Biosynthesis of 4-Thiouridine in tRNA in the Methanogenic Archaeon Methanococcus maripaludis†§

Received for publication, July 27, 2012, and in revised form, August 16, 2012. Published, JBC Papers in Press, August 17, 2012, DOI 10.1074/jbc.M112.405688

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Background: Bacterial ThiI catalyzes 4-thiouridine biosynthesis by using a rhodanese-like domain for sulfur transfer.

Results: ThiI in methanogenic archaea employs a conserved CXXC motif to generate persulfide and disulfide intermediates for sulfur transfer.

Conclusion: Methanogens possess a unique sulfur relay strategy.

Significance: Sulfur metabolism in methanogens is a model for the evolution of sulfur metabolism in the anaerobic sulfide-rich environments common on ancient earth.

4-Thiouridine (s^4U) is a conserved modified nucleotide at position 8 of bacterial and archaeal tRNAs and plays a role in protecting cells from near-UV killing. *Escherichia coli* employs the following two enzymes for its synthesis: the cysteine desulfurase IscS, which forms a Cys persulfide enzyme adduct from free Cys; and ThiI, which adenylates U8 and transfers sulfur from IscS to form s^4U. The C-terminal rhodanese-like domain (RLD) of ThiI is responsible for the sulfurtransferase activity. The mechanism of s^4U biosynthesis in archaea is not known as many archaea lack cysteine desulfurase and an RLD of the putative ThiI. Using the methanogenic archaeon *Methanococcus maripaludis*, we show that deletion of ThiI (MMP1354) abolished the biosynthesis of s^4U but not of thiamine. MMP1354 complements an *Escherichia coli ΔthiI* mutant for s^4U formation, indicating that MMP1354 is sufficient for sulfur incorporation into s^4U. In the absence of an RLD, MMP1354 uses Cys and Cys located in the PP-loop pyrophosphatase domain to generate persulfide and disulfide intermediates for sulfur transfer. *In vitro* assays suggest that S^2- is a physiologically relevant sulfur donor for s^4U formation catalyzed by MMP1354 (K_m for Na_2S is ~1 mM). Thus, methanogenic archaea developed a strategy for sulfur incorporation into s^4U that differs from bacteria; this may be an adaptation to life in sulfide-rich environments.

The 4-thiouridine (s^4U) modification at position 8 of tRNAs is conserved in bacteria and archaea (1). In *Escherichia coli*, this modification was found in about 70% of the bulk tRNAs (2), and its level in some tRNA species varied depending on the growth rate (3). A *Salmonella typhimurium* mutant that lacked s^4U was more sensitive to broadband near-UV killing, suggesting that this modification protects the cells from near-UV light (4). In response to near-UV irradiation, s^4U in tRNAs serves as a photosensor (λ_max of s^4U is 334 nm) by cross-linking with the nearby cytosine at position 13 (5–7). These cross-linked tRNAs are poor substrates for aminoacylation (6, 8), leading to the accumulation of uncharged tRNAs (9). This effect mimics amino acid starvation and triggers the stringent response through the synthesis of ppGpp and AppppGpp, which inhibit cell growth and induce the expression of specific proteins that enhance cell survival after the stress (2, 4, 7, 10).

Two enzymes in bacteria are required for the biosynthesis of s^4U, the cysteine desulfurase IscS and ThiI (11–15). IscS is a pyridoxal 5'-phosphate-dependent enzyme that liberates sulfur from free cysteine, resulting in a cysteine persulfide in its active site and free alanine (16–18). This IscS persulfide then donates sulfur for the biosynthesis of s^4U, other thiolated nucleosides in tRNAs, and sulfur-containing cofactors (e.g. Fe-S clusters, thiamine, and molybdopterin) (14, 19–23). For the synthesis of s^4U, the IscS persulfide serves as a S^0 donor to generate a persulfide on ThiI, which catalyzes the adenylation of tRNA U8. This ThiI persulfide serves as the proximal sulfur donor for the thiolation of U8.

The *E. coli* ThiI contains four domains as follows: the N-terminal ferredoxin-like domain (NFLD); the thiouridine synthases, RNA methyltransferase, and pseudouridine synthases domain (THUMP domain); the PP-loop pyrophosphatase domain (PP-loop domain); and the C-terminal rhodanese-like domain (RLD). The NFLD and THUMP domains bind tRNA by recognizing the acceptor-stem region (24–26); the
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PP-loop domain adenylylates the 4-carbonyl group of tRNA U8 at the expense of ATP (27, 28); and the RLD transfers sulfur (29, 30). During the sulfur transfer, the catalytically essential Cys\(^{\text{256}}\) located in the RLD receives sulfur from IscS to form the Thi persulfide (29–31). After donation of the terminal sulfur of the Thi persulfide to tRNA U8, Cys\(^{\text{256}}\) forms a disulfide bond with Cys\(^{\text{244}}\) located in the PP-loop domain (30, 32, 33). Presumably, the Cys\(^{\text{256}}\)–Cys\(^{\text{244}}\) disulfide needs to be reduced to complete the enzymatic cycle. In *E. coli* and *Salmonella enterica*, the RLD of Thi also participates in the sulfur transfer for thiamine biosynthesis (34–36). However, Thi homologs in many other bacteria lack the C-terminal RLD and are not necessary for thiamine biosynthesis (37–39). A recent report demonstrated that the *Bacillus subtilis* Thi, which lacks the RLD, cooperates with a specialized cysteine desulfurase NifZ to synthesize s\(^4\)U (37). However, the participation of this Thi in sulfur transfer is still unclear.

The presence of s\(^4\)U in archaeal tRNAs has been demonstrated in *Thermoproteus neutrophilicus* (40) and a number of methanogenic archaea, including *Methanococcus vannielii*, *Methanococcus maripaludis*, *Methanothermococcus thermolithotrophicus*, *Methanocaldococcus igneus*, *Methanocaldococcus jannaschii*, and *Methanococcoides burtonii* (41–43). However, some fundamental questions remain about the mechanism of sulfur transfer to generate s\(^4\)U in archaea. First, the sulfur donor is not known. Many methanogens and the sulfur-dependent hyperthermophilic archaea do not encode a recognizable cysteine desulfurase in their genomes (44). The methanogens also use a RNA-dependent pathway for cysteine biosynthesis (45) and produce a much smaller pool of free cysteine in comparison with *E. coli* (46). These archaea use sulfide instead of cysteine as the sulfur source for Fe-S cluster and methionine biosynthesis (46). These results suggest that cysteine may also not serve as a sulfur source for s\(^4\)U biosynthesis. Second, although most archaea have Thi homologs, many homologs do not have the C-terminal RLD. Therefore, it is not known if Thi participates in sulfur transfer. In this study, we used *M. maripaludis*, an obligately anaerobic methane-producing archaean, as the model organism to study the biosynthesis of s\(^4\)U in archaea. The *M. maripaludis* Thi (MMP1354), which lacks RLD, is essential for s\(^4\)U but not for thiamine biosynthesis. Furthermore, a conserved CXXC motif located in the PP-loop domain is essential for both *in vitro* and *in vivo* activities of s\(^4\)U formation. These two cysteines form persulfide and disulfide intermediates during sulfur transfer. Finally, sulfide is a sufficient sulfur donor *in vitro*. Based upon these results, a model of s\(^4\)U biosynthesis in methanogenic archaea is proposed.

**EXPERIMENTAL PROCEDURES**

**Media and Culture Conditions of *M. maripaludis*—** *M. maripaludis* was grown in 28-ml aluminum sealed tubes with 275 kilopascals of H\(_2\)/CO\(_2\) (4:1, v/v) at 37 °C in 5 ml of MCNA (minimal medium + 10 mM sodium acetate, reduced with 3 mM l-cysteine), McNACoM (McNA reduced with 3 mM coenzyme M instead of cysteine), or McC (McNA + 0.2% (w/v) casamino acids + 0.2% (w/v) yeast extract) medium as described previously (47). The 100-ml cultures were grown in 1-liter bottles pressurized to 138 kilopascals with H\(_2\)/CO\(_2\) (4:1, v/v). Antibiotics were not included when comparing the growth of the wild-type and mutants. The inocula were 0.1 ml of cultures (~10\(^6\) cells) grown in McNA or McNACoM medium. Puromycin (2.5 μg/ml) or neomycin (500 μg/ml in plates and 1 mg/ml in broth) was added when needed. Before inoculation, 3 ml of sodium sulfide was added as the sulfur source. When grown with elemental sulfur as the sulfur source, 0.1 g of S\(_0\) was added to 5 ml of medium before autoclaving.

**Mutagenesis of mmp1354 in *M. maripaludis*—** The replacement of the *thil* gene (mmp1354) with a puromycin resistance cassette was made by transformation of the wild-type *M. maripaludis* strain S2 with pJ1A03-mmp1354, which was constructed from the integration vector pJ1A03 as described (48–50). The transformants were plated on McC medium plus puromycin. Puromycin-resistant isolates were restreaked on the same medium, and isolated colonies were then transferred to broth cultures containing 5 ml of McC medium plus puromycin. The genotype of the Δmmp1354 mutant (S620) was confirmed by Southern hybridization (data not shown).

For complementation of S620, the mmp1354 gene was cloned into the shuttle vector pMEV2 (49), and the resulting plasmid, pMEV2-mmp1354, was transformed into S620. The transformants were plated on McC medium plus puromycin and neomycin. The complemented strain expressing the wild-type MMP1354 was named S624. The mutations of MMP1354 (including C78A, C265A, C268A, and C348A), were constructed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA).

**Cloning, Expression, and Purification of Recombinant MMP1354—** The *thil* gene from *M. maripaludis* (mmp1354) was cloned into the vector pQE2 (Qiagen), which introduced an N-terminal His\(_6\) affinity tag. The mutations of MMP1354, including four single mutants (C78A, C265A, C268A, and C348A), one double mutant (C265A/C268A), and one triple mutant (C265A/C268A/C348A), were constructed using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA).

The resulting plasmids were individually transformed into the *E. coli* SG13009[pREP4] strain (Qiagen) for expression of recombinant proteins. The transformed cells were grown in 1 liter of Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin at 37 °C with shaking until they reached an absorbance at 600 nm of 0.6–0.8. Isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 mM to induce overnight production of recombinant proteins at 25 °C. For anaerobic protein purification, the harvested *E. coli* cells were transferred into the anaerobic chamber (atmosphere of 95% N\(_2\) and 5% H\(_2\)) and resuspended in 20 ml of buffer A (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole (pH 7.4)). The cells were disrupted by addition of 2 ml of BugBuster (Novagen). RNA and DNA were digested with 10 μl of benzonase (Sigma) by incubation at room temperature for 30 min. The cell lysate was then centrifuged at 100,000 × g for 30 min at 4 °C. The supernatant was applied to 1 ml of TALON metal affinity resin (Clontech) equilibrated with buffer A. Proteins bound to the column were eluted with 10 ml of buffer B (20 mM sodium phosphate, 0.5 M NaCl, 200 mM imidazole (pH 7.4)). The elution fractions of the desired proteins were analyzed by SDS-PAGE, dialyzed against buffer C.
(50 mM of HEPES-NaOH, 150 mM KCl, 10 mM MgCl₂, 40% (v/v) glycerol (pH 7.0)), concentrated with a 30-kDa cutoff centrifugal filter (Millipore), and stored at −80 °C until use. Protein concentrations were determined with the bicinchoninic acid assay (51). No spectroscopic features indicative of Fe-5 clusters were present.

Complementation of the E. coli ΔthiI Mutant with mmp1354—The E. coli ΔthiI mutant strain JW0413 (from the Keio collection (52)) was transformed with the plasmid pQE2-mmp1354. The transformants were grown in LB or M9 medium supplemented with 1 mM thiamine in the presence of 100 µg/ml ampicillin and 25 µg/ml kanamycin. The expression of MMP1354 in the complemented strain (JW0413-MMP1354) was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside and confirmed by Western blotting with the monoclonal mouse anti-His tag antibody (Sigma).

Isolation, Digestion, and Nucleoside Analysis of total tRNAs—The total tRNAs were isolated from M. maripaludis by a modification of the method of Gupta (53). M. maripaludis strains were grown in 200 ml of MCC medium to an absorbance of ~1.0 at 600 nm. The cells were collected by centrifugation at 10,000 × g for 30 min at 4 °C and suspended in 0.5 ml of 10 mM Tris-HCl buffer (pH 7.7). Then the cell suspension was mixed with 0.5 ml of phenol and centrifuged at 16,000 × g for 10 min at 4 °C. The aqueous phase was transferred to a new microcentrifuge tube and mixed with 0.1 volume of cold 20% (w/v) potassium acetate (pH 5.0). The nucleic acids were precipitated with 2 volumes of ethanol at −80 °C for 30 min and collected by centrifugation. Low molecular weight nucleic acids were extracted twice from the pellet with 0.1 ml of cold 1 M NaCl, precipitated again with 2 volumes of ethanol at −80 °C for 30 min, and collected by centrifugation. The pellet was dissolved in 0.1 ml of the buffer containing 10 mM Tris-HCl (pH 7.5) and 5 mM MgCl₂ and incubated with 10 units of RNase-free DNase (New England Biolabs) for 30 min at 37 °C. The solution was then extracted with 1 ml of chloroform, and the tRNAs were precipitated with 0.1 volume of cold 20% (w/v) potassium acetate (pH 5.0) and 2 volumes of ethanol at −80 °C for 30 min and collected by centrifugation. The pellet was dissolved in 0.4 ml of 0.5 M Tris-HCl buffer (pH 8.8) and incubated for 1 h at 37 °C to deacylate aminoacyl-tRNAs. The tRNAs were precipitated again as described above. The pellet was dissolved in 0.5 ml of 0.3 M sodium acetate (pH 7.0) on ice, followed by dropwise addition of 0.27 ml of cold isopropyl alcohol. The mixture was raised to room temperature and centrifuged at 16,000 × g for 10 min at room temperature. The tRNAs in the supernatant were precipitated with dropwise addition of 0.98 volume of isopropyl alcohol and collected by centrifugation at room temperature. The tRNAs were rinsed with ethanol and dried under vacuum.

The tRNAs were digested by the method of Mesbah et al. (54). About 70–250 µg of tRNAs were dissolved in 70 µl of HPLC water, heated in a boiling water bath for 2 min, and placed immediately on ice. Then 5 µl of 0.3 M sodium acetate (pH 5.1), 5 µl of 20 mM zinc sulfate, and 1 unit of S1 nuclease (Sigma) were added to each sample. The samples were incubated at 37 °C for 2 h. The nucleic acids were then dephosphorylated with 1 unit of alkaline phosphatase (Sigma) in 10 mM glycine-HCl (pH 8.0–8.5) at 37 °C for 2 h.

The digested nucleic acids were analyzed with a Waters 2695 separation HPLC system. For each tRNA preparation, 90 µl of sample (20 µg of tRNA) were loaded onto an Altima C-18 reversed phase column and eluted with the buffer containing 20 mM triethylamine phosphate and 7.5% methanol (pH 5.1) at 30 °C. The nucleosides were monitored at 260 nm, and s₄U (A₅₃ at 334 nm) was monitored at 330 nm.

Determination of the Persulfide Content of MMP1354 by Fluorescent Labeling—The anaerobically purified protein (~1 nmol) in 100 µl of buffer C (50 mM HEPES-NaOH, 150 mM KCl, 10 mM MgCl₂ (pH 7.0)) was incubated anaerobically with 20 nmol of the fluorescent dye N-(iodocetaminoethyl)-1-naphthylthamine-5-sulfonic acid (1.5–1-AEDANS) at 37 °C for 30 min to derivatize thiol groups in the protein (55). The persulfide content of MMP1354 was then determined as described (56).

Sulfur Transfer Assay with Radioactive Sulfur—The procedure was carried out in the anaerobic chamber with an atmosphere of 95% N₂ and 5% H₂. The maltose-binding protein-tagged-IscS (5 µM) was incubated with MMP1354 (20 µM) in the presence of L-[³⁵S]cysteine (150 µCi) at 37 °C in the reaction buffer containing 50 mM HEPES-NaOH (pH 7.3), 150 mM KCl, 10 mM MgCl₂, and 10 µM pyridoxal 5'-phosphate for 30 min. The reaction was stopped by addition of nonreducing SDS loading dye. The protein mixture was then separated by SDS-PAGE, and the radioactivity retained on the gel was followed by autoradiography.

Identification of the Persulfide Modification on MMP1354 by Mass Spectrometry—The anaerobically purified recombinant MMP1354 (50 µM) in the 0.1 mM ammonium bicarbonate/formic acid buffer (pH 7.2) was digested with 20 µg/ml trypsin (Prozyma) at 37 °C overnight. The digestion was stopped with 1% (v/v) formic acid, and the samples were dried in vacuum. The peptides obtained from the trypsin digestion were analyzed with LC-MS/MS as described (56).

Assay of in Vitro Formation of s₄U—The M. jannaschii tRNA⁸⁰C⁸⁰ substate was synthesized in vitro 17-RNA polymerase run-off transcription as described (57). Before use, the tRNA transcripts were folded by heating at 80 °C for 5 min, cooling down slowly to 45 °C, and adding 5 mM MgCl₂ before placing on ice. The M. jannaschii substate was used because the M. maripaludis tRNA⁸⁰C⁸⁰ transcripts failed to fold properly (57).

The in vitro formation of s₄U was performed anaerobically. The tRNA⁸⁰C⁸⁰ transcripts (20 µM) were incubated with MMP1354 (50 nM) at 37 °C for 10–120 min in the buffer containing 50 mM HEPES-NaOH (pH 7.0), 150 mM KCl, 10 mM MgCl₂, and 2 mM ATP in the presence of 5 mM sodium sulfide, sodium thiosulfate, sodium thiophosphate, or L-cysteine as the sulfur donor. The reaction was stopped by addition of an equal volume of the formamide loading dye. The tRNA thiolation was then analyzed with the [(N-acryloylamo)phenyl]mercuric chloride (APM)-retardation gel (58). The thiolated and unmodified tRNA⁸⁰C⁸⁰ (2.5 µg per lane) were separated by electrophoresis in 12% urea-polyacrylamide gels supplemented with 9 µg/ml APM. The tRNAs were visualized by staining with 0.1% (w/v) toluidine in 40% (v/v) methanol and 1% (v/v) acetic acid.
RESULTS

Phylogenetic Distribution and Conserved Cys Residues of Archaeal ThiI—ThiI homologs are conserved in most archaea with completely sequenced genomes except some members of the Thaumarchaeota (Cenarchaeum symbiosum A, Nitrosopumilus spp., and Candidatus “Nitrosoarchaeum limnia”), the halarchaea (Haloquadratum walsbyi and Halorubrum lacsprofundi), and the Sulfolobales (Metallosphaera spp.). The NFLD, the THUMP domain, and the PP-loop domain are present in most archaeal ThiI homologs. However, the C-terminal rhodanese-like domain (RLD) is only present in the Thermoplasmatales and the Thermoproteales orders. The conserved PP-loop motif (SGGXDS) and Cys residues are indicated. Most ThiI homologs from methanogens contain three conserved Cys residues at the PP-loop domain, with two of them arranged in a CXXC motif and the third one corresponding to Cys344 of E. coli ThiI. The only exception is the M. burtonii ThiI, which does not have the third conserved Cys. The genomes of the Thermococcales orders usually encode multiple ThiI homologs. For example, Pyrococcus furiosus has three ThiI homologs (locus tags: PF1288, PF1487, and PF1835). PF1288 has the NFLD, THUMP domain, and the PP-loop domain with three conserved Cys; PF1487 has the same three domains but does not have the PP-loop motif and conserved Cys in the PP-loop domain; and PF1835 has only the PP-loop domain with three conserved Cys.

MMP1354 Is Required for s4U Formation but Not for Thiamine Biosynthesis in M. maripaludis—The physiological function of the M. maripaludis ThiI (MMP1354) was investigated by construction and characterization of a Δmmp1354 mutant (S620). This mutant had no growth defects in minimal medium in the absence of thiamine, suggesting that MMP1354 is not an essential enzyme under the tested growth conditions.

The involvement of MMP1354 in s4U biosynthesis was examined by reverse phase HPLC analysis of the nucleosides derived from total tRNA digestion. In wild-type cells cultivated with completely sequenced genomes except some members of the Thaumarchaeota (Cenarchaeum symbiosum A, Nitrosopumilus spp., and Candidatus “Nitrosoarchaeum limnia”), the halarchaea (Haloquadratum walsbyi and Halorubrum lacsprofundi), and the Sulfolobales (Metallosphaera spp.). The NFLD, the THUMP domain, and the PP-loop domain are present in most archaeal ThiI homologs. However, the C-terminal rhodanese-like domain (RLD) is only present in the Thermoplasmatales and the Thermoproteales orders. The conserved PP-loop motif (SGGXDS) and Cys residues are indicated. Most ThiI homologs from methanogens contain three conserved Cys residues at the PP-loop domain, with two of them arranged in a CXXC motif and the third one corresponding to Cys344 of E. coli ThiI. The only exception is the M. burtonii ThiI, which does not have the third conserved Cys. The genomes of the Thermococcales orders usually encode multiple ThiI homologs. For example, Pyrococcus furiosus has three ThiI homologs (locus tags: PF1288, PF1487, and PF1835). PF1288 has the NFLD, THUMP domain, and the PP-loop domain with three conserved Cys; PF1487 has the same three domains but does not have the PP-loop motif and conserved Cys in the PP-loop domain; and PF1835 has only the PP-loop domain with three conserved Cys.

MMP1354 Complements an E. coli ΔthiI Mutant for s4U Formation—To determine whether archaeal ThiI without the RLD is functional in E. coli, a complementation test of an E. coli ΔthiI mutant strain (JW0413) with mmp1354 was performed. The JW0413 mutant requires thiamine for growth and is unable to form s4U. Upon complementation, the strain expressing MMP1354 (JW0413-MMP1354) still required thiamine for growth (data not shown), suggesting that MMP1354 was unable to transfer sulfur for thiamine biosynthesis in E. coli. This observation agreed with the proposal that the RLD of ThiI is necessary and sufficient for thiamine biosynthesis (35). In contrast, strain JW0413-MMP1354 contained s4U in its tRNAs (Fig. 3), suggesting that MMP1354 without RLD was sufficient for s4U biosynthesis in E. coli.

Important Cys Residues of MMP1354 for In Vivo s4U Formation—To identify the Cys residues necessary for in vivo s4U formation in M. maripaludis, five of the nine cysteines of MMP1354 were individually altered to Ala. These mutated cysteines included Cys78, which is located in the THUMP domain and conserved in methanococci, and the three Cys residues located in the PP-loop domain and conserved among all methanogens, i.e., Cys265, Cys268, and Cys348. Although the tRNAs from M. maripaludis cells expressing the C78A or the C348A variant possessed only moderate decreases in their s4U levels, cells expressing the C265A or the C268A variant had dramatic decreases on the s4U level, containing ~10% of the wild-type level or no detectable amount of s4U, respectively (Table 1). These results indicated that Cys265 and Cys268 are important for the in vivo activity of s4U biosynthesis and that Cys78 and Cys348 have minor contributions.

MMP1354 Forms a Persulfide Enzyme Adduct—The E. coli ThiI forms a persulfide intermediate on the essential Cys456 during sulfur transfer (29–33). Because both Cys265 and Cys268 were important for MMP1354 activity, three methods were applied to investigate whether MMP1354 also forms a persulfide intermediate.
with 100-fold excess of DTT. If persulfide is present in the protein, the fluorophore would be released from the protein into solution upon reduction with DTT. In three independent treatments of 1 nmol of wild-type protein, 87 ± 2 pmol of the fluorophore were released (Table 2). This suggests that ~9% of the purified protein contained persulfide if only one Cys residue forms persulfide per protein molecule. The C78A variant had similar amounts of persulfide as the wild-type protein, whereas the C265A, C268A, and C348A variants contained no detectable amount of persulfide (Table 2). This result suggests that Cys265, Cys268, and Cys348 are essential for the generation or stabilization of the persulfide in MMP1354.

Second, the generation of persulfide by MMP1354 was followed by a sulfur transfer assay with [35S]Cys as the sulfur source (31). Anaerobically purified recombinant MMP1354 was incubated with [35S]Cys in the absence or presence of the maltose-binding protein-tagged E. coli IscS. The protein(s) was then subjected to SDS-PAGE analysis under nonreducing conditions, and the sulfur transfer from [35S]Cys to protein(s) was monitored by autoradiography. MMP1354 was not labeled with [35S]Cys alone, demonstrating the absence of cysteine desulfurase activity (lane 2 of Fig. 4). However, when wild-type MMP1354 together with IscS were incubated with [35S]Cys, both proteins were radiolabeled (lane 3 of Fig. 4), indicating that MMP1354 can accept [35S]S from IscS. Addition of the reducing agent β-mercaptoethanol (1%, v/v) to the protein mixture removed the radiolabel (lane 10 of Fig. 4), suggesting that [35S]S was attached to both proteins as persulfide. Although an IscS homolog is not encoded in M. maripaludis, this experiment suggested that a persulfide can be generated on MMP1354 with a persulfide sulfur donor. Alteration of Cys265, Cys268, or Cys348 to Ala abolished the [35S]S labeling of MMP1354 (lanes 4–9 of Fig. 4), which further confirmed that these three cysteines are essential for the generation or stabilization of the persulfide under in vitro conditions.

Third, the location of persulfide in MMP1354 was analyzed with mass spectrometry. The anaerobically purified recombinant protein was digested with trypsin, and the resulting peptides were then analyzed by LC-MS/MS (Fig. 5). For the peptide 36687, the precursor ions can be observed with −2 Da (mass of −2H) and +30 Da (mass of −2H + 1S) shift. Analysis of the MS/MS spectra of the −2 Da shifted precursor ion matched the expected amino acid sequence when the the-

### Table 1

| MMP1354 variant | Level of s4U (a) |
|-----------------|-----------------|
| Wild type       | 92 ± 6          |
| C78A            | 85 ± 7          |
| C265A           | 11 ± 5          |
| C268A           | ND              |
| C348A           | 62 ± 10         |

(a) The level of s4U in total tRNAs from wild-type M. maripaludis (strain S2) cells was set as 100%. The values are average ± S.D. from three independent cultures of the wild-type, C265A, and C268A variants and from five independent cultures of the C78A and C348A variants.

First, the amount of persulfide in the recombinant MMP1354 expressed in E. coli was measured with a fluorescent labeling assay (17). The protein was purified anaerobically in the absence of reducing agents; the free sulfhydryl groups of the purified protein were labeled with I-EDANS, a fluorescent derivative of iodoacetamide (55); after removal of unreacted fluorescent dye by centrifugal filtration, the protein was treated

![Image](image-url)

**FIGURE 2. Analysis of s4U in M. maripaludis tRNAs.** Total tRNAs were prepared and digested as described under “Experimental Procedures.” Nucleosides derived from tRNAs of the wild-type strain S2 (blue), of the Δmmp1354 strain S620 (red), of the complemented strain S624 (S620 complemented with MMP1354 expressed from a shuttle vector) (green), and 0.02 nmol of s4U standard (black) were analyzed by reverse phase HPLC and monitored at 260 nm (A) and 330 nm (B). The UV absorbance at 330 nm allows specific determination of s4U (λmax of s4U is 334 nm).

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**FIGURE 3. UV-visible spectra of E. coli tRNAs.** The tRNAs were isolated from the wild-type strain K12 (blue), the ΔthiI mutant strain JW0413 (green), and the complemented strain (JW0413 complemented with MMP1354) (red). The tRNAs of each sample were at the concentration of 10 μg/μl as determined with UV absorbance at 260 nm.

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![Image](image-url)
4-Thiouridine Biosynthesis in Methanococcus maripaludis

**TABLE 2**

| MMP1354 variant | Amount of persulfide | Amount of persulfide/mL of protein |
|-----------------|----------------------|-----------------------------------|
| Wild type       | 0.087 ± 0.002        |                                   |
| C78A            | 0.073 ± 0.005        |                                   |
| C265A           | ND                   |                                   |
| C268A           | ND                   |                                   |
| C348A           | ND                   |                                   |
| C265A/C268A     | ND                   |                                   |
| C265A/C268A/C348A | ND             |                                   |

* The values are the average ± S.D. from three independent measurements.

**FIGURE 4.** Sulfur transfer from the *E. coli* cysteine desulfurase IscS to MMP1354. The *E. coli* IscS (5 μM) lane 1, the wild-type MMP1354 (20 μM, lane 2), and the two proteins together (lane 3) were incubated with 150 μM [35S]Cys at 37°C for 30 min, and then the incubation mixtures were analyzed by SDS-PAGE under nonreducing conditions. The positions of maltose-binding protein-tagged IscS (~90 kDa) and His-tagged MMP1354 (~45 kDa) are labeled. Lanes 4–9, the *E. coli* IscS was incubated with the MMP1354 variants C78A, C265A, C268A, C348A, C265A/C268A, and C265A/C268A/C348A, respectively. Lane 10, the *E. coli* IscS and wild-type MMP1354 reaction mixture (as in lane 3) was analyzed by SDS-PAGE under reducing conditions with 1% (v/v) β-mercaptoethanol.

Theoretical masses were corrected with an intra-peptide disulfide between Cys265 and Cys268 (Fig. 5A). The fragmentation of the +30 Da shifted precursor ion matched the modification as a trisulfide linkage between Cys265 and Cys268 (Fig. 5B). This fragmentation pattern was similar to that observed for the *M. jannaschii* O-phosphoseryl-tRNA: Cys-tRNA synthase, which contained a trisulfide linkage in a CXXC motif, presumably resulting from an oxidation (loss of dihydrogen) of a Cys persulfide and a Cys thiol or from a reaction between two persulfide groups to expel a bisulfide (56). The trisulfide linkage was possibly formed either in vitro or during the protein purification and the mass spectrometry processes due to the instability of persulfide. No modification was observed for the peptide containing Cys348. Overall, the mass spectrometry analysis of MMP1354 suggests that Cys265 and Cys268 easily form a disulfide bond and one of them carries a sulfane sulfur in a proportion of the protein.

In Vitro Formation of s^{4}U by MMP1354 with Sulfide as the Sulfur Donor—The formation of s^{4}U by MMP1354 with different sulfur donors was tested using *M. jannaschii* tRNA as a substrate. As shown by APM-retardation gel analysis, only Na_{2}S resulted in significant thiolation of tRNA-Cys (Fig. 6A). The *K_{m*} for Na_{2}S was 1.0 ± 0.2 mM, which was within the range of intracellular free sulfide concentration in methanococci of 1–3 mM (59). This result suggests that MMP1354 has a much higher affinity for sulfide than *E. coli* Thil (*K_{m*} > 20 mM) (26), and sulfide is a physiologically relevant sulfur donor for s^{4}U biosynthesis in *M. maripaludis*. Furthermore, unlike the *E. coli* Thil that requires exogenous reductant for multiple turnovers (32), the addition of DTT inhibited the s^{4}U formation by MMP1354 (Fig. 6B). This result suggests that an oxidized form of MMP1354 (presumably with a disulfide) is important to initiate the catalysis and the sulfur donor is a S^{2−} equivalent. Alteration of Cys265, Cys268, or Cys348 to Ala abolished tRNA thiolation (Fig. 6C), suggesting that these three cysteines are required for the in vitro activity of MMP1354.

**DISCUSSION**

Proposed Model of s^{4}U Formation in M. maripaludis—The data presented here demonstrate that MMP1354 requires two conserved Cys residues (Cys265 and Cys268), which are arranged in a CXXC motif in the PP-loop domain, for both in vitro and in vivo activities to generate s^{4}U in tRNAs. Furthermore, both cysteines are essential for the formation of a persulfide enzyme adduct and also readily form a disulfide linkage, as identified by mass spectrometry. The recruitment of the CXXC motif for sulfur transfer resembles the sulfur relay mechanism of O-phosphoseryl-tRNA: Cys-tRNA synthase, which catalyzes the conversion of tRNA-bound O-phosphoserine to cysteine in methanogenic archaea (56). Based upon these findings, a model for catalysis by MMP1354 is proposed in Fig. 7. First, a sulfur donor (equivalent of S^{2−}) attacks the Cys265–Cys268 disulfide linkage to generate a persulfide on either Cys265 or Cys268, leaving the other Cys as a free thiol. Then, a thiolate derived from deprotonation of the free thiol attacks the bridging sulfur of the persulfide to liberate the terminal sulfur. After donation of the sulfur (in the −2 oxidation state) to form s^{4}U, the disulfide (with both sulfurs in the −1 oxidation state) is consequently regenerated. No exogenous electron donor or acceptor is required for the catalytic cycle.

The *in vivo* and *in vitro* characterization of MMP1354 yielded apparently contradictory results on the importance of Cys348, which is at an equivalent position as Cys344 in *E. coli* Thil. *In vivo*, the alteration of Cys348 to Ala had a moderate effect on the s^{4}U level, suggesting an important but not essential role. However, *in vitro* the C348A variant protein had no detectable activity for s^{4}U formation. The absence of this Cys in the homolog from *M. burtonii* supports the conclusion that this residue is nonessential. Possibly, Cys348 functions to stabilize the persulfide intermediate. Under *in vitro* conditions, the loss of this function could be replaced by some other components in the *M. maripaludis* cells or compensated for in some other fashion. Although this scenario could account for the discrepancy, further investigations are required to clarify the function of Cys348.

Structural Insights into the Sulfur Transfer by MMP1354—The structure model of MMP1354 based upon the structure of *B. anthracis Thil* in complex with AMP (24) supports the proposed model of sulfur transfer. First, Cys265 and Cys268 are located in the vicinity of the binding site of AMP (supplemental Fig. S2A). In addition, Cys268 is adjacent to a highly conserved Lys or Arg residue (Arg265 of *B. anthracis* Thil), which is in contact with the phosphate group of AMP (24). Therefore, these two catalytic cysteines are presumably located near the adenylated form of U. Second, Cys265 and Cys268 are at the N terminus of an α-helix, which is a conserved location of a CXXC motif in the thiol:disulfide oxidoreductase superfamily (60). In this superfamily, the CXXC motif is essential for the catalysis of...
FIGURE 5. Identification of disulfide and sulfane sulfur modification in MMP1354 by LC-MS/MS. Identified fragment b- and y-ions derived from the tryptic peptide 26DKYTCLYCK (theoretical $M_{r}^{+} = 569.19$) are labeled in the spectra. A, MS/MS fragmentation spectra of the $-2$-Da shifted precursor ion ($M_{r}^{+} = 568.79$). Detected fragment b- and y-ions containing Cys$^{265}$ and Cys$^{268}$ with $-2$ Da shift are underlined. B, MS/MS fragmentation spectra of the $+30$-Da shifted precursor ion ($M_{r}^{+} = 584.37$). Detected fragment b- and y-ions containing Cys$^{265}$ and Cys$^{268}$ with $+30$ Da shift are underlined.

FIGURE 6. Analysis of in vitro formation of s$^4$U by MMP1354 with APM-retardation gel analysis. A, wild-type MMP1354 (50 nM) was incubated with the unmodified tRNA$^{Cys}$ transcripts (20 μM) together with 5 mM sodium sulfide (lanes 1–4), sodium thiosulfate (lanes 5–8), sodium thiophosphate (lanes 9–12), or L-cysteine (lane 13–16) for 0, 10, 30, and 120 min, respectively. The reactions with cysteine were performed as a separate experiment where the positive control with sodium sulfide is not shown. B, wild-type MMP1354 was incubated with the unmodified tRNA$^{Cys}$ transcripts together with 5 mM sodium sulfide for 10 min in the presence (lane 1) or absence (lane 2) of 1 mM DTT. C, wild-type, C78A, C265A, C268A, and C348A MMP1354 (lanes 1–5, respectively) were incubated with the unmodified tRNA$^{Cys}$ transcripts together with 5 mM sodium sulfide for 10 min.
referred to Cys265 or Cys268 (Cys265 shown in the model here) to generate a persulfide enzyme adduct. Then the thiolate formed by the other Cys attacks the bridging sulfur of the persulfide to liberate the terminal sulfur as a formal equivalent of S₂ for s⁴U biosynthesis. The intramolecular disulfide is consequently regenerated.

The thiamine levels in methanogens are about 5-fold lower compared to other organisms (61), but it is unclear whether the low levels of thiamine in methanogens are due to a lack of thioredoxin or to the absence of thioredoxin-dependent pathways. This conclusion is supported by the observation that several genes encoding the thioredoxin system are present in the methanococcal genomes. Thus, methanococci apparently have a different strategy for sulfur transfer from IscS to U8; Thi in B. subtilis cooperates with a specialized cysteine desulfurase NifZ to transfer sulfur presumably through a persulfide (37), although the catalytic cysteine has not been identified; and Thi in methanogens and some other archaea uses a conserved CXXC motif in the PP-loop domain to generate a persulfide for sulfur transfer. Consistent with the importance of the CXXC motif in archaeal Thi, s⁴U is not observed in the tRNAs from Haloferax volcanii (64) and Solfolobus solfataricus (65), which possess Thi homologs lacking the CXXC motif. Given the similar sulfur transfer mechanisms of methanogen Thi and O-phosphoseryl-tRNA:Cys-tRNA synthase, the utilization of a CXXC motif to generate a persulfide is possibly a common scheme of sulfur chemistry in methanogens. Third, MMP1354 has much higher affinity for sulfide than E. coli Thi. The Kₘ value of MMP1354 for NaN₂S²⁻ is ~1 mM and is within the range of the intracellular level of free sulfide, suggesting that sulfide is a physiologically relevant sulfur donor. Methanococci are well adapted to live in sulfide-rich environments as they lack many targets of sulfide toxicity (46). They also use sulfide instead of free cysteine as the sulfur source for Fe-S cluster biosynthesis (46), which suggests that sulfide plays an important role in sulfur traffic in methanogens. Therefore, it is likely that sulfide replaces cysteine and cysteine desulfurase to generate persulfide on some sulfur carrier proteins. Presumably, methanogens have evolved explicit mechanisms to control the specificities of sulfur incorporation mediated with sulfide and persulfide under anaerobic conditions. These processes may provide a paradigm for studying ancient sulfur metabolism on the early anoxic earth.

**Acknowledgments**—We are grateful to James A. McCluskey for insightful discussions and comments on tRNA modification in archaea. We thank Dan Su for synthesizing APM. We thank Markus Englert, Kara Ford, and Randon Prather for technical assistance. We thank Chenguang Fan and Jae-Hyeong Ko for helpful suggestions on the manuscript.

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