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The putative tRNA 2-thiouridine synthetase Ncs6 is an essential sulfur carrier in *Methanococcus maripaludis*

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Abstract

Thiolation of carbon-2 of uridine located in the first position of the anticodons of $tRNA^{Gln}_{UUG}$, $tRNA^{Glu}_{UUC}$, and $tRNA^{Lys}_{UUU}$ is a conserved RNA modification event requiring the 2-thiouridine synthetase Ncs6/Ctu1 in archaea and eukaryotes. Ncs6/Ctu1 activates uridine by adenylation, but its role in sulfur transfer is unclear. Here we show that Mmp1356, the Ncs6/Ctu1 homolog in the archaeon *Methanococcus maripaludis*, forms a persulfide enzyme adduct with an active site cysteine; this suggests that Mmp1356 directly participates in sulfur transfer as a persulfide carrier. Transposon mutagenesis shows that Mmp1356 is likely to be an essential protein.

Keywords

2-thiouridine; tRNA modification; sulfur; methanogen; archaea

1. Introduction

2-Thiouridine (s^2U) derivatives are present as the first anticodon base (position 34) in $tRNA^{Gln}_{UUG}$, $tRNA^{Glu}_{UUC}$, and $tRNA^{Lys}_{UUU}$ in all three domains of life. This RNA modification enhances aminoacylation kinetics [1-3] and improves translation efficiency and accuracy [4-6]. The lack of s^2U in yeast causes a pleotropic phenotype leading to defects in invasive growth [7], hypersensitivity to rapamycin, caffeine, or oxidative stress [8], and inability to maintain normal metabolic cycles [9]. These observations suggest that 2-thiolation of U_{34} plays essential roles in challenging growth environments.

The s^2U biosynthetic pathway recruits a series of proteins for sulfur transfer. In bacteria, the sulfur relay starts with the generation of a protein-bound Cys persulfide by the cysteine desulfurase IscS employing free Cys as the sulfur donor [10,11]. Then the sulfur is transferred sequentially to TusA, TusBCD, and TusE as persulfide [12]. Finally, the persulfide is transferred to the bacterial s^2U synthetase (MnmA), a PP-loop ATPase domain-

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containing protein. MnmA activates U_{34} through adenylation and functions as the proximal sulfur donor for U_{34} thiolation [13]. In eukaryotic cytosol, the sulfur also derives from free Cys activated by a cysteine desulfurase (Nfs1) [14]. Then the persulfide is transferred to a rhodanese domain-containing protein (Tum1/Yor251c), which subsequently transfers the persulfide to the rhodanese domain of Uba4/MOCS3, an E1-like protein [15]. Uba4/MOCS3 activates and then thiolates an ubiquitin-like (Ubl) protein Urm1 to form a thiocarboxylate, which probably donates sulfur to generate s^2U [8,15-19]. The eukaryotic s^2U synthetase (Ncs6/Ctu1), which also contains a PP-loop like the bacterial MnmA, binds tRNA and activates U_{34} by adenylation [18,20]; however, its role in sulfur transfer is unclear.

The archaeal s^2U biosynthetic pathway probably resembles that present in the eukaryotic cytosol. This assumption is based upon the observation that mutagenesis of SAMP2 (an archaeal Ubl protein) or UbaA (an E1-like protein that actives SAMPs) in *Haloferax volcanii* prevents formation of s^2U in $tRNA^{Lys}_{UUU}$ [21]. This demonstrates that an Ubl protein similar to Urm1 is required for archaeal s^2U biosynthesis. However, the direct evidence that SAMP2 serves as sulfur donor remains to be determined. Furthermore, the enzymes Nfs1 and Tum1 that are involved in the early steps of the eukaryotic sulfur transfer are missing in *Methanococcus maripaludis* and many other archaea [22]. In addition, the methanococcal Uba4 homolog lack the rhodanese domain required for S transfer. Therefore, the nature of the sulfur source for archaeal tRNA thiolation is unclear. Here we show that the putative 2-thiouridine synthetase (Mmp1356, an Ncs6/Ctu1 homolog) in *M. maripaludis* can function as a sulfur carrier by forming a persulfide group. This suggests that Mmp1356 directly participates in sulfur transfer and may be the proximal sulfur donor for tRNA thiolation.

2. Materials and methods

2.1. Media and culture conditions for M. maripaludis growth

M. maripaludis was grown in 28-ml aluminum seal tubes with 275 kPa of H_2:CO_2 (4:1, v/v) at 37 °C in 5 ml McC medium as described previously [23]. The 1.5 L cultures were grown in 2-L bottles in formate medium [24] buffered with 0.2 M glycylglycine (pH 7.0). Neomycin (500 μg/ml in plates and 1 mg/ml in broth) was added when needed. Before inoculation, 3 mM of sodium sulfide was added as the sulfur source.

2.2. Cloning, expression, and purification of recombinant Mmp1356

The *M. maripaludis mmp*1356 with a C-terminal His_6-tag was cloned into the vector pCDFDuet-1 (Novagen) for expression in the *E. coli* Rosetta 2(DE3) strain. The same construct was cloned into the vector pQE2 (Qiagen) for expression in the *E. coli* strains from the Keio collection BW25113 (parent strain), JW2514 (*ΔiscS*), JW2513 (*ΔiscU*), and JW1670 (*ΔsufS*) [25]. The mutations of Mmp1356 (C142S, C145S, and C233S) were constructed using the QuikChange mutagenesis kit (Agilent).

For expression of recombinant Mmp1356, the transformed *E. coli* cells were grown in 1 L of Luria-Bertani (LB) medium at 37 °C with shaking until they reached an absorbance at 600 nm of 0.6~0.8. Then 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce overnight production 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 resupended in 10 ml buffer A (50 mM sodium HEPES, 0.3 M NaCl, 5 mM $MgCl_2$, 20 mM imidazole, pH 7.5). The cells were disrupted by addition of 1 ml BugBuster (Novagen). The cell lysate was centrifuged at 100,000 × g for 30 min at 4 °C, and 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 buffer B (50 mM sodium HEPES, 0.3 M

NaCl, 5 mM $MgCl_2$, 0.2 M imidazole, pH 7.5) and dialyzed against buffer C (50 mM sodium HEPES, 0.3 M NaCl, 5 mM $MgCl_2$, 20% [v/v] glycerol, pH 7.5). The purified proteins were stored at −80 °C until use.

2.3. Identification of modifications of Mmp1356 by mass spectrometry

The anaerobically purified recombinant Mmp1356 was digested with trypsin as described [26]. Tryptic peptides were separated on a Waters nanoACQUITY column (75μm × 250mm eluted at 300nl/min, 80min run time) followed by tandem MS analysis on an Orbitrap Elite mass spectrometer at the Keck MS & Proteomics Resource Laboratory at Yale University. The peptides were identified by MASCOT search with the following parameters: trypsin digestion with two possible missed cleavages, peptide mass tolerance of 15 parts-per-million, fragment mass tolerance of 0.2 Da, and variable Cys modifications of disulfide (−1.0078 Da; needs two Cys with this modification to form a disulfide), persulfide (+31.9721 Da), and trisulfide (+29.9564 Da; needs one Cys with this modification and an unmodified Cys to form a trisulfide). Confidence level was set to 95% within the MASCOT search engine for protein hits.

2.4. Sulfur transfer assay with radioactive sulfur

The sulfur transfer assay was carried out anaerobically as described [26]. The maltose-binding protein (MBP) tagged-IscS (80 μM) was incubated with Mmp1356 (40 μM) in the presence of L-[^{35}S]cysteine (0.3 mM) at 37 °C in the reaction buffer containing 50 mM sodium HEPES (pH 7.3), 150 mM KCl, 10 mM $MgCl_2$, and 10 μM PLP for 30 min. The reaction was stopped by addition of non-reducing SDS loading dye. The proteins mixture was then separated by SDS-PAGE, and the radioactivity retained on the gel was followed by autoradiography.

2.5. Affinity purification and protein identification by mass spectrometry

The N-terminal His_6-tagged *mmp1356* was cloned into the vector pMEV2 for expression in *M. maripaludis* strain S2 [27]. For affinity purification, the *M. maripaludis* cells anaerobically harvested from 1.5 L cultures were resupended in 10 ml buffer D (20 mM sodium HEPES, 0.1 M NaCl, 5 mM $MgCl_2$, 20 mM imidazole, pH 7.5). The cells were disrupted by two times freezing (−80 °C) and thawing. DNA and RNA were digested with 10 U of Benzonase Nuclease (Sigma). Then the cell lysate was centrifuged at 100,000 × g for 30 min at 4 °C, and the supernatant was applied to 1 ml of TALON Metal Affinity Resin (Clontech) equilibrated with buffer D. Proteins bound to the column were eluted with buffer E (20 mM sodium HEPES, 0.1 M NaCl, 5 mM $MgCl_2$, 0.2 M imidazole, pH 7.5). The purified proteins were applied to SDS-PAGE and silver stained. The protein bands were excised, in-gel digested with trypsin, and analyzed by LCMS/MS at Keck MS & Proteomics Resource Laboratory at Yale University.

3. Results and discussion

3.1. Mmp1356 contains conserved residues of the eukaryotic s^2U synthetase

Mmp1356 is the most closely related homolog of the eukaryotic s^2U synthetase (Ncs6/Ctu1) in *M. maripaludis* (Fig. 1A). It shares 31% identity with the *Saccharomyces cerevisiae* Ncs6 and 27% identify with the *Thermus thermophilus* TtuA. Both Ncs6/Ctu1 and TtuA belong to the group II of the TtcA tRNA 2-thiolation enzyme family, which has five conserved CXXC/H motifs and a signature PP-loop motif [28]. Structural and mutational studies of *Pyrococcus horikoshii* TtuA reveal that three conserved cysteines of the putative active site are necessary for the 2-thiolation activity [29]. Multiple sequence alignments recognized

that the CXXC and PP-loop motifs as well as the active site cysteines are all conserved in Mmp1356 (Fig. 1B); thus Mmp1356 is a putative 2-thiouridine synthetase.

3.2. Mmp1356 is a sulfur carrier

The requirement of three cysteines at the active site of the group II TtcA family resembles that of the tRNA 4-thiouridine synthetase (ThiI) [30] and of Sep-tRNA:Cys-tRNA synthase (SepCysS) [26] in methanogenic archaea. Since ThiI and SepCysS form a persulfide enzyme adduct for sulfur transfer [26], the group II TtcA probably also participates in sulfur transfer with a similar reaction mechanism to that of ThiI and SepCysS. To examine whether Mmp1356 is a sulfur carrier, the modifications of the anaerobically purified recombinant Mmp1356 expressed in *E. coli* was analyzed by LC-MS/MS. For the peptide $_{136}$NLTMNPCSFCGVIR$_{149}$, the precursor ions were observed with −2 Da (mass of −2H) and +30 Da (mass of −2H + 1S) shifts (Fig. 2A). Analysis of the MS/MS spectra matched them with intra-peptide disulfide and trisulfide modifications at $C_{142}XXC_{145}$, respectively (Fig. 2B). Due to the instability of a Cys persulfide, the detected trisulfide presumably resulted from oxidation (loss of dihydrogen) of a Cys persulfide and a nearby Cys thiol. This modification pattern is similar to that observed for SepCysS [26] and ThiI [30], suggesting that a fraction of the purified Mmp1356 carries an oxygen-sensitive persulfide group at an active site cysteine.

The *E. coli* cysteine desulfurase IscS is a versatile sulfur donor [31-34]. It generates a persulfide group from free Cys and donates sulfur for the biosynthesis of multiple cofactors (*e.g.* Fe-S clusters, thiamine, and molybdopterin) and thionucleotides (*e.g.* 2-thiouridine and 4-thiouridine). Because IscS can deliver persulfide to diverse downstream sulfur carrier proteins, it is possible that the persulfide of the recombinant Mmp1356 came from IscS. To test this possibility, Mmp1356 was expressed in an *E. coli ΔiscS* strain. As control experiments, Mmp1356 was also expressed in the deletion strains of *iscU* (a scaffold protein that receives sulfur from IscS for Fe-S cluster assembly) and *sufS* (a cysteine desulfurase that functions as the sulfur donor for Fe-S cluster biosynthesis under stress conditions). Although the disulfide modification of $C_{142}XXC_{145}$ was present in all cases, the trisulfide modification was not detected in Mmp1356 purified from the *ΔiscS* strain. This suggests that IscS generates the persulfide on Mmp1356 when expressed in *E. coli*. In contrast, the trisulfide modification was present in both the *ΔiscU* and *ΔsufS* strains, suggesting that this process is independent of Fe-S cluster assembly.

To further confirm that *E. coli* IscS delivers sulfur to Mmp1356, Mmp1356 was incubated with [^{35}S]Cys in the absence or presence of IscS (Fig. 3). The protein(s) was then subjected to SDS-PAGE analysis under non-reducing conditions. Mmp1356 was only radiolabeled in the presence of IscS (lane3 of Fig. 3), indicating that Mmp1356 can accept ^{35}S from IscS. The radiolabeling was sensitive to the reductant 2-mercaptoethanol (lane 4 of Fig. 3), suggesting that ^{35}S was attached to IscS and Mmp1356 as persulfide. Mutations of Cys142, Cys145, or Cys233 in Mmp1356 to Ser markedly reduced its ^{35}S-labelling (lanes 5-7 of Fig. 3), indicating that these three cysteines are essential for the generation or stabilization of the persulfide under *in vitro* conditions.

Overall, these results imply that the $C_{142}XXC_{145}$ motif of Mmp1356 can form a persulfide enzyme adduct. Therefore, Mmp1356 probably directly participates in sulfur transfer and functions as the proximal sulfur donor for tRNA thiolation. Although an IscS homolog is not encoded in *M. maripaludis*, the capability of IscS to donate sulfur to Mmp1356 suggests that Mmp1356 can receive sulfur from a protein sulfur donor or sulfide directly, resembling the case of ThiI in methanogens [30].

3.3. Mmp1356 interacts with Mmp1357 in vivo

To identify the physiological partner proteins of Mmp1356, the His_6-tagged Mmp1356 was overexpressed in *M. maripaludis* for a pull-down experiment. Affinity purification followed by MS analysis demonstrated that Mmp1357 (~ 10 kDa) co-purified with Mmp1356 (~ 37 kDa). They appeared as major components of two >200 kDa and one ~ 65 kDa protein bands separated by SDS-PAGE (Fig. 4). This result suggests that Mmp1356 interacts with Mmp1357 *in vivo* and they are likely to be covalently linked into large molecular mass conjugates. Other proteins co-purified specifically with Mmp1356 (identified with > 40% coverage in MS analysis) included 2-phosphosulfolactate phosphatase (Mmp0161), uracil phosphoribosyltransferase (Mmp0680), and DNA-directed RNA polymerase subunit D (Mmp1322). These proteins may directly or indirectly associate with Mmp1356.

Mmp1357 is homologous to the archaeal Ubl protein SAMP, and it is encoded upstream of the *mmp1356* gene. According to the transcriptomic tiling array data [35], *mmp1357* and *mmp1356* are most likely to be co-transcribed, suggesting that they represent an operon. This genetic association is present in several archaeal lineages [36], including some species of the methanogens (*Methanoregula*, *Methanocorpusculum*, *Methanococcus*, and *Methanosphaerula*), the crenarchaeotes (*Aeropyrum*, *Hyperthermus*, *Pyrolobus*, *Staphylothermus*, and *Stygioglobus*), and the korarchaeotes (*Korarchaeum*). These associations suggest that SAMPs are often functionally linked to tRNA thiolation [36,37].

The functional link of Mmp1356 and Mmp1357 is supported by their physiological interaction, which has also been observed for their homologs in haloarchaea [38], *Thermus thermophilus* [39], and mammalian cells [40]. In all these cases, the C-terminal Gly of the Ubl proteins are appended to Lys residues of the 2-thiolation enzymes via isopeptide bonds, similar to ubiquitylation. In *H. volcanii*, 29 proteins are conjugated (sampylated) with SAMP2, including homologs of Uba4, Tum1, and Ncs6 associated with the s^2U biosynthetic pathway [38]. Furthermore, SAMP2 also sampylates its own Lys to form polymeric chains [38]. Deletion of SAMP2 causes retarded growth at high temperatures and lack of s^2U in tRNA [21]; this provides direct evidence that SAMP is required for s^2U biosynthesis. However, whether SAMP can be thiolated to form a C-terminal thiocarboxylate and donate sulfur to tRNA are unclear. The *Δsamp2* phenotype permits two interpretations: (i) SAMP acts as a sulfur carrier; or (ii) sampylation of s^2U synthetase controls the thiolation activity.

3.4. Mmp1356 is likely to be essential in M. maripaludis

A whole-genome analysis of gene function by the Tn-seq methodology has recently been completed in *M. maripaludis* [41]. Two libraries of mutants were generated for *M. maripaludis* using a derivative of the Tn*5* transposon comprising puromycin resistance [41]. Because *M. maripaludis* is polyploid and contains 30-55 copies of the chromosome per cell [42], the transposon was inserted into both essential and nonessential genes during growth with puromycin selection [41]. When puromycin was removed, transposon insertions in essential genes were quickly replaced by the wild-type alleles [41]. This is because gene conversion rapidly homogenizes the genome to prevent accumulation of heterologous genes [42]. Using this method, 526 genes were classified as possibly essential or strongly advantageous for growth in rich medium, corresponding to 30% of the genome [41]. The genomic region of *mmp1357-mmp1356* (1335578-1336834) contained 37 unique insertions at T0 (growth with puromycin for 20 generations) and only one insertion at T2 (growth without puromycin for 14 generations). This suggests that (i) *mmp1356* is likely to be essential and (ii) the upstream *mmp1357* is either essential or falsely appeared essential because of polarity.

3.5. Concluding remarks and future prospects

Our biochemical characterizations of Mmp1356, a putative s^2U synthetase, indicate that it functions as a sulfur carrier. This provides a new perspective of the function of the Ncs6/Ctu1 enzymes, suggesting that they serve as a sulfur donor for s^2U biosynthesis. Three cysteines at the active site of Mmp1356 are required to generate a persulfide enzyme adduct, resembling the archaeal ThiI and SepCysS. This suggests that these enzymes may use a common mechanism for sulfur transfer. The functional link of Mmp1356 with an archaeal ubiquitin-like protein (Mmp1357) is supported by their conserved genetic association and physiological interaction. However, whether Mmp1357 forms a thiocarboxylate group and directly participates in sulfur transfer remains to be determined. Finally, knowledge of the nature of the physiological sulfur donor used to form the persulfide on Mmp1356 and the ultimate sulfur source for archaeal s^2U biosynthesis awaits future investigations.

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Highlights

- Mmp1356 is a putative 2-thiouridine synthetase and acts as a sulfur carrier.
- Three conserved cysteines of Mmp1356 are required for sulfur transfer.
- Mmp1356 interacts with the ubiquitin-like protein Mmp1357 *in vivo*.
- Mmp1356 is likely to be an essential protein in *Methanococcus maripaludis*.

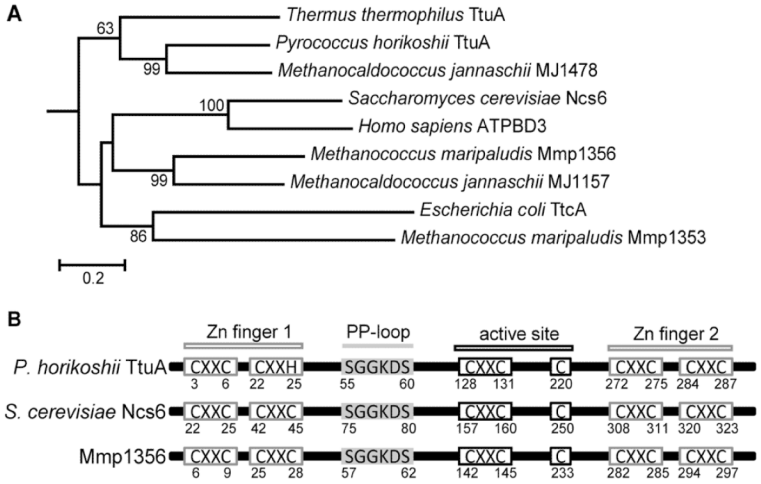

Fig. 1.

(A) Phylogeny of tRNA 2-thiolation enzymes. The sequence-based phylogenetic tree was constructed with the Minimum-Evolution algorithm of MEGA5. Bootstrap analysis was performed with 1,000 replicates, and values greater than 60% are labeled on the nodes. The *E. coli* 2-thiouridine synthetase MnmA was used as an outgroup. TtuA, Ncs6/ATPBD3, and TtcA are involved in the 2-thiolation reactions of tRNA nucleotides T_{54}, U_{34}, and C_{32}, respectively. (B) Conserved motifs and residues of the group II of the TtcA family. Four CXXC/H motifs are involved in Zn-binding, and three cysteines are located at the putative active site. The numbers under the schemes are the amino acid positions of the *P. furiosus* TtuA (locus tag: PH0300), *S. cerevisiae* Ncs6 (locus tag: YGL211W), and Mmp1356.

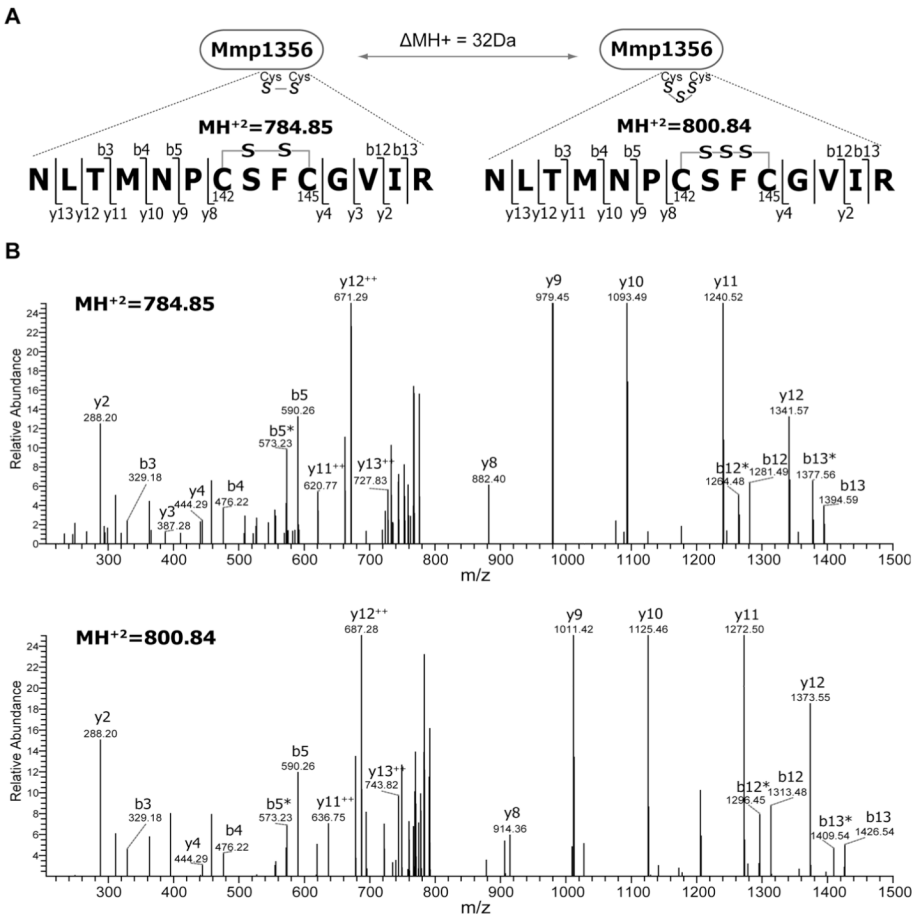

Fig. 2.

Identification of sulfur modifications of Mmp1356 by LC-MS/MS. (A) Two precursor ions with a mass difference of 32 Da were observed for the tryptic peptide $_{136}$NLTMNPCSFCGVIR$_{149}$ (theoretical MH^{+2} =785.86 with an oxidized Met). (B) MS/MS fragmentation spectra of the −2 Da (upper panel) and the +30 Da (lower panel) precursor ions. The detected b- and y- ions are labeled in the spectra. * indicates immonium ions.

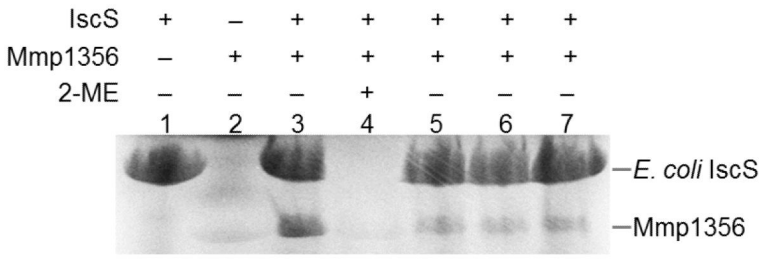

Fig. 3.

Persulfide formation on Mmp1356. The *E. coli* IscS (lane 1), the wild-type Mmp1356 (lane 2), and the two proteins together (lane 3) were incubated with 300 μM [^{35}S]Cys at 37 °C for 30 min, and then the incubation mixtures were analyzed by SDS-PAGE under non-reducing condition. Lane 4, the IscS and Mmp1356 mixture (as in lane 3) was analyzed by SDS-PAGE under reducing condition with 1% (v/v) 2-mercaptoethanol (2-ME). Lane 5-7, the *E. coli* IscS was incubated with the C142S, C145S, and C233S variants of Mmp1356, respectively. The positions of the MBP-tagged IscS (~ 75 kDa) and the His-tagged Mmp1356 (~ 37 kDa) are labeled.

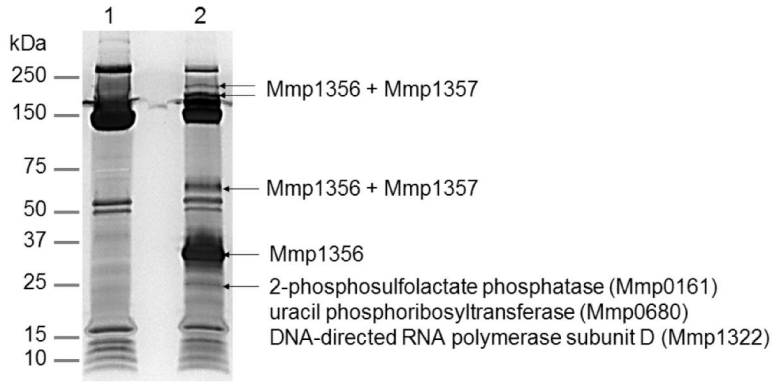

Fig. 4.

Pull-down of Mmp1356 expressed in *M. maripaludis*. The proteins purified by metal affinity chromatography from *M. maripaludis* cells transformed with the empty vector (lane 1) or the shuttle vector expressing His_6-tagged Mmp1356 (lane 2) were analyzed with SDS-PAGE and stained with silver. The protein bands marked with arrows in lane 2 were excised, digested with trypsin, and analyzed by LC-MS/MS. The corresponding regions in lane 1 were analyzed as controls. The proteins specifically identified in lane 2 with > 40% coverage are listed.