16 h with 30 mL of 50 mM Na_2S to give HcyS-COSH or with 30 mL of 50 mM DL-homocysteine to yield HcyS-DL-homocysteine. Proteins were buffer exchanged into 100 mM Tris, 150 mM NaCl, 2 mM TCEP, 30% glycerol, pH 8.0 by dialysis using a Novagen D-tube dialyzer Maxi (MWCO 3.5 kDa) and stored as frozen aliquots at −80 °C.

Activity Assay for HcyD (Putative Metalloprotease). 200 μL of 611 μM HcyS-Ala were treated with 6 μL of 2.4 mM HcyD and 3 μL of 10 mM $ZnSO_4$ at room-temperature for 2 h. The samples were desalted into 200 μL of 50 mM NH_4OAc. 200 μL of acetonitrile and 2 μL of HCOOH were then added and the sample was then analyzed for HcyS formation by positive-mode ESI-MS.

For alanine detection, HcyS-Ala and HcyD were buffer exchanged twice into 50 mM potassium phosphate, pH 8.0 using Biorad biospin 6 columns. Eighty-five microliters of 28 mM HcyS-Ala was treated with 100 μL of 1.62 mM HcyD at room-temperature for 2 h. The sample was freeze-dried and redissolved in D_2O. The proteins were removed using YM-3 microcon (washed extensively with D_2O to remove glycerol before loading the protein) and the filtrate was analyzed by ^1H NMR on a Varian 500 MHz spectrometer.

Activity Assay for HcyF (Putative HcyS Adenylating Enzyme). Thirty microliters of 3.98 mM HcyS-Ala, 23 μL of 1.76 mM HcyF, 30 μL of 0.88 mM HcyD, 3 μL of 10 mM ATP and 6 μL of 10 mM $MgCl_2$ were mixed and incubated at room-temperature for 15 min. The reaction was quenched with an equal volume of 12 M urea, the proteins were removed using a YM-10 microcon and the samples analyzed for AMP formation by HPLC (Agilent 1200, Supelco supelcosil 15 cm × 4.6 mm, 3 μm LC-18-T column) using the following gradient at a flow rate of 1 mL/min: solvent A is water, solvent B is 100 mM potassium phosphate, pH 6.6, solvent C is methanol. 0 min: 100% B; 7 min: 10% A, 90% B; 12 min: 25% A, 60% B, 15% C; 17 min: 25% A, 10% B, 65% C; 19 min: 100% B, 25 min: 100% B. Controls lacking HcyF, HcyD and HcyS were similarly run and analyzed.

Preparation of Reduced Methyl Viologen. Methyl viologen (149.5 mg) was dissolved in 12.5 mL of 50 mM potassium phosphate, pH 8.0 in a 15 mL centrifuge tube. 10% Pt on activated carbon (15 mg) was added. Argon was bubbled through the solution for 5 min followed by hydrogen for 30 min. The sample was quickly sealed with parafilm and centrifuged for 5 min to remove the catalyst. The supernatant containing the reduced methyl viologen was then transferred to a new 15 mL centrifuge tube in an oxygen free glovebox. The concentration of the reduced methyl viologen was measured at 600 nm (extinction coefficient 1.3 × 10^4 $M^{-1}cm^{-1}$).[16]

Sir-Mediated HcyS-COSH Formation Detected using Lissamine Rhodamine Sulfonyl Azide and LC-MS. Thirty microliters of 3.98 mM HcyS-Ala, 30 μL of 1.33 mM HcyF, 10 μL of 1.61 mM HcyD and 9 μL of 379 μM Sir were mixed. The sample was then transferred to a glovebox and allowed to stand for 2 h to allow for the sample to become anerobic. Thirty microliters of 10 mM ATP, 4 μL of 1 M $MgCl_2$, 2.5 μL of 100 mM Na_2SO_3 and 100 μL of 3.5 mM reduced methyl viologen were then added. After 15 min, the sample was quenched by exposing to air with shaking.

For gel analysis, the sample was buffer exchanged into 100 μL of 50 mM NH_4OAc, 6 M urea, pH 6.0 using a Biorad biospin 6 column and then treated with 7.4 μL of 15 mM lissamine rhodamine sulfonyl azide for 15 min in the dark at 26 °C.[17] The sample was then desalted by chloroform/methanol precipitation and analyzed by SDS-PAGE (15% tris-glycine gel). The fluorescence image of the labeled protein in the gel was obtained on a Typhoon Trio imager (excitation: 532 nm green laser; emission: 580-nm band-pass filter (580 BP 30)). A similar sample, lacking HcyD, was prepared as a control.

For MS analysis, the sample was heated at 100 °C for 5 min and left to cool to room-temperature for an hour. The precipitated

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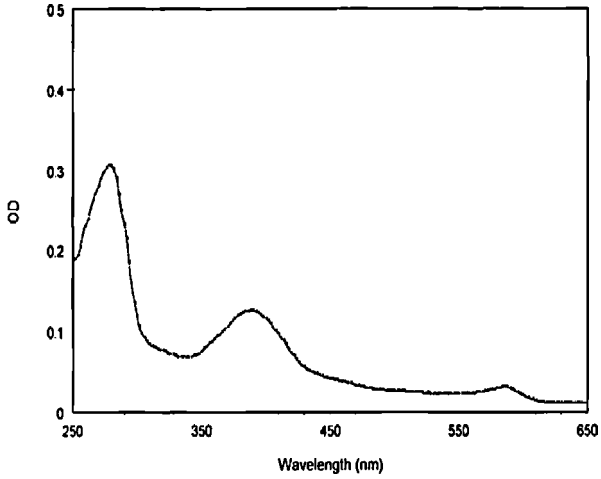

Figure 4. Absorbance spectrum of Sir with its maxima at 388 and 590 nm characteristic of a ferredoxin-sulfite reductase.

protein was removed by filtration. The filtrate, containing small proteins, was then concentrated and buffer exchanged into 50 mM NH_4OAc, pH 6.0 using a Biorad biospin 6 column and analyzed by LC-MS.

HcyS-COSH is the Sulfur Donor for Homocysteine Biosynthesis. Ninety-five microliters of 159 μM HcyS-COSH and 11 μL of 1.4 mM MetY were incubated with 1.3 μL of 10 mM *O*-acetyl-L-serine or *O*-acetyl-L-homoserine. The samples were incubated at room-temperature for 1 h. They were then buffer exchanged into 50 mM NH_4OAc, pH 6.0 and analyzed by MALDI-MS. The time-period of incubation was later reduced to 2 min to avoid formation of the 7912 Da adduct (Supporting Information, Figure 3s).

Sulfide at High Concentrations is also a Sulfur Source for Homocysteine Biosynthesis. Eighty microliters of 1.4 mM MetY (in 50 mM potassium phosphate, pH 8.0) were mixed with 320 μL of 50 mM potassium phosphate, 80% D_2O, pH 8.0 along with 50 μL each of 100 mM sodium sulfide and 100 mM *O*-acetyl-L-homoserine. The sample was incubated for 1 h at 26 °C. An identical sample lacking MetY was prepared as a control. Samples were freeze-dried, redissolved in 100% D_2O and analyzed by ^1H NMR on Varian 300 MHz instrument.

Reaction of HcyS-COSH with MetZ. Two and nine-tenths microliters of 1.4 mM MetZ and 95 μL of 42 μM HcyS-COSH were incubated with the appropriate substrate −0.4 μL of 10 mM *O*-acetyl-L-serine, 0.4 μL of 10 mM *O*-acetyl-L-homoserine and 0.4 μL of 10 mM *O*-succinyl-L-homoserine for 3 min at room-temperature. The samples were buffer exchanged into 50 mM NH_4OAc, pH 6.0 and analyzed by MALDI-MS.

HcyD-Catalyzed Homocysteine Release from HcyS-Homocysteine. Ninety-five microliters of 337 μM HcyS-(DL)-homocysteine were mixed with 3.6 μL of 880 μM HcyD and incubated at room-temperature for 5 min. The sample was then treated with 98.6 μL of 9 M urea, buffer exchanged into 50 mM NH_4OAc, pH 6.0 using a Biorad biospin 6 chromatography column and analyzed by MALDI-MS. An identical reaction mixture lacking HcyD was prepared as a control.

For small molecule analysis, 190 μL of 42 μM HcyS-COSH and 8 μL of 1 mM MetY were mixed, buffer-exchanged into 50 mM potassium phosphate, pH 8.0 and concentrated to 100 μL using a Millipore ultrafree centrifugal filter device (NMWL = 5000 Da). Four-tenths microliters of 250 mM TCEP and 0.8 μL of 10 mM *O*-acetyl-L-homoserine were then added. After incubation at room-temperature for 5 min, 10 μL of 76 μM HcyD in 50 mM potassium phosphate, pH 8.0 was added. After an additional 5 min at room-temperature, protein was removed using a YM-3 microcon and the

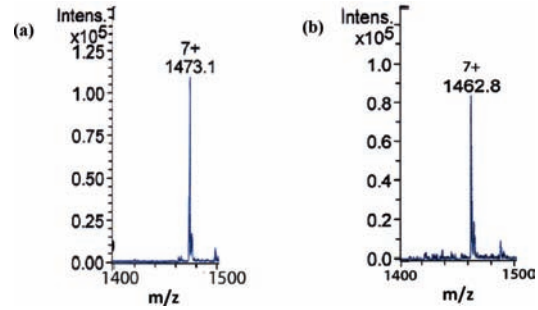

Figure 5. ESI-MS analysis of the HcyD-catalyzed removal of alanine from HcyS-Ala (+7 charge state). (a) HcyS-Ala (observed deconvoluted mass, 10304.98 Da; expected mass, 10304 Da; error, 0.01%) (b) HcyS formed after treatment of HcyS-Ala with HcyD. (observed deconvoluted mass, 10235.61 Da; expected mass, 10232.92 Da; error, 0.03%).

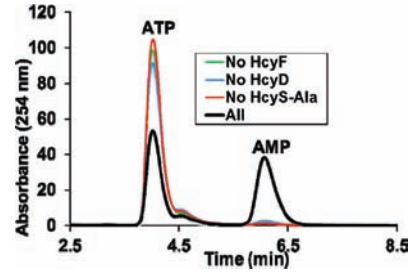

Figure 6. Adenylating activity of HcyF. HcyF adenylates HcyS and the resulting acyl-adenylate undergoes hydrolysis in the absence of bisulfide to give AMP.

sample was treated with methanolic o-phthalaldehyde[18−20] (1 mM final concentration) and analyzed immediately by HPLC (Agilent 1200 using Supelco supelcosil LC-18-T (15 cm ×4.6 mm, 3 μm)) and the following gradient at a flow rate of 1 mL/min: solvent A is water, solvent B is 100 mM potassium phosphate, pH 6.6, solvent C is methanol; 0 min, 100% B; 7 min, 10% A, 90% B; 12 min, 25% A, 60% B, 15% C; 17 min, 25% A, 10% B, 65% C; 19 min, 100% B; 25 min, 100% B.

HcyD Catalyzed Hydrolysis of HcyS-COSH. Ninety-five microliters of 42 μM HcyS-COSH were mixed with 3.6 μL of 88 μM HcyD and incubated at room-temperature for 5 min. The sample was then treated with 99.5 μL of 9 M urea, buffer exchanged into 50 mM NH_4OAc, pH 6.0 using a Biorad biospin 6 chromatography column and analyzed by MALDI-MS. An identical reaction mixture lacking HcyD was prepared as a control.

WS0269 (MetE)-Catalyzed Conversion of Homocysteine to Methionine. One-hundred microliters of 169 μM of WS0269 (annotated as MetE) were incubated with 20 μL of 50 mM potassium phosphate, pH 8.0, 1.7 μL of saturated 100 mM 5-DL-methyltetrahydrofolate, 1.7 μL of 10 mM $MgSO_4$ and 1.7 μL of 100 mM DL-homocysteine.[21] The sample was incubated at room-temperature for 1 h. The protein was then removed by passing the samples through a Millipore ultrafree centrifugal filter device (NMWL = 5000 Da). 150 μL of the flow-through was treated with 50 μL of o-phthalaldehyde reagent (1 mL of 37 mM *o*-phthalaldehyde in MeOH, 4 mL of 0.1 M boric acid, pH 9.3, 162 μL of 3-mercaptopropionic acid were mixed together and the pH was adjusted to 9.3 with NaOH). After 5 min at room-temperature, 20 μL of 1 M potassium phosphate, pH 6.0 were added and the reaction

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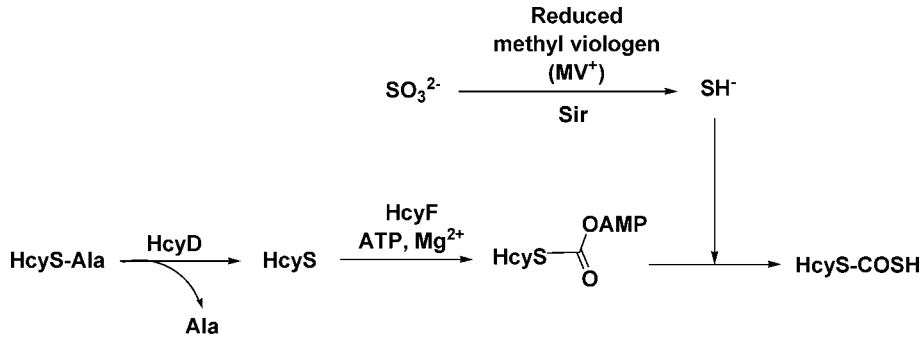

Figure 7. Reconstitution of HcyS-COSH biosynthesis using methyl viologen as the electron donor and sulfite as the sulfur source.

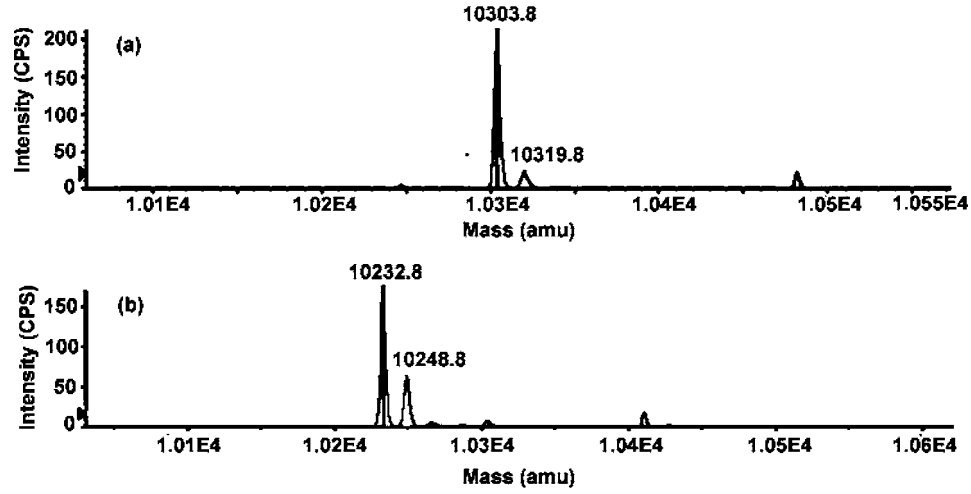

Figure 8. Reconstitution of HcyS-COSH biosynthesis from HcyS-Ala monitored by LC-MS analysis. (a) Reconstitution reaction mixture from which HcyD was omitted showing that HcyS-Ala remains unmodified. (b) Full reaction mixture showing conversion of HcyS-Ala (observed mass, 10303.8 Da; expected mass, 10304 Da; error, 0.002%) to HcyS-COSH (observed mass, 10248.8 Da; expected mass, 10248.92 Da; error, 0.001%) via the intermediacy of HcyS (observed mass, 10232.8; expected mass, 10232.92 Da; error, 0.001%).

mixture was analyzed by HPLC (Agilent 1200 using Phenomenex Gemini 5 μ C18 110A (15 cm $\times$4.6 mm, 5 μm)) using the following gradient at a flow rate of 1 mL/min: solvent A is water, solvent B is 100 mM potassium phosphate, pH 6.6, solvent C is methanol; 0 min, 100% B; 7 min, 10% A, 90%B; 12 min, 25% A, 60% B, 15% C; 17 min, 25% A, 10% B, 65% C; 19 min, 100% B; 25 min, 100% B.

3. Results

Growth and Overexpression. The *sir*, *hcyD*, *hcyF*, *hcyS-ala*, *metY*, *metZ* and *WS0269* (*metE*) genes (Table 1) were cloned into the THT vector. All the proteins, except Sir, were overexpressed in *E. coli* BL21 (DE3) in LB medium. The *sir* gene was overexpressed in the methionine-auxotroph *E. coli* B834 (DE3). In this strain, Sir, annotated as ferredoxin-sulfite reductase, was coexpressed with siroheme synthase (cloned into pACYDuet) and the IscS-cluster assembly proteins (cloned into pDB1282) in M9 medium. Coexpression with these proteins is essential for good reconstitution of active Sir, which utilizes siroheme and [4Fe-4S] cofactors. All proteins were purified on a Ni-NTA affinity column. Sir was purified aerobically (this [4Fe-4S] containing protein did not require anaerobic purification) at 4 °C. It had the characteristic absorbance of a siroheme-[4Fe-4S] cluster protein[22] (Figure 4). MetY and MetZ were yellow suggestive of bound PLP. SDS-PAGE analysis of all the homocysteine biosynthetic proteins is given in the Supporting Information, Figure 1s.

(22) Koguchi, O.; Tamura, G. *Agric. Biol. Chem.* **1988**, *52*, 373.

HcyD is a Metalloprotease Involved in C-Terminal Processing of HcyS-Ala. HcyD removes the C-terminal alanine from HcyS-Ala in the presence of exogenous Zn^{2+}. ESI-MS analysis of the reaction mixture demonstrates the conversion of HcyS-Ala to HcyS (observed mass change: 69.37 Da, expected mass change upon removal of alanine: 71.04 Da, Figure 5). ^{1}H NMR analysis of the small-molecule product confirmed the release of alanine from HcyS-Ala (Supporting Information, Figure 2s).

HcyF Activates HcyS-COOH by Adenylation. HcyF was shown to adenylate HcyS-COOH, generated by HcyD catalyzed hydrolysis of HcyS-Ala. The adenylating reaction was monitored by the release of AMP from the unstable HcyS-COAMP. AMP formation was dependent on HcyF, HcyS-Ala and HcyD (Figure 6).

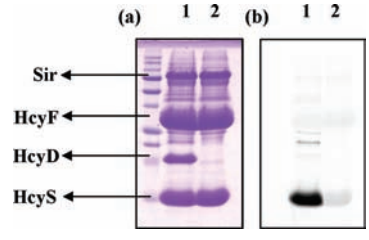

Figure 9. SDS-PAGE analysis of HcyS-COSH formation after labeling with lissamine rhodamine sulfonyl azide (a) Coomassie image (b) Fluorescent image. (Lane 1) Sample containing all components—HcyD, HcyF, HcyS-Ala, Sir, SO_3^{2-}, reduced methyl viologen (electron donor), ATP, Mg^{2+}. (Lane 2) Same as Lane 1 except HcyD was omitted from the reaction mixture. PMT voltage: 400 V.

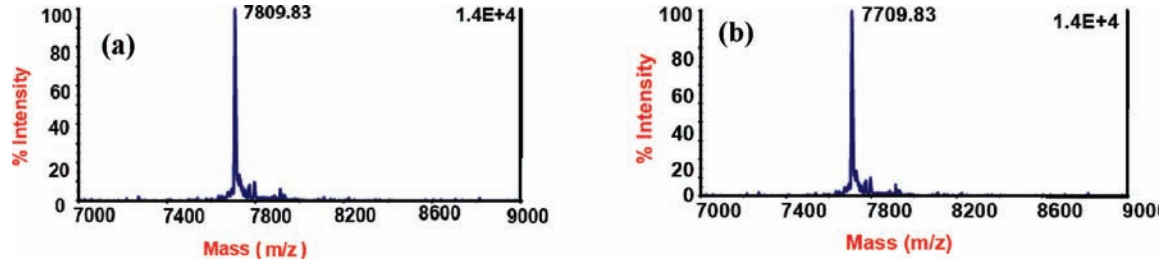

Figure 10. MALDI-MS analysis of the MetY-catalyzed homocysteine biosynthesis (a) HcyS-COSH in the presence of MetY and *O*-acetyl-L-homoserine, forms HcyS-homocysteine (observed mass, 7809.83 Da; expected mass, 7805.96 Da; error, 0.05%; net mass change of protein, 100 Da). (b) HcyS-COSH (observed mass, 7709.83 Da; expected mass, 7704.82 Da; error, 0.07%) in the presence of MetY and *O*-acetyl-L-serine, 1 h incubation time, showing that HcyS-COSH cannot transfer sulfide to *O*-acetyl-L-serine. HcyS-COSH and HcyS-homocysteine (see below) do not have the His_6-tag and hence, their masses are different from the His-tagged HcyS used in other experiments in this work.

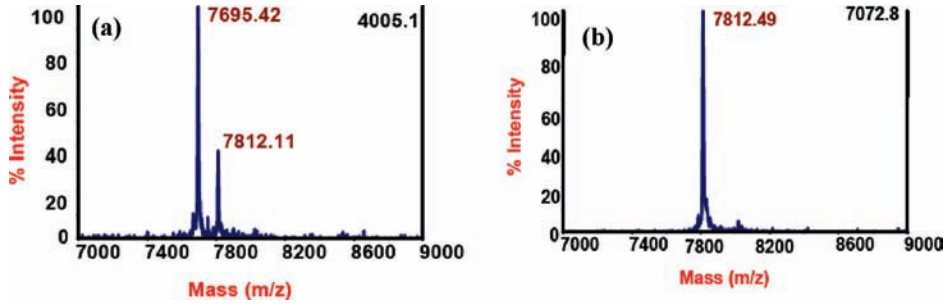

Figure 11. MALDI-MS analysis of HcyD treated HcyS-DL-Homocysteine: (a) HcyS-DL-Homocysteine (observed mass, 7812.11 Da; expected mass, 7805.96 Da; error, 0.08%) in the presence of HcyD is converted to HcyS-homocysteine to HcyS-COOH (observed mass, 7695.42 Da; expected mass, 7688.82 Da; error, 0.09%; net mass change, 116.69 Da). (b) Reference sample of HcyS-DL-homocysteine (observed mass, 7812.49 Da; expected mass, 7805.96 Da; error, 0.08%).

Sir Catalyzes the Formation of HcyS-COSH from Sulfite. The formation of HcyS-COSH from HcyS-Ala requires HcyD catalyzed removal of alanine, HcyF-catalyzed adenylation and AMP displacement by sulfide generated by the Sir-catalyzed reduction of SO_3^{2-} (Figure 7). LC-MS analysis of the reconstitution reaction mixture demonstrated that this conversion was successfully achieved (Figure 8).

HcyS-COSH formation was also detected using the thiocarboxylate specific fluorescent reagent lissamine rhodamine sulfonyl azide.[17] The resulting fluorescent protein was analyzed by SDS-PAGE and scanned for fluorescence using a Typhoon trio (Figure 9).

HcyS-COSH is the Sulfur Source for MetY-Catalyzed Homocysteine Biosynthesis. WS1012, annotated as a putative *O*-acetyl-L-homoserine sulfhydrylase (MetY), catalyzed the reaction of HcyS-COSH with *O*-acetyl-L-homoserine to form HcyS-Homocysteine (Figure 10a). MetY did not accept *O*-acetyl-L-serine as a substrate even after extended incubation time (Figure 10b). Longer incubation time or higher concentrations of *O*-acetyl-L-homoserine resulted in the formation of a higher molecular weight adduct (7911 Da), consistent with homolanthionine formation (Supporting Information, Figure 3s).[23] MetY can also use sulfide to convert *O*-acetyl-L-homoserine to homocysteine (Supporting Information, Figure 4s).

WS1015, annotated as a putative *O*-acetyl/succinyl-L-homoserine sulfhydrylase (MetZ), did not catalyze the reaction of HcyS-COSH with *O*-acetyl-L-homoserine or *O*-succinyl-L-homoserine to form HcyS-Homocysteine (Supporting Information, Figure 5s).

HcyD Catalyzes the Release of Homocysteine from HcyS-Homocysteine. HcyS-homocysteine was prepared by cleaving the HcyS-intein construct with (DL)-homocysteine. HcyD catalyzes the release of homocysteine from HcyS-homocysteine as

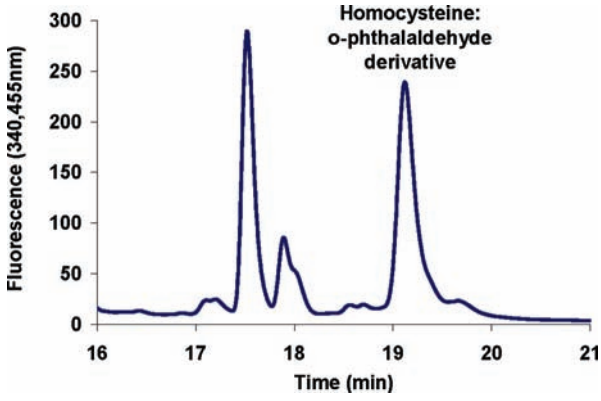

Figure 12. Characterization of the amino acid released from HcyS-homocysteine by *o*-phthalaldehyde derivatization. The peak at 19.1 min comigrated with a reference homocysteine/*o*-phthalaldehyde adduct.

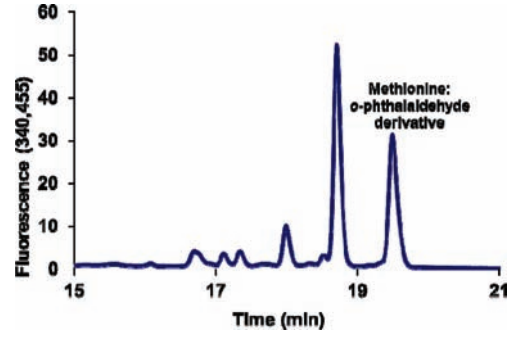

Figure 13. WS0269 is a methyltetrahydrofolate dependent methionine synthase. HPLC analysis of the reaction mixture after derivitization with *o*-phthalaldehyde. The peak at 19.5 min comigrated with a reference methionine/*o*-phthalaldehyde adduct.

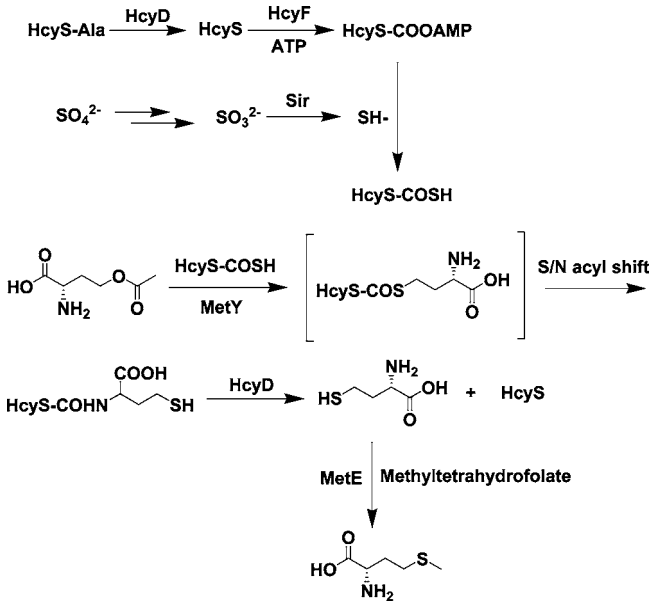

Figure 14. New protein-thiocarboxylate-mediated methionine biosynthesis in *Wolinella succinogenes.*

shown by MALDI-MS analysis of the reaction mixture (Figure 11a). This reaction proceeded to greater than 50% indicating that in addition to alanine (see above) HcyD can remove both isomers of homocysteine. HcyD also catalyzes the hydrolysis of HcyS-COSH (Supporting Information, Figure 6s).

To further characterize the released amino acid, HcyS-L-homocysteine was prepared by reacting HcyS-COSH with *O*-acetyl-L-homoserine in the presence of MetY and then treated with HcyD. The resulting reaction mixture was then treated with *o*-phthalaldehyde to yield a fluorescent homocysteine derivative (Supporting Information, Figure 7s) which was analyzed by HPLC[18−20] (Figure 12). This detected product was identified as the homocysteine derivative by coelution with a reference homocysteine/*o*-phthalaldehyde adduct.

WS0269 is a Methionine Synthase. WS0269 (MetE) catalyzes the methyl transfer from methyltetrahydrofolate to homocysteine to form methionine (Figure 13). Methionine production was assayed by HPLC after derivatization with *o*-phthalaldehyde/mercaptopropionic acid.

4. Discussion

Thiocarboxylated proteins are important intermediates in a variety of biochemical sulfide transfer reactions. For ThiS-COSH, MoaD-COSH, and Urm1p, the thiocarboxylate sulfur is derived from cysteine in a cysteine desulfurase catalyzed reaction.[4,9,10] The sulfur donor for QbsE, PdtH and CysO is unknown. The identification of a putative protein thiocarboxylate dependent methionine biosynthetic gene cluster in *Wolinella succinogenes*, also encoding the enzymes involved in the reduction of sulfate to sulfide, suggested that sulfite reductase might function as the sulfide source for protein thiocarboxylate formation. The successful reconstitution of the new methionine biosynthetic pathway described in Figure 14 supports this conclusion. In this pathway, the carboxy terminal alanine is removed from HcyS-Ala in a reaction catalyzed by HcyD. HcyF then catalyzes the adenylation of HcyS. HcyS acyl-adenylate undergoes nucleophilic substitution by bisulfide produced by Sir to form HcyS thiocarboxylate, which adds to *O*-acetylhomoserine to give HcyS-homocysteine in a reaction catalyzed by MetY. This reaction is likely to proceed via a PLP-mediated substitution followed by an S/N acyl shift analogous to cysteine biosynthesis in *M.tuberculosis*.[5] In that pathway, CysO-thiocarboxylate is converted to CysO-cysteine upon treatment with the PLP-dependent *O*-phospho-L-serine sulfhydrylase and *O*-phospho-L-serine.[5,24] HcyD mediated hydrolysis liberates homocysteine. A final methylation, catalyzed by MetE, completes the methionine biosynthesis.

Several features of this pathway merit further comment. All known thiocarboxylate forming proteins have a Gly-Gly C-terminus in their active form (Figure 15). HcyS-Ala ends in a Gly-Gly-Ala sequence that requires processing by the HcyD metalloprotease. The physiological significance of this processing event is unknown. Pathway regulation is one possibility. We have previously observed analogous carboxy terminal processing: QbsD removes Cys-Phe from the carboxy terminal of QbsE during thioquinolobactin biosynthesis in *P. fluorescens*[6] and Mec^{+} removes cysteine from CysO-Cys during cysteine biosynthesis in *M. tuberculosis*.[5] HcyD shows broad substrate tolerance and also catalyzes the removal of both isomers of homocysteine from HcyS-homocysteine as well as sulfide from HcyS-COSH.

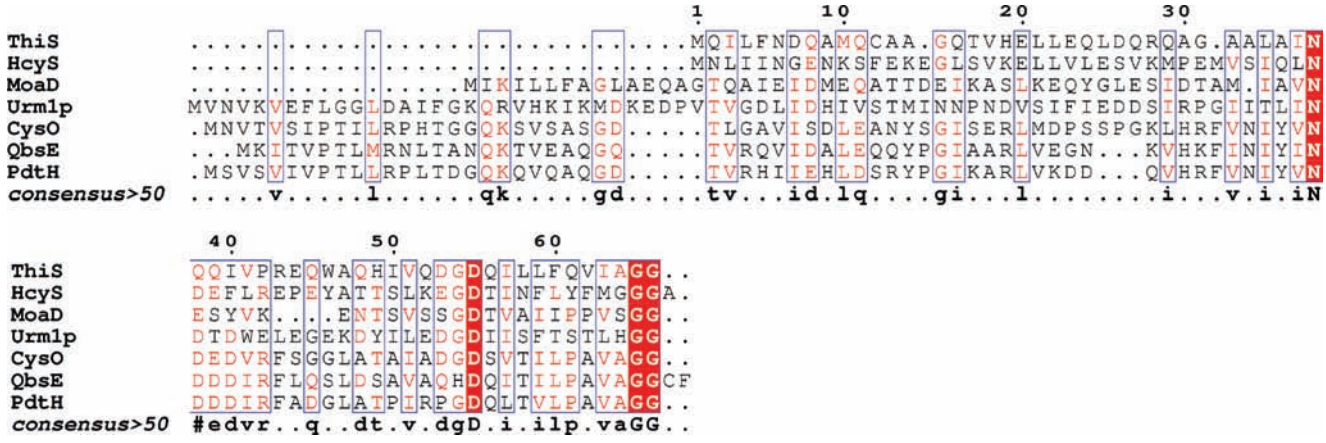

Figure 15. Sequence alignment of biochemically characterized thiocarboxylate-forming proteins (done using Escript 2.2). All have a diglycyl C-terminus in their active form. Both HcyS-Ala and QbsE are activated by carboxy terminal processing. Sequence alignment of putative HcyS-like proteins from various organisms is shown in Supporting Information, Figure 8s.

We assayed for formation of HcyS thiocarboxylate using an MS based assay as well as a recently developed reagent for the detection of protein thiocarboxylates in bacterial proteomes involving a click reaction with lissamine rhodamine B sulfonyl azide.[17] The fact that Sir is clustered with the other methionine biosynthetic genes suggests that it is functioning as the sulfur source for methionine biosynthesis *in vivo* in *Wolinella succinogenes*. However, we do not have evidence at this time for sulfide channeling between Sir and the HcyF/HcyS complex. Kinetics studies to address this are in progress.

(23) Kromer, J. O.; Heinzle, E.; Schroder, H.; Wittmann, C. *J. Bacteriol.* **2006**, *188*, 609.
(24) O'Leary, S. E.; Jurgenson, C. T.; Ealick, S. E.; Begley, T. P. *Biochemistry* **2008**, *47*, 11606.

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Supporting Information Available: Additional sequence analysis, SDS-PAGE of purified proteins, reaction schemes, reaction mixture, and product spectra and the primers used for cloning. This material is available free of charge via the Internet at http://pubs.acs.org.

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