BIOCHEMISTRY
including biophysical chemistry & molecular biology

Rapid Report

pubs.acs.org/biochemistry

Homocysteine Is Biosynthesized from Aspartate Semialdehyde and Hydrogen Sulfide in Methanogenic Archaea

Kylie D. Allen,[†] Danielle V. Miller,[†] Benjamin J. Rauch,[‡] John J. Perona,[‡,§] and Robert H. White*[,†]

[†]Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, United States

[‡]Department of Biochemistry and Molecular Biology, Oregon Health and Science University, 3181 Southwest Sam Jackson Park Road, Portland, Oregon 97239-3098, United States

[§]Department of Chemistry, Portland State University, 1719 Southwest 10th Avenue, Portland, Oregon 97201, United States

Supporting Information

ABSTRACT: The biosynthetic route for homocysteine, an intermediate in methionine biosynthesis, is unknown in some methanogenic archaea because homologues of the canonical required genes cannot be identified. Here we demonstrate that *Methanocaldococcus jannaschii* can biosynthesize homocysteine from aspartate semialdehyde and hydrogen sulfide. Additionally, we confirm the genes involved in this new pathway in *Methanosarcina acetivorans*. A possible series of reactions in which a thioaldehyde is formed and then reduced to a thiol are proposed. This represents a novel route for the biosynthesis of homocysteine and exemplifies unique aspects of sulfur chemistry occurring in prebiotic environments and in early life forms.

Methionine biosynthesis in methanogenic archaea has remained a mystery since the genome of *Methanocaldococcus jannaschii* was sequenced in 1996.[1] In the classical pathway, Met is derived from homoserine (Figure 1, blue). The genes required for homoserine biosynthesis are present in methanogens; however, genes known to be involved in the conversion of homoserine to homocysteine (Hcy) (*met*A, *met*B, *met*C, and *met*Y) are missing from most methanogenic genomes.[2] A few methanogens, including the metabolically diverse *Methanosarcina acetivorans*, do possess *met*A and *met*Y, which were likely acquired through horizontal gene transfer from bacteria.[3] The C-terminal half of cobalamin-independent methionine synthase (MetE, MJ1473), the final enzyme in the pathway that methylates Hcy to generate Met, is encoded in methanogenic genomes, and the activity of this enzyme from *Methanothermobacter thermautotrophicus* has been demonstrated.[4] However, a major gap still exists in the methanogenic pathway upstream of Hcy.

Isotopic feeding experiments in *Methanococcus maripaludis* demonstrated that the sulfur in Met was derived from inorganic sulfide and not cysteine.[5] Additionally, the substrate for Hcy biosynthesis was proposed to be homoserine phosphate on the basis of the presence of genes for synthesizing homoserine-P (Figure 1) and an increase in Hcy concentration with the addition of homoserine-P to *M. jannaschii* cell extracts.[6] However, none of the recombinant putative PLP-dependent enzymes from *M. jannaschii* catalyzed the conversion of homoserine-P and HS^- to Hcy (R. H. White, unpublished data), indicating that this may be a side reaction and not biochemically relevant. Recently, a bioinformatics and genetic study revealed three new genes likely to be involved in Hcy biosynthesis in *Me. acetivorans*;[7] however, the substrates and mechanism involved remain unknown. Here, we sought to explore how Hcy is biosynthesized in methanogens using *M. jannaschii* and *Me. acetivorans* cell extracts and stable isotopically labeled precursors.

For analysis of thiol-containing compounds, the monobromobimane derivatives were generated, partially purified by preparative TLC, and analyzed by LC−ESI-MS (see the Supporting Information for details). As a baseline measurement and validation of the method, we determined the concentration of Hcy in *M. jannaschii* cell extracts measured by isotope dilution analysis to be 40 μM (Table 1, experiment 1). This value is comparable to that previously reported in *Met. maripaludis*[5] and is in the range for the values reported in bacteria.[8]

Aspartate semialdehyde (Asa) is a common intermediate in lysine, threonine, and methionine biosynthesis in all organisms, and also in aromatic amino acid biosynthesis in methanogens[9] (Figure 1). We reasoned that Hcy could be biosynthesized directly from Asa, rather than through homoserine or activated homoserine derivatives. Incubation of *M. jannaschii* cell extracts with Asa and HS^- in the presence of water containing 18% D_2O increased the concentration of Hcy to 200 μM, and 15% of this was labeled with one deuterium (Table 1, experiment 3; specific activities for this and following experiments are reported in Table S1 of the Supporting Information). The discrepancy in deuterium incorporation is due to unlabeled Hcy already present in the cell extract. This result is consistent with Hcy arising from Asa and HS^- with one hydrogen derived from water. In control experiments without cell extracts, no Hcy formation was observed (Table 1, experiment 2), demonstrating that the reaction is dependent on enzymes from *M. jannaschii*.

When the experiment was conducted with $H^{34}S^-$, 340 μM Hcy was observed, and this was almost completely labeled with ^{34}S (Table 1, experiment 4). The increase in the level of Hcy production compared to that in experiment 3 is likely due to

Received: February 5, 2015
Revised: May 1, 2015
Published: May 4, 2015

DOI: 10.1021/acs.biochem.5b00118
Biochemistry 2015, 54, 3129−3132

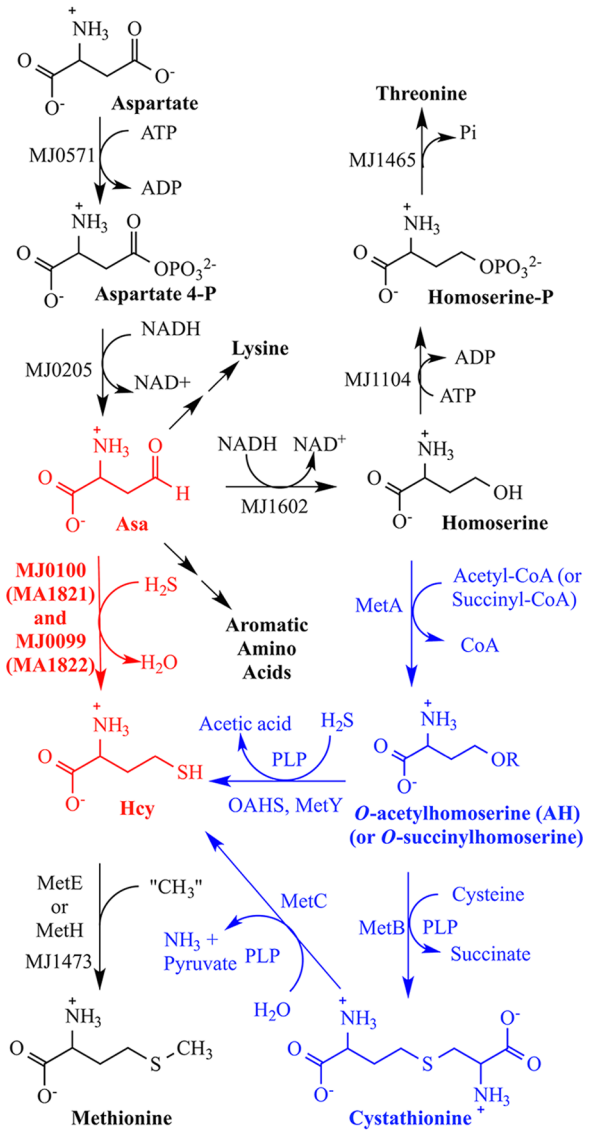

Figure 1. Hcy/Met biosynthetic pathways. Blue indicates the canonical pathway, and red represents the new step in methanogens identified in this work. Black depicts the reactions common to all organisms.

the increased amount of provided sulfide (Table S1). To confirm that Asa is the precursor to Hcy, the experiment was performed with [3,3-$^2H_2$]Asa. We found that 68% of the Hcy contained two deuteriums with none containing one deuterium, demonstrating that Asa is a precursor to Hcy and also that no deuteriums on C-3 of the Asa are exchanged or lost during its conversion into Hcy.

Taken together, our results demonstrate that Hcy is derived from Asa and HS^- in *M. jannaschii*. Both genes previously identified in *Me. acetivorans* to be involved in Hcy biosynthesis (MA1821 and MA1822)[7] have homologous genes in *M. jannaschii* (MJ0100 and MJ0099, respectively) and are also found in all other methanogens. To confirm the role of MA1821/MA1822 in Hcy biosynthesis, we conducted experiments with *Me. acetivorans* cell extracts and the previously generated knockout strains.[7] The wild-type cells contain two routes for Hcy biosynthesis: a canonical bacterial pathway from *O*-acetylhomoserine using *O*-acetylhomoserine sulfhydrylase (OAHS, MetY) and the new pathway described here involving

Table 1. Incorporation of Precursors into Hcy in *M. jannaschii* (MJ) and *Me. acetivorans* (MA) Cell Extracts[a]

experiment	extent of labeling[b]	[Hcy] (μM)[c]
(1) MJ cell extract	na[d]	40
(2) extraction buffer, Asa, HS^-	na[d]	<0.7
(3) MJ cell extract, 18% ^2H_2O, Asa, HS^-	15% $^2H_1$	200
(4) MJ cell extract, Asa, $H^{34}S^-$	89% ^{34}S	340
(5) MJ cell extract, [3,3-$^2H_2$]Asa, HS^-	68% $^2H_2$	130
(6) MA wild-type cell extract, Asa, $H^{34}S^-$	92% ^{34}S	22
(7) MA Δ*oahs*Δ*ma*1821-22 cell extract, Asa, $H^{34}S^-$	na[d]	nd[e]
(8) MA Δ*oahs* cell extract, Asa, $H^{34}S^-$	67% ^{34}S	28
(9) MA Δ*ma*1821-22 cell extract, AH, $H^{34}S^-$	99% ^{34}S	1000
(10) MA Δ*oahs* cell extract, AH, $H^{34}S^-$	48% ^{34}S	12
(11) MA Δ*ma*1821-22 cell extract, Asa, $H^{34}S^-$	74% ^{34}S	13

[a]Experimental conditions are described in the Supporting Information. [b]The measured isotopic abundances for monobromobimane-Hcy positive ions at *m/z* 326, 327, and 328 are reported in Table S1. [c]Methods for calculations are described in the Supporting Information. [d]Not applicable. [e]Too low to measure accurately.

MA1821 and MA1822 (Figure 1, red). In WT cell extracts with $H^{34}S^-$ and Asa added, 22 μM Hcy that contained 92% ^{34}S was observed (Table 1, experiment 6), while cell extracts lacking both OAHS and MA1821/MA822 (Δ*oahs*Δ*ma*1821-22) did not contain any detectable Hcy under the same conditions (Table 1, experiment 7). In cells with only the OAHS gene deleted (Δ*oahs*), production of Hcy from $H^{34}S^-$ was observed (Table 1, experiment 8), indicating that MA1821 and MA1822 are indeed involved in Hcy biosynthesis.

To support the idea that Asa is the direct precursor for MA1821/MA1822-dependent Hcy biosynthesis, we tested *O*-acetylhomoserine (AH) as a substrate in two of the deletion strains. In Δ*ma*1821-22 cell extracts, significant Hcy production was observed (Table 1, experiment 9), while Δ*oahs* cell extracts had less than half the activity when AH was added instead of Asa (in Table 1, compare experiments 8 and 10), indicating that AH is not the direct substrate for MA1821/22-dependent Hcy biosynthesis. Similarly, Δ*ma*1821-22 cell extracts showed far less activity when Asa was added instead of AH (in Table 1, compare experiments 9 and 11). Because low levels of activity were observed in experiments 10 and 11, the cell extracts are likely capable of interconverting Asa and AH. However, because the other required substrates are not provided for this conversion [NADH and acetyl-CoA for Asa to AH and CoA and NAD^+ for AH to Asa (Figure 1)], the extracts cannot maintain the level of activity observed when the correct substrate is provided.

Taken together, these experiments prove that MA1821 and MA1822 are involved in Hcy biosynthesis in a pathway that involves sulfide and strongly suggest that Asa is the direct precursor (Figure 1, red). Future studies will be required to unambiguously determine the true substrates in this new route. The significantly larger amount of production of Hcy from AH in Δ*ma*1821-22 cell extracts (Table 1, experiment 9) indicates that the OAHS-dependent pathway is more efficient and thus likely the preferred route. Most methanogens do not have this option, however, because they lack the required genes.

MA1821 (MJ0100) does not have an annotated function but is composed of two cystathionine β-synthase (CBS) domains at the C-terminus and a DUF39 domain of unknown function at the N-terminus. Crystal structures of the CBS domain pair of

DOI: 10.1021/acs.biochem.5b00118
Biochemistry 2015, 54, 3129−3132

MJ0100 demonstrated that this domain binds *S*-adenosyl-L-methionine (SAM) and 5′-methylthioadenosine (MTA), which induce a conformational change consistent with regulatory function.[10] This supports the idea that MA1821 is involved in Hcy biosynthesis; when SAM and/or MTA are present in excess, they could function as feedback inhibitors to block MA1821 catalysis for Hcy production and suppress downstream SAM biosynthesis. It was also determined that the CBS domains are not absolutely required for activity, indicating that the DUF39 domain harbors the catalytic function.[7]

Two conserved cysteines (C54 and C131 in MA1821) are present in the DUF39 domain. One possible mechanism consistent with sulfur reactivity in enzyme active sites is that these cysteines may be present as a disulfide, which reacts with HS^- to form a persulfide anion (Figure 2). The terminal thiol

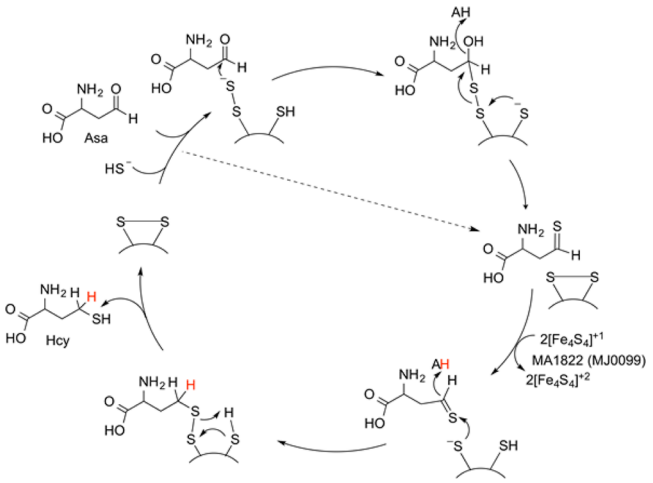

Figure 2. Proposed mechanism for biosynthesis of Hcy from Asa and HS^-.

of this persulfide could then undergo an addition reaction to the aldehyde of Asa to produce the disulfide hemiacetal adduct. A thiolate anion from the other cysteine would then react with the disulfide and expel water from the adduct to form the thioaldehyde. After reduction of the resulting disulfide bond, the thiol pair would reduce the thioaldehyde to Hcy with incorporation of hydrogen (red) from water. One of these cysteines (C54) was shown to be essential, while the other (C131) was not essential,[7] indicating that the first is involved in persulfide formation and the latter can be replaced with an alternate thiol. Dimerization or higher-order multimerization of MA1821 to create an assembly in which the C54 residues from different subunits are juxtaposed could explain this discrepancy.

It is also important to note that, while the sulfur atom from sulfide is clearly incorporated into Hcy, there is presently no evidence of direct binding of sulfide to the MA1821 protein. An alternative possibility is that MA1821 associates with a donor protein from which it accepts a persulfide sulfur relayed as sulfane (S^0). Additionally, another mechanism could involve reaction of HS^- with Asa to generate the thioaldehyde directly instead of going through the initial persulfide intermediate (Figure 2, dotted arrow). Future experiments with the purified enzymes and isolation of the proposed thioaldehyde and persulfide intermediates will be required to shed light on this intriguing enzymatic mechanism.

MA1822 (MJ0099) is annotated as a ferredoxin of unknown function. We propose that it may be involved in the reduction of the disulfide formed in MA1821 during the conversion of Asa to Hcy (Figure 2). Reduction of cystines by Fe−S clusters is well-documented in enzymes such as ferredoxin:thioredoxin reductase[11] and heterodisulfide reductase, where a redox-active disulfide is cleaved in sequential one-electron steps to two cysteines by an active site $[Fe_4S_4]$ cluster (Table S2 of the Supporting Information, Scheme 1).[12]

Biochemical (Table S2) and chemical (Table S3 of the Supporting Information) precedent for some of these proposed reaction steps is sparse; however, there are a few known examples supporting this mechanism. Thiols are known to readily react with aldehyde groups to form hemithioacetals (Table S2, Scheme 2).[13] In the glyoxalase I reaction, the hemithioacetal substrate is converted to a thioester,[14] while in the 3-hydroxy-3-methylglutaryl-CoA reductase reaction, a hemithioacetal intermediate is formed and a thiol is released.[15] Elimination of water to generate a thioaldehyde has limited biochemical or chemical precedent, perhaps because of the instability of such structures.[16] One biochemical example is in the phosphopantothenoylcysteine decarboxylase reaction, in which a thioaldehyde is formed by flavin-catalyzed oxidation (Table S2, Scheme 3).[17,18] Another example similar to what we propose here is 4-thiouridine biosynthesis, in which a persulfide-modified cysteine residue reacts with the pyrimidine substrate to form a disulfide bond between the substrate and the enzyme. A second active site cysteine then reacts with the first to release a thiopyrimidine intermediate (Table S2, Scheme 4).[19]

In conclusion, the work presented here represents a new route for the biosynthesis of Hcy. This transformation is chemically identical to that reported for the biosynthesis of coenzyme M and is also likely for coenzyme B biosynthesis.[20] In our view, these pathways are hallmarks of primitive metabolism because they depend on HS^-, which was abundant when life was first evolving in hot anaerobic oceans.[21]

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and tables of model biochemical and chemical reactions. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00118.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: rhwhite@vt.edu.

Funding

This work was supported by National Science Foundation Grant MCB0722787 to R.H.W. and by the Agricultural Experiment Station Hatch Program (CRIS Project No. VA-135981).

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Bult, C. J., et al. (1996) *Science 273*, 1058−1073.
(2) Selkov, E., Maltsev, N., Olsen, G. J., Overbeek, R., and Whitman, W. B. (1997) *Gene 197*, GC11−GC26.
(3) Galagan, J. E., et al. (2002) *Genome Res. 12*, 532−542.
(4) Schroder, I., and Thauer, R. K. (1999) *Eur. J. Biochem. 263*, 789−796.
(5) Liu, Y., Sieprawska-Lupa, M., Whitman, W. B., and White, R. H. (2010) *J. Biol. Chem. 285*, 31923−31929.

DOI: 10.1021/acs.biochem.5b00118
Biochemistry 2015, 54, 3129−3132

(6) White, R. H. (2003) *Biochim. Biophys. Acta 1624*, 46−53.
(7) Rauch, B. J., Gustafson, A., and Perona, J. J. (2014) *Mol. Microbiol. 94*, 1330−1342.
(8) Kessler, D. (2006) *FEMS Microbiol. Rev. 30*, 825−840.
(9) White, R. H. (2004) *Biochemistry 43*, 7618−7627.
(10) Lucas, M., Encinar, J. A., Arribas, E. A., Oyenarte, I., Garcia, I. G., Kortazar, D., Fernandez, J. A., Mato, J. M., Martinez-Chantar, M. L., and Martinez-Cruz, L. A. (2010) *J. Mol. Biol. 396*, 800−820.
(11) Walters, E. M., Garcia-Serres, R., Naik, S. G., Bourquin, F., Glauser, D. A., Schurmann, P., Huynh, B. H., and Johnson, M. K. (2009) *Biochemistry 48*, 1016−1024.
(12) Duin, E. C., Madadi-Kahkesh, S., Hedderich, R., Clay, M. D., and Johnson, M. K. (2002) *FEBS Lett. 512*, 263−268.
(13) Lienhard, G. E., and Jencks, W. P. (1966) *J. Am. Chem. Soc. 88*, 3982−3994.
(14) Cameron, A. D., Ridderstrom, M., Olin, B., Kavarana, M. J., Creighton, D. J., and Mannervik, B. (1999) *Biochemistry 38*, 13480−13490.
(15) Tabernero, L., Bochar, D. A., Rodwell, V. W., and Stauffacher, C. V. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 7167−7171.
(16) Okuma, K. (2002) *Sulfur Rep. 23*, 209−241.
(17) Kupke, T., Uebele, M., Schmid, D., Jung, G., Blaesse, M., and Steinbacher, S. (2000) *J. Biol. Chem. 275*, 31838−31846.
(18) Strauss, E., and Begley, T. P. (2001) *J. Am. Chem. Soc. 123*, 6449−5640.
(19) Mueller, E. G. (2006) *Nat. Chem. Biol. 2*, 185−194.
(20) Grochowski, L. L., and White, R. H. (2010) Biosynthesis of the Methanogenic coenzymes. In *Comprehensive Natural Products II: Chemistry and Biology* (Begley, T. P., Ed.) pp 711−748, Elsevier Ltd., New York.
(21) Martin, W., Baross, J., Kelley, D., and Russell, M. J. (2008) *Nat. Rev. Microbiol. 6*, 805−814.

DOI: 10.1021/acs.biochem.5b00118
Biochemistry 2015, 54, 3129−3132