S-Inosyl-1-L-Homocysteine Hydrolase, a Novel Enzyme Involved in S-Adenosyl-1-Methionine Recycling

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ABSTRACT
S-Adenosyl-1-homocysteine, the product of S-adenosyl-1-methionine (SAM) methyltransferases, is known to be a strong feedback inhibitor of these enzymes. A hydrolase specific for S-adenosyl-1-homocysteine produces 1-homocysteine, which is remethylated to methionine and can be used to regenerate SAM. Here, we show that the annotated S-adenosyl-1-homocysteine hydrolase in *Methanocaldococcus jannaschii* is specific for the hydrolysis and synthesis of S-inosyl-1-homocysteine, not S-adenosyl-1-homocysteine. This is the first report of an enzyme specific for S-inosyl-1-homocysteine. As with S-adenosyl-1-homocysteine hydrolase, which shares greater than 45% sequence identity with the *M. jannaschii* homolog, the *M. jannaschii* enzyme was found to copurify with bound NAD⁺ and has *Kₘ* values of 0.64 ± 0.4 mM, 0.0054 ± 0.006 mM, and 0.22 ± 0.11 mM for inosine, 1-homocysteine, and S-inosyl-1-homocysteine, respectively. No enzymatic activity was detected with S-adenosyl-1-homocysteine as the substrate in either the synthesis or hydrolysis direction. These results prompted us to redesignate the *M. jannaschii* enzyme an S-inosyl-1-homocysteine hydrolase (SIHH). Identification of SIHH demonstrates a modified pathway in this methanogen for the regeneration of SAM from S-adenosyl-1-homocysteine that uses the deamination of S-inosyl-1-homocysteine to form S-inosyl-1-homocysteine.

IMPORTANCE
In strictly anaerobic methanogenic archaea, such as *Methanocaldococcus jannaschii*, canonical metabolic pathways are often not present, and instead, unique pathways that are deeply rooted on the phylogenetic tree are utilized by the organisms. Here, we discuss the recycling pathway for S-adenosyl-1-homocysteine, produced from S-adenosyl-1-methionine (SAM)-dependent methylation reactions, which uses a hydrolase specific for S-inosyl-1-homocysteine, an uncommon metabolite. Identification of the pathways and the enzymes involved in the unique pathways in the methanogens will provide insight into the biochemical reactions that were occurring when life originated.

The recycling of S-adenosyl-1-methionine (SAM)-derived metabolites in *Methanocaldococcus jannaschii* was recently shown to use a novel enzyme, 5′-deoxyadenosine deaminase (DadD) (1). DadD deaminates three SAM-derived enzymatic products (5′-methylthioadenosine, 5′-deoxyadenosine, and S-adenosyl-1-homocysteine) to produce the inosine analogs (Fig. 1) (1). The canonical pathway for recycling S-adenosyl-1-homocysteine (SAH) produced from SAM-dependent methyltransferases (2–4) proceeds in a three-step recycling pathway back to SAM (Fig. 2A). SAH is first hydrolyzed to produce adenosine and homocysteine using S-adenosyl-1-homocysteine hydrolase (SAHH). Homocysteine is then methylated by methionine synthase to produce methionine (5). The last step in the pathway is the generation of SAM by combining the adenosyl moiety of ATP with methionine to produce SAM, pyrophosphate, and phosphate (Fig. 2), catalyzed by SAM synthase (6). A similar pathway has been established in *M. jannaschii* but with the addition of one extra step involving the deamination of SAH to form S-inosyl-1-homocysteine (SIH), catalyzed by DadD (Fig. 2B).

The production of SIH prompted our search for an enzyme able to metabolize this uncommon metabolite. An annotated SAHH encoded by MJ1388, which shares greater than 45% sequence identity with other characterized SAHHS (Fig. 3), was identified as possibly metabolizing SIH based on the structural similarities between SAH and SIH (Fig. 2). Here, we show that the annotated SAHH from *M. jannaschii* is specific for SIH and failed to either hydrolyze or synthesize SAH, prompting the reassignment of the MJ1388-encoded enzyme as an S-inosyl-1-homocysteine hydrolase (SIHH).

MATERIALS AND METHODS
Chemicals. All chemicals were purchased from Sigma-Aldrich unless otherwise noted.

**Enzymatic production of S-inosyl-1-homocysteine.** The enzymatic preparation of S-inosyl-1-homocysteine was done by incubating 1 mM S-adenosyl-1-homocysteine with 18.2 ng of DadD (the MJ1541 gene product) overnight at 25°C in 1 ml of 50 mM 1,3-bis[tris(hydroxymethyl)amino]propane (BIS TRIS propane) buffer (pH 9.0) (1). Under these conditions, 1 mM SAH was completely converted to 1 mM SIH, as measured by high-performance liquid chromatography (HPLC).

Cloning, overexpression, and purification of the *M. jannaschii* MJ1388 gene product in *E. coli*. The MJ1388 gene (UniProt accession number Q58783) was amplified by PCR from genomic DNA using oligonucleotide primers MJ1388-Fwd, 5′-GGTCTATAGTGAAGTTAGGG

**Received** 28 January 2015  **Accepted** 22 April 2015  **Accepted manuscript posted online** 27 April 2015


Editor: W. W. Metcalf
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AC-3′, and MJ1388-Rev, 5′-GCTGGATCCTTAAGTTCTTCTC-3′. PCR amplification was performed as described previously (7) using a 55°C annealing temperature. The purified PCR product was digested with NdeI and BamHI restriction enzymes and ligated into compatible sites in vector pT7-7. The sequence of the resulting plasmid, pMJ1388, was verified by DNA sequencing. pM1388 was transformed into *Escherichia coli* strain BL21-CodonPlus(DE3)-RIL cells (Stratagene). The transformed cells were grown in LB medium (200 ml) supplemented with 100 μg/ml ampicillin at 37°C with shaking until they reached an optical density at 600 nm (OD₆₀₀) of 1.0. Recombinant-protein production was induced by addition of lactose to a final concentration of 28 mM (7). After an additional 2 h of culture, the cells (200 ml) were harvested by centrifugation (4,000 × g; 5 min) and frozen at −20°C. Induction of the desired protein was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of total cellular proteins.

The frozen *E. coli* cell pellet containing the desired protein (−0.4 g [wet weight]) was suspended in 3 ml of extraction buffer [50 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) (pH 7.0), 10 mM MgCl₂, 20 mM dithiothreitol (DTT)] and lysed by sonication. After centrifugation (14,000 × g; 5 min), the resulting recombinant protein was found to remain soluble in the cell extract after heating for 10 min at 80°C followed by centrifugation (8,100 × g; 20 to 30 min). This heating step allowed the purification of the recombinant enzymes from the majority of *E. coli* proteins, which denature and precipitate under these conditions. Purification of the desired protein was performed by anion-exchange chromatography of the 80°C soluble fractions on a MonoQ HR column (1 by 8 cm; Amersham Bioscience) using a linear salt gradient from 0 to 1 M NaCl in 25 mM Tris buffer (pH 7.5) over 55 min at a flow rate of 1 ml/min. One-milliliter fractions were collected, and fractions containing the desired protein were identified through SDS-PAGE analysis of the individual fractions. Matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS) was used to confirm the identity of the MJ1388 gene product, as previously described (1). The protein concentration was determined by Bradford analysis (8).

**Measurement of the native molecular mass of SIHH.** The native molecular mass of SIHH was determined by size exclusion chromatography as described previously, using a Superose 12 HR column (9) with the following standards: blue dextran (2,000 kDa), alcohol dehydrogenase (150 kDa), conalbumin (77.5 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (14 kDa).

**Initial characterization of the SIHH-catalyzed reaction.** All assays were done in a 100-μl reaction volume with the final concentrations of reagents indicated. The linear dependence of the reaction rate on the amount of enzyme was determined by incubating various amounts of SIHH (0 to 11 μg) with 0.2 mM L-homocysteine and inosine in 25 mM Tris buffer, pH 7.5, for 10 min at 70°C and measuring the amount of SIH produced. The effect of temperature on SIHH (5.5 μg) activity was tested at 37, 70, and 80°C by preincubating SIHH at each temperature for 10 min in 25 mM Tris, pH 7.5, prior to adding 0.2 mM inosine and 0.2 mM L-homocysteine, followed by incubation of the reaction mixture at each fixed temperature for another 10 min. The results for the preincubated SIHH assay mixtures were compared with those for control assays (no

FIG 1 The first step in recycling SAM-derived enzymatic products in *M. jannaschii.*
Shimadzu HPLC system equipped with a photodiode array detector and a centrifugation (7,500 g) reactions were stopped with the addition of 5 mM for the inosine-containing compounds using an ε at 16.8 min, SAH at 18.8 min, and adenosine at 21.8 min. Quantitation of temperature activation through the comparison of enzymatic activities with and without preincubation at the indicated temperatures. A time course of the SIHH-catalyzed condensation of 0.2 mM inosine and 0.2 mM L-homocysteine was performed at 70°C in 25 mM Tris, pH 7.5, using 5.5 μM of enzyme in a 500-μl assay volume. Aliquots (100 μl) were removed after 0, 10, 20, 30, and 40 min and assayed for SIH formation.

After about 2 weeks of daily freeze-thaw cycles (20°C to room temperature [RT]) of the purified SIHH-containing solution, it was found that SIHH became less active. The addition of a final concentration of 20 mM DTT to the solution was found to fully restore the activity of the enzyme, and therefore, DTT was included in the assay mixture.

The specificity of the enzyme for the D versus the L configuration of homocysteine was determined by incubating SIHH with 0.2 mM inosine and either 0.2 mM Dl-homocysteine or 0.2 mM L-homocysteine (Hcy) in the standard assay. The pH optimum of SIHH (1.1 μg) was determined over the pH range of 6.5 to 11.5, using 50 mM sodium phosphate buffer in 0.5-pH-unit increments for both directions of the reaction. In the synthesis direction, the assay mixture contained 0.2 mM inosine, 0.2 mM L-homocysteine, and 20 mM DTT, and in the hydrolysis direction, the assay mixture contained 0.2 mM SIH, 0.23 mM NAD⁺, and 20 mM DTT. All reactions were stopped with the addition of 5 μl 2 M HCl, and after a brief centrifugation (−7,500 × g; 2 min), the supernatant was analyzed by HPLC.

The HPLC analyses of substrates and products were performed on a Shimadzu HPLC system equipped with a photodiode array detector and a C₁₈ reverse-phase column (Kromasil; 250 by 4.6 mm; 5-μm particle size) operated at RT. The elution profile consisted of 5 min at 95% sodium acetate buffer (25 mM; pH 6.0; 0.02% NaN₃) and 5% methanol followed by a linear gradient to 50% sodium acetate buffer-50% methanol over 25 min at 0.5 ml/min. Under these conditions, SIH eluted at 15.3 min, inosine at 16.8 min, SAH at 18.8 min, and adenosine at 21.8 min. Quantitation was based on absorbance at 260 nm for the adenosine-containing and 248 nm for the inosine-containing compounds using an ε of 14,900 M⁻¹ cm⁻¹ for adenosine and an ε of 12,300 M⁻¹ cm⁻¹ for inosine (10). Quantitation was based on peak areas.

**Standard assays for SIHH activity.** The standard enzymatic assay used to measure the synthesis of SIH contained 1.1 μg SIHH, 0.2 mM inosine, 0.2 mM Hcy, and 20 mM DTT in a 100-μl total volume of 50 mM sodium phosphate buffer (pH 7.0). The reaction mixtures were incubated at 70°C for 20 min, and the reactions were stopped with the addition of 5 μl 2 M HCl. After brief centrifugation (−2 min; −7,500 × g), the samples were analyzed by HPLC. The standard assay for measurement of the hydrolysis of SIH is the same as that described here, but with the addition of 0.23 mM NAD⁺.

**Determining the substrate specificity of SIHH.** The activity of SIHH for the synthesis of SIH was tested with either inosine or adenosine and Hcy using equal molar concentrations of adenosine or inosine and Hcy (each at 0.2 mM) with 1.1 μg of SIHH, 20 mM DTT in a 100-μl total volume of 50 mM sodium phosphate buffer, pH 7.0, with 0.23 mM NAD⁺ when added. The assay mixture was then incubated at 70°C for 20 min, followed by addition of 5 μl 2 M HCl to stop the reaction and by a brief centrifugation (−2 min; −7,500 × g) before HPLC analysis.

The hydrolysis of SIH or SAH was tested under various conditions (see Table 2). The equilibrium constant between SAH, adenosine, and Hcy for the synthesis of SIH has been previously reported to favor the synthesis of SAH in vitro, and as a result, the hydrolysis of SAH could be observed only upon the addition of adenosine deaminase to remove the adenosine (2). The removal of adenosine was required in order to measure the hydrolysis of SAH; however, for SIHH, the hydrolysis of SIH could be monitored without the removal of inosine. The assay was performed with a final concentration of 0.1 mM SIH or SAH, 1.1 μg SIHH, 20 mM DTT, with or without 0.1 mM NAD⁺, in a 100-μl total volume of 50 mM sodium phosphate buffer at pH 7.0. The assay mixture was incubated at 70°C for 20 min, and the assay was stopped with the addition of 5 μl 2 M HCl, followed by brief centrifugation, and then analyzed for adenosine, SAH, inosine, and SIH by HPLC.

![Diagram of Purine Metabolism](http://jb.asm.org/Downloadedfrom)
Determination of the kinetic parameters for SIHH. The kinetic parameters of the SIHH-catalyzed condensation of inosine with Hcy were determined by using the standard assay parameters while varying the concentrations of inosine (0, 0.2, 0.4, 0.6, and 0.8 mM) at 0.04, 0.06, and 0.08 mM Hcy. The kinetics of SIH hydrolysis was determined with an excess of NAD\(^+\)/NADH (0.23 mM) and 20 mM DTT in 100 mM 50 mM sodium phosphate buffer, pH 7.0, and concentrations of SIH from 0, 0.05, 0.07, 0.1, and 0.2 mM. The kinetic experiment mixtures were then incubated at 70°C for 20 min, followed by 5 ml 2 M HCl and brief centrifugation (7,500 g; 2 min), and then analyzed by HPLC.

Determination of the amount of NAD\(^+\)/NADH bound to SIHH. In an effort to determine the amount of NAD\(^+\) or NADH that was bound to the purified SIHH, the methods described by Yuan and Borchardt (11) were used. To 400 ml of the MonoQ fraction containing 1.4 mg of SIHH, 1.2 ml 97% ethanol was added to precipitate the protein, which was pelleted by centrifugation (7,500 g; 10 to 15 min). The supernatant was evaporated to dryness with a stream of N\(_2\) gas while being heated in a water bath of 100°C. The resulting residue was dissolved in 200 ml water and analyzed by HPLC as described above. Known concentrations of NAD\(^+\), eluting at 13.3 min with a \(A_{\text{max}}\) of 260 nm, and NADH, eluting at 14.3 min with a \(A_{\text{max}}\) of 340 nm, were used to determine the ratio of NAD\(^+\) and/or NADH that was bound to the recombinantly expressed and purified SIHH.

Preparation of apo-SIHH. The generation of apo-SIHH was done by modifying the methods outlined by Yuan et al. (12). Saturated ammonium sulfate (2 ml) was gradually added to 100 ml of a stirred solution of 1.4-mg/ml SIHH in 25 mM Tris, 0.45 M NaCl buffer (pH 7.5) from precipitation, and stirring continued at RT for 30 min. After centrifugation (8,100 g for 10 min), the supernatant was removed and the precipitated protein was redissolved in 100 ml of 25 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA and 5 mM DTT, and the above-mentioned steps were repeated. Following centrifugation, the protein was redissolved in 100 ml of 25 mM potassium phosphate buffer, pH 7.2. The amount of protein recovered was determined by Bradford analysis (8).

Activity of the generated apo-SIHH for SIH synthesis was performed by incubating 0.2 mM inosine, 0.2 mM Hcy, 0.42 g apo-SIHH, or untreated SIHH with and without 0.2 mM NAD\(^+\) in a 100-ml volume of 50 mM sodium phosphate buffer, pH 7.0, with 20 mM DTT, and the above-mentioned steps were repeated. Following centrifugation, the product was reanalyzed with 0.1 mM of SIH, pH 7.2, containing 1 mM DTT. The amount of protein recovered was determined by Bradford analysis (8).

Activity of the generated apo-SIHH for SIH hydrolysis was performed under the same conditions but with replacement of the 0.2 mM inosine and 0.2 mM Hcy with 0.1 mM SIH. The reactions were stopped with the addition of 5 ml 2 M HCl, followed by brief centrifugation and analysis by HPLC.

RESULTS

Cloning, overexpression, and purification of the M. jannaschii MJ1388 gene product in E. coli. The MJ1388-derived protein was efficiently expressed in E. coli, as measured from the SDS-PAGE analysis of the total proteins present in the E. coli cells after expression. Single new band, with a molecular mass of 46 kDa, that was not observed in the control cells was identified. MALDI-MS analysis of the tryptic peptides derived from this band showed five

FIG 3 Sequence alignment of SIHH with homologues. The residues highlighted in blue are those involved in substrate binding, those highlighted in green are involved in binding the NAD\(^+\), and the boldface residues are not highly conserved for either substrate or NAD\(^+\) binding. The general base histidine is indicated by an asterisk, and the threonines implicated in phosphorylation are marked by red brackets. The UniProt accession numbers for the sequence alignment are as follows (in order from the top down): Q58783, M. jannaschii; CP7826, Methanocaldococcus fervens; O27673, Methanothermobacter thermotrophicus; Q6LYR8, Methanococcus maripaludis; C9RID4, Methanocaldococcus vulgaris; O28279, Archaeoglobus fulgidus; Q4IAZ7, Sulfolobus acidocaldarius; PS0251, Pyrococcus furiosus; O67240, Aquifex aeolicus; P9WG33, Mycobacterium tuberculosis (PDB 2ZIZ); Q4Q124, Leishmania major (PDB 3G1U); and P23526, Homo sapiens (PDB 3NJ4).
unique peptides expected to arise from SIHH, confirming the presence of the desired protein in the band. After sonication and centrifugation of the *E. coli* cells, SDS-PAGE analysis of the soluble and insoluble material demonstrated that most of the expressed MJ1388 protein was found in the soluble extract. Heating portions of the resulting crude soluble extract at different temperatures indicated that SIHH remained soluble up to 80°C based on SDS-PAGE analysis. Thus, the first step in purification of the native enzyme was heating of the sonicated cell extract to 80°C for 20 min prior to purification of the SIHH on MonoQ. This separation produced one peak of MJ1388 activity eluting at 0.4 M NaCl.

**MALDI-MS analysis of the tryptic peptides showed four of the same five unique peptides reported above from analysis of the excised band of the SDS-PAGE of the crude extract of SIHH.**

**Native molecular mass of SIHH.** The monomeric molecular mass of SIHH was measured as 49 kDa by SDS-PAGE analysis, agreeing with the predicted molecular mass of 46 kDa. Using size exclusion chromatography, two peaks were identified that contained SIHH on the basis of their elution volumes; the major peak was the tetrameric form, and the minor peak was the dimeric form of SIHH. The ratio of the tetramer form to the dimer form of SIHH was 4:1 based on their 280-nm absorbance.

**Initial characterization of the SIHH-catalyzed reaction.** SIHH was 25% more active in 50 mM sodium phosphate buffer, pH 7.5, than in 25 mM Tris buffer, pH 7.5. Phosphate buffer was tested because it has been shown that the presence of phosphate can have an effect on SAHH activity and it is the buffer most often used in characterizing SAHH in other organisms (13). SIHH activity was activated when SIHH was incubated for 10 min at 70°C prior to being assayed. The activity of SIHH without preincubation retained only 35% of the activity of preincubated SIHH (Table 1). This indicates that preincubation at 70°C increased the observed activity. SIHH activity at 37°C and 80°C after preincubation for 10 min versus assay at 37°C and 80°C alone demonstrated minimal change compared with the SIHH activity following preincubation at 70°C (Table 1). These results suggest that 70°C is the optimal temperature to measure SIHH activity. A time course of the enzymatic activity at 70°C was measured and showed a linear increase in product formation up to 20 min.

SIHH did show a slight preference for L-homocysteine in the synthesis direction; however, it retained 94% activity when assayed with the same concentration of D,L-homocysteine. This indicates that the D isomer present in the D,L-homocysteine is not a strong inhibitor of SIHH. SIHH had no measurable activity at either pH 6.5 or pH 11 and showed maximal activity at pH 7.0 to 7.5 and pH 9.6 (Fig. 4, squares). The activity of SIHH in the hydrolysis direction showed essentially the same activity over the same pH range (Fig. 4, triangles).

**Substrate specificity of SIHH.** The activity of SIHH was found to be specific for Hcy and inosine versus Hcy and adenosine. No activity was observed for the synthesis of SAH from adenosine and Hcy. SIHH was found to use only Hcy and inosine as substrates, and the addition of excess NAD⁺ (0.2 mM) increased enzymatic activity by ~2-fold (Table 2). Hydrolysis of SAH with and without the addition of excess NAD⁺ in the presence of SIHH was not successful. However, the hydrolysis of SIH was dependent upon the addition of excess NAD⁺ to the assay mixture (Table 2). At this point, we are unable to explain why the addition of excess NAD⁺ allowed for the hydrolysis of SIH.

**Determination of the kinetic parameters of SIHH.** Our kinetic data are summarized in Table 3. During the analysis of the kinetic constants, it was found that at high concentrations of Hcy

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**FIG 4** pH profile of SIHH activity. The squares represent the pH profile in the synthesis of SIH, and the triangles represent the pH profile in the hydrolysis of SIH in the presence of 0.23 mM NAD⁺.
TABLE 2 Comparison of activities of apo-SIHH and holo-SIHH with and without excess NAD$^+$

<table>
<thead>
<tr>
<th>Reaction and condition</th>
<th>Substrate</th>
<th>Apo-SIHH</th>
<th>Holo-SIHH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesis of SIH$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No NAD$^+$</td>
<td>0.63</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>NAD$^+$ (0.2 mM)</td>
<td>0.63</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Hydrolysis of SIH$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No NAD$^+$</td>
<td>0.33</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>NAD$^+$ (0.1 mM)</td>
<td>0.33</td>
<td>0.55</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Each sample contained 0.4 μg of SIHH. ND, no detectable activity.

$^b$ Incubated with 0.2 mM inosine and 0.2 mM Hcy.

TABLE 3 Kinetic parameters of SIHH

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (±SD) (mM)</th>
<th>$V_{max}$ (±SD) (s$^{-1}$)</th>
<th>$K_{cat}$/$K_m$ (s$^{-1}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inosine$^a$</td>
<td>0.64 ± 0.4</td>
<td>0.83 ± 0.21</td>
<td>1.3 $\times$ 10$^4$</td>
</tr>
<tr>
<td>Hcy$^b$</td>
<td>3.5 $\times$ 10$^{-3}$ ± 0.006</td>
<td>0.83 ± 0.21</td>
<td>1.52 $\times$ 10$^5$</td>
</tr>
<tr>
<td>SIH$^c$</td>
<td>0.22 ± 0.11</td>
<td>0.42 ± 0.06</td>
<td>1.9 $\times$ 10$^3$</td>
</tr>
</tbody>
</table>

$^a$ Determined without the addition of NAD$^+$ to the assay mixture.

$^b$ Determined in the presence of 0.23 mM NAD$^+$.

Synthesis of SIH to be 2.5 times SAH. There are two established pathways to prevent inhibition of the methyltransferase reactions by SAH. In the first pathway, found only in Bacteria, a methylthioadenosine/S-adenosylhomocysteine nucleosidase cleaves the nucleotide to produce adenosine and S-ribosylhomocysteine (5, 20, 21). S-ribosylhomocysteine is recycled by an S-ribosemiocysteine lyase to produce homocysteine and 4,5-dihydroxy-2,3-pentanedione (5, 20, 21). 4,5-Dihydroxy-2,3-pentanedione is an important precursor for autoinducer-2, which is involved in quorum sensing (5). In the second pathway, an SAHH hydrolizes off the nucleoside to produce homocysteine and adenosine (5, 20) (Fig. 2A). SAHH homologues are almost ubiquitously found in Eukarya and Archaea, leading to the conclusion that the direct conversion of SAH to Hcy occurs in these organisms (5).

M. jannaschii is a strict anaerobic methanogenic archaeal organism that is deeply rooted on the phylogenetic tree and has been a source for the identification of novel pathways and enzymes involved in its metabolism (22–24). The establishment of these pathways and the enzymes involved has provided insight into the biochemical reactions that were occurring when life first originated. Annotation of an SAHH homologue in the M. jannaschii genome would indicate direct hydrolysis of SAH to Hcy and adenosine; however, we have recently shown that a different pathway must proceed due to the lack of an adenosine deaminase in methanogens. In M. jannaschii, DadD was found to deaminate SAH to SIH with a $k_{cat}$/$K_m$ of $4.4 \times 10^6$ M$^{-1}$ s$^{-1}$ (1) (Fig. 1). SIH is not a typical metabolite and has been identified only in Streptomyces flocculus (25). Here, we show that the annotated SAHH in M. jannaschii (MJ1388) does not accept SAH as a substrate and instead exclusively uses SIH. In this reaction, SIH is hydrolyzed to Hcy and inosine for SAM recycling and purine metabolism (Fig. 2B). This suggests that SIH is a central metabolite for SAM recycling in M. jannaschii.

SIHH is the first of two enzymes we have identified that are responsible for recycling the Hcy moeity of SAH into methionine. The Hcy generated is then methylated to form methionine (26, 27). The C-terminal portion of the cobalamin-independent methionine synthase (MetE) is encoded in M. jannaschii (MJ1473), and the activity of the homologous enzyme from Methanobacterium thermautotrophicus has been demonstrated (28) (Fig. 2). Therefore, the Hcy generated from SIHH can be methylated to generate methionine.

Our characterization of M. jannaschii SIHH indicates that its mechanism is the same as the established mechanism for SAHH. Here, NAD$^+$ oxidizes the 3’ hydroxyl to a carbonyl, and the active-site base then abstracts the C-4’ hydrogen, eliminating Hcy. Conjugate addition of hydroxide to the resulting α,β-unsaturated ketone, followed by rearrangement and protonation of the enolate ion, generates the 3’-keto compound that is reduced by the NADH to inosine (16, 29–32). The general base has been established in Mycobacterium tuberculosis as H363 (H293 in M. jannaschii) (30), which has been found to be highly conserved (Fig. 3, July 2015 Volume 197 Number 14 Journal of Bacteriology
In addition to conservation of the general base, other residues involved in substrate and cofactor binding were found to be conserved (Fig. 3), further supporting the idea that the mechanism is the same for SIHH and SAHH. Sequence alignments of SIHH and SAHHS show that the active-site residues involved with binding of the substrate are highly conserved (Fig. 3, blue). As a result, there is no clear indication as to what residues may be involved in causing SIHH to be specific for SIH rather than SAH. Further studies of the active site to determine what residues confer the differences in specificity for SIH versus SAH would be interesting.

Interestingly, a group of three threonine residues (T159 to T161 [M. jannaschii numbering]) involved in cofactor binding are also highly conserved (Fig. 3, red brackets), and in M. tuberculosis SAHH, the conserved threonines were identified as a site of posttranslational modification by phosphorylation and were required for enzymatic activity (33). Phosphorylation of the conserved threonines, in the NAD\(^+\) binding pocket, generated an inactive enzyme by preventing NAD\(^+\)\ binding (33). The conservation of these threonines could indicate a site of posttranslational modification by phosphorylation in M. jannaschii.

In the characterization of SIHH, we found that there are two pH maxima for activity in the synthesis of SIH activity (Fig. 4, squares). The pH profile for the hydrolysis of SIH did not show a clear optimum and instead showed activity across the whole pH range tested (Fig. 4, triangles). The SAHH from *Plasmodium falciparum* and human enzymes for the synthesis of SAH activity were found to have maximal activity at approximately pH 7.5 (4); however, the maximal pH was found to be between 8.0 and 10.5 for both the synthesis and hydrolysis of SAH for the SAHH from *Alicicilgenes fœcæalis* (34). Interestingly, rat liver SAHH was found to have two different pH optima, depending on the direction of the reaction: the hydrolysis of SAH had a pH optimum between 6.4 and 7.2, and the synthesis of SAH had an optimum pH above 7.8 (35).

Identification of SIHH in *M. jannaschii* represents a reoccurring theme in *M. jannaschii* purine salvage pathways. Salvage of the nitrogen-rich purine ring, in this case hypoxanthine (HX), is carried out by several enzymes in *M. jannaschii*: DaD, which deaminates adenosine, 5′-deoxyadenosine, and methylthioadenosine; SAH (1) (Fig. 1), an adenine deaminase that deaminates adenine to HX (D. Miller, unpublished data); a purine nucleoside phosphorylase that phosphorylates the HX base from methylthioinosine, 5′-deoxyinosine, inosine, and 2′-deoxyinosine (Miller, unpublished); and now SIHH, which hydrolyzes SIH to produce inosine and Hcy. Recently, Armenta-Medina et al. have postulated that inosine predated guanosine as the original base (36), which is supported by (i) the widespread conservation of the *de novo* IMP-biosynthetic pathway in all three domains of life (36), (ii) an RNA polymerase II from *E. coli* that retains activity with IMP (37), and (iii) an acetyl-coenzyme A (CoA) synthetase (ADP forming) from *Pyrococcus furiosus* that is 2-fold and 3-fold more active with IDP and ITP as substrates than with ADP and ATP, respectively (38). Taken together, these data suggest that HX-containing nucleosides and nucleotides potentially predated guanine-containing nucleotides and nucleosides. This is also supported by the annotated adenine phosphoribosyltransferase (encoded by MJ1655) and its homologues from *M. thermautrotrophicus* and *Methanococcus voltae*, which were all demonstrated to be specific for HX and guanine, showing no activity toward adenine (39, 40; Miller, unpublished). The presence of these enzymes demonstrates that *M. jannaschii*, and possibly other methanogens, is unable to use adenine for the salvage of purines and require conversion to HX prior to salvage of the nitrogen-rich purine ring.

In summary, we demonstrate that SIHH in *M. jannaschii* is required for the recycling of SAH produced from SAM-dependent methyltransferases in a four-step process (Fig. 2B) versus the canonical three-step pathway (Fig. 2A). This is only the second report of SIH serving as a metabolite, which likely represents a primitive pathway for SAM recycling in methanogens.

**ACKNOWLEDGMENTS**

We thank W. Keith Ray for performing the mass spectrometry experiments and Kylie Allen for her assistance in editing the manuscript.

The mass spectrometry resources are maintained by the Virginia Tech Mass Spectrometry Incubator, a facility operated in part through funding by the Fralin Life Science Institute at Virginia Tech and by the Agricultural Experiment Station Hatch Program (CRIS Project number VA-135981). This work was supported by National Science Foundation grant MCB1120346. Funding for this work was also provided, in part, by the Virginia Agricultural Experiment Station and the Hatch Program of the National Institute of Food and Agriculture, U.S. Department of Agriculture.

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