An ancient type of MnmA protein is an iron–sulfur cluster-dependent sulfurtransferase for tRNA anticodons

NAOKI SHIGI,1 MASAKI HORITANI,2 KENJYO MIYAUCHI,3 TSUTOMU SUZUKI,3 and MISAO KUROKI1

1Biotechnology Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan
2Faculty of Agriculture, Department of Applied Biochemistry and Food Science, Saga University, 1 Honjo-machi, Saga 840-8502, Japan
3Department of Chemistry and Biotechnology, Graduate School of Engineering, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

ABSTRACT

Transfer RNA (tRNA) is an adaptor molecule indispensable for assigning amino acids to codons on mRNA during protein synthesis. 2-thiouridine (s2U) derivatives in the anticodons (position 34) of tRNAs for glutamate, glutamine, and lysine are post-transcriptional modifications essential for precise and efficient codon recognition in all organisms. s2U34 is introduced either by (i) bacterial MnmA/eukaryote mitochondrial Mtu1 or (ii) eukaryote cytosolic Ncs6/archaeal NcsA, and the latter enzymes possess iron-sulfur (Fe–S) cluster. Here, we report the identification of novel-type MnmA homologs containing three conserved Cys residues, which could support Fe–S cluster binding and catalysis, in a broad range of bacteria, including thermophiles, Cyanobacteria, Mycobacteria, Actinomycetes, Clostridium, and Helicobacter. Using EPR spectroscopy, we revealed that Thermus thermophilus MnmA (TtMnmA) contains an oxygen-sensitive [4Fe–4S]-type cluster. Efficient in vitro formation of s2U34 in tRNALys and tRNAGln by holo-TtMnmA occurred only under anaerobic conditions. Mutational analysis of TtMnmA suggested that the Fe–S cluster is coordinated by the three conserved Cys residues (Cys105, Cys108, and Cys200), and is essential for its activity. Evolutionary scenarios for the sulfurtransferases, including the Fe–S-containing Ncs6/NcsA s2U thiouridylases and several distantly related sulfurtransferases, are proposed.

Keywords: biosynthesis; iron–sulfur cluster; post-transcriptional modification; sulfurtransferase; tRNA

INTRODUCTION

During translation of genomic information into proteins, tRNAs deliver amino acids to their respective mRNA codons. There are numerous post-transcriptional chemical modifications in tRNA that are important for precise codon recognition and stabilization of the tRNA tertiary structure (Ducheler et al. 2016; Boccaletto et al. 2018; Hori et al. 2018). Position 34 (the anticodon wobble base) of glutamate, glutamine, and lysine tRNAs is universally modified to 5-methyl-2-thiouridine derivatives (km5s2U) (Fig. 1A; Armengod et al. 2014; Schaffrath and Leidel 2017). These amino acids are encoded by two degenerate codons ending in purine in the two-codon boxes that specify two amino acids by the difference in the third codon bases. Because of the steric hindrance between the bulky 2-thiocarbonyl group and the 2′-hydroxyl group of s2U, km5s2U preferentially adopts the C3′-endo form of ribose puckering (Yokoyama et al. 1985; Agris et al. 1992). km5s2U base-pairs more tightly with purines than with pyrimidines, are thus required for efficient codon selection (Ashraf et al. 1999; Vendeix et al. 2012; Rodriguez-Hernandez et al. 2013; Rozov et al. 2016; Ranjan and Rodnina 2017), and for preventing misreading of near cognate codons (Agris et al. 1973; Yokoyama et al. 1985; Johansson et al. 2008) and frame shifting (Urbonavicius et al. 2001). The absence of the 2-thio modification leads to ribosome stalling at A-ending codons in mRNAs, and abnormal translation speed causes protein misfolding and aggregation (Nedialkova and Leidel 2015). In patients with myoclonus epilepsy with ragged-red fibers, the lack of the U34 modification in mutant mitochondrial tRNA54s induces abnormal translation leading to disease development (Yasukawa et al. 2001). The 2-thio modification in the m5s2U( s2T) at position 54 in the T-loop also plays a role in translation at high temperatures. In some thermophilic bacteria and archaea, nearly all of the tRNA species possess m5s2U54
Fe–S cluster-dependent MnmA

(Watanabe et al. 1974; Kowalak et al. 1994), in contrast to the conserved m^5^-U (rT) 54 in mesophiles. 2-thiolation of m^5^-s^2^-U54 is required for survival at high temperatures (Shigi et al. 2006a). m^5^-s^2^-U54 base-pairs with m^1^-A58 and stacks with G53 and \( \Psi \) 55. \( \Psi \) 55 and C56 base-pair with G18 and G19, respectively. The A-form helix formed by these interactions in the tRNA core is stabilized by the intrinsic rigidity of the m^5^-s^2^-U structure (Watanabe et al. 1974; Horie et al. 1985).

The biosynthesis of tRNA sulfur modifications involves “sulfur trafficking systems” that begin with cysteine desulfurases and “modification enzymes” that directly incorporate sulfur into tRNAs (Shigi et al. 2014, 2018). For s^2^-U34 synthesis, two types of modification enzymes have been discovered: (i) bacterial MnmA (Hagervall et al. 1998; Kambampati and Lauhon 2003; Ikeuchi et al. 2006)/eukaryotic mitochondrial Mtu1 (Umeda et al. 2005) and (ii) eukaryotic cytosolic Ncs6 (Bjork et al. 2007; Dewez et al. 2008; Nakai et al. 2008; Leidel et al. 2009; Noma et al. 2009)/archaeal NcsA (Chavarria et al. 2014). For s^2^-U54 synthesis, we identified TtuA, which is closely related to the Ncs6/NcsA family (Shigi et al. 2006a,b, 2008, 2016; Chen et al. 2017). These RNA sulfurtransferases contain a characteristic signature motif (SxGxDS/T, PP-loop), which binds to the pyrophosphate moiety of ATP (Bork and Koonin 1994). These PP-loop ATP pyrophosphatases first activate their substrates via adenylation and a subsequent nucleophilic attack by the activated sulfur (e.g.,

![FIGURE 1. Two types of MnmA tRNA s^2^-U34 sulfurtransferases. (A) Reaction mechanism of MnmA. The PP-loop motif in MnmA is involved in the formation of an adenylated intermediate, and subsequent nucleophilic attack by activated sulfur (depicted as “S”) generates s^2^-U and releases AMP. (B) Structures of the catalytic centers of representative RNA sulfurtransferases. Structures of EcMnmA (PDB: 2deu), TmMnmA model generated by SWISS-MODEL, and TtTtuA (PDB: 5b4e) were rendered by the PyMol program (DeLano Scientific). Important residues and ligands in these proteins are depicted and labeled. The Fe with a free coordination site in TtTtuA is labeled with an asterisk (*). The flexible loops with a cysteine residue are colored blue. (C) Conserved sequence motifs in the catalytic domains of MnmAs from several representative bacteria and eukaryotes. These sequences were aligned with the CLUSTAL X2 program (Larkin et al. 2007) and visualized with the GeneDoc program. The complete alignment is shown in Supplemental Figure S1. Schematic representation of the conserved motifs in the catalytic domains of EcMnmA, TmMnmA, and TtTtuA is depicted below the alignment, along with other domains. (D) Phylogenetic tree of several MnmAs drawn with the CLUSTAL X2 and iTOL programs (Letunic and Bork 2016) based on the sequence alignment in C.}
bisulfide, protein-persulfide, or protein-thio-carboxylate; depicted as “S”) generates s^2U (Fig. 1A; Ikeuchi et al. 2006; Shigi et al. 2008; Arragain et al. 2017; Chen et al. 2017). One sharp difference in these sulfurtransferases is the cofactor dependence: Only the Ncs6/NcsA/TtuA proteins require iron-sulfur (Fe–S) clusters for their activity. In this study, we found that MnmA homologs in various species possess characteristic conserved Cys residues reminiscent of Fe–S cluster binding structures. We revealed that one such MnmA in Thermus thermophilus (TtMnmA) possess three CxxC motifs, and we refer to them as D- and C-type MnmAs, respectively. C-type MnmAs were found mainly in thermophilic bacteria such as Thermus thermophilus, Thermotoga maritima, and Aquifex aeolicus, in Actinobacteria such as Mycobacterium, Corynebacterium, Streptomyces, and Actinomyces, and in Cyanobacteria. In Firmicutes, both types of MnmAs were identified, including D-type MnmAs in Bacillus, Staphylococcus, and Streptococcus and C-type MnmAs in Clostridium. Although D-type MnmAs were most commonly identified in Proteobacteria (e.g., in Escherichia and Pseudomonas), a C-type MnmA variant lacking the region containing the first CC motif was found in Campylobacter and Helicobacter. We could not find C-type MnmAs in eukaryotic mitochondria and chloroplasts. Of note, both types of MnmAs can be found within the same genus (e.g., in Clostridium), suggesting that horizontal gene transfer would have occurred in the MnmA family. For example, C-type MnmAs were identified in C. acidi-butylicum, C. botulinum, C. butyricum, C. difficile, C. perfringens, and C. tetani, while D-type MnmAs were detected in C. acetidigarinis, C. cadaveris, C. cellulovorans, C. putrefaciens, and C. thermopalmarium.

Comparison of sequences and structures of MnmA and TtTuA

Thermus thermophilus MnmA (TtMnmA) possess three conserved Cys residues in CxxC—C motifs (Cys105, Cys108, and Cys200) rather than in DxxC—C motifs (Asp99, Cys102, and Cys199) in Escherichia coli MnmA (EcMnmA), which are required for s^2U34 synthesis (Fig. 1B,C; Numata et al. 2006). MnmA consists of the catalytic amino-terminal domain and the central and carboxy-terminal domains involved in tRNA recognition (Fig. 1C, bottom panel). In the EcMnmA amino-terminal domain (Fig. 1B, top panel; Numata et al. 2006), the PP-loop, the Asp99 and Cys102 residues in the rigid α-helix, and Cys199 in a flexible loop (shown in blue), are close together in the catalytic center. A sulfate molecule is bound to the PP-loop of EcMnmA that mimics the pyrophosphate liberated following the adenylation step. Numata et al. (2006) speculated that Asp99 acts as an acid/base to protonate/deprotonate the adenylated U34, while the ATP bound to the PP-loop supports the formation of an adenylated intermediate. In one of the two proposed mechanisms, the Sy of the persulfide formed on Cys199 is attacked by Cys102, and a liberated bisulfide nucleophilically substitutes the adenylate group to form s^2U34. In another type of s^2U thiouridylase TtTuA/Ncs6, there are conserved CxxC—C motifs (consisting of Cys130, Cys133, and Cys222 in TtTtuA) in the catalytic domain, and this domain is sandwiched between the amino- and carboxy-terminal Zn-finger domains, which may contribute to tRNA binding (Fig. 1C, bottom panel; Nakagawa et al. 2013; Chen et al. 2017). Cys130 and Cys133 are located in the rigid α-helix, while Cys222 is present in a flexible loop region (Fig. 1B, bottom panel). These three Cys residues bind a [4Fe–4S] cluster with a free coordination site on one of the iron atoms (Arragain et al. 2017; Chen et al. 2017). We have proposed that this iron atom binds either sulfur-carrier proteins (e.g., TtB-COSH) or bisulfide, and that the sulfur atom is ultimately incorporated into s^2U54.

We built a homology model of TtMnmA (Fig. 1B, middle panel) by SWISS-MODEL (Waterhouse et al. 2018) using EcMnmA as a template. The sequence identity (43%) between TtMnmA and EcMnmA was sufficient for model building and QMEAN Z-score of TtMnmA model was −1.20, indicating the high reliability of this model (Benkert et al. 2011). Strikingly, the model of the TtMnmA catalytic center has an architecture composed of a PP-loop and three Cys residues located in similar positions to those in TtTuA (Fig. 1B, bottom panel). Considering this significant similarity, we speculated that MnmA homologs with CxxC—C motifs can bind Fe–S clusters, which are required for the sulfurtransferase activity.

T. thermophilus tRNAs contain mmn^5s^2U

The residues at position 34 of the Glu, Gln, and Lys tRNAs are universally modified to 5-methyl-2-thiouridine derivatives in bacteria and eukaryotes. 5-methylaminomethyl-2-thiouridine (mmn^5s^2U) and 5-carboxymethylaminomethyl-2-thiouridine (cmnm^5s^2U) are found in bacterial tRNAs (Carbon et al. 1968; Oashi et al. 1970; Hagervall et al. 1987). We analyzed the total nucleoside of T. thermophilus tRNA mixtures by LC/MS (Supplemental Fig. S2). Both mmn^5s^2U (m/z = 304.1) and mmn^5U (m/z = 288.1) were detected, and their MS/MS spectra further confirmed...
their identities (Supplemental Fig. S2A), based on a comparison with E. coli RNA (Supplemental Fig. S2B). Neither cmnm<sup>S</sup>S<sup>U</sup> (m/z = 348.1) nor cmnm<sup>S</sup>U (m/z = 332.1) was detected in T. thermophilus tRNA.

**T. thermophilus MnmA can bind an oxygen-labile [4Fe–4S]-type cluster**

We investigated T. thermophilus MnmA from a spectroscopic standpoint. TtMnmA expressed in E. coli and purified under aerobic conditions did not yield an ultraviolet-visible (UV–VIS) spectrum which indicates the presence of a Fe–S cluster. When aerobically purified apo-TtMnmA was incubated with iron and sulfide under anaerobic conditions, a brown solution of the TtMnmA protein was obtained and exhibited a UV–VIS spectrum with a shoulder at ~410 nm (Fig. 2A), which is characteristic of [4Fe–4S] clusters in proteins (Arragain et al. 2017; Chen et al. 2017). The Fe content of the reconstituted TtMnmA sample was 3.72 ± 0.03 mol per mol of protein (n = 3), further supporting the presence of a [4Fe–4S]-type Fe–S cluster.

To clarify the properties of the Fe–S center in detail, we analyzed reconstituted MnmA by electron paramagnetic resonance (EPR) spectroscopy. Reconstituted MnmA displayed some small signals around g ~ 2 at 12 K that are consistent with a [3Fe–4S]<sup>1+</sup> cluster and radicals (Fig. 2B[a]). After reduction with excess dithionite, the absorbance at ~410 nm decreased (Fig. 2A). The reduced MnmA showed slightly rhombic EPR signals with principle g = [2.04, 1.93, 1.90] (g<sub>ω</sub> = 1.96) (Fig. 2B[a]), and these signals were not observed at 40 K (Supplemental Fig. S3A), suggesting that MnmA has a [4Fe–4S]-type cluster like those of TtuA (Arragain et al. 2017; Chen et al. 2017), aconitase (Empigate et al. 1983), and radical S-adenosylmethionine enzymes (Broderick et al. 2014; Hanson and Berliner 2017). The signal was fitted well with the simulated [4Fe–4S]<sup>1+</sup> cluster (dotted line) and the concentration of the [4Fe–4S]<sup>1+</sup> cluster was estimated to ~30 μM based on comparison with double integration of the signal for the Cu<sup>2+</sup> standard. The weak signal intensity might reflect the difficulty to reduce the cluster with dithionite (Wong et al. 1977; Vollmer et al. 1983; Henshaw et al. 2000). When ferricyanide was added to the reconstituted MnmA, the absorbance at around 410 nm was also decreased (Fig. 2A). EPR signal centered at g ~ 2 appeared (Fig. 2B[a]), and this signal was present at 40 K (Supplemental Fig. S3B).

The T. thermophilus MnmA Fe–S cluster is required for s<sup>2</sup>U synthesis in tRNAs

Because TtMnmA can bind an oxygen-labile [4Fe–4S]<sup>2+</sup> cluster, we investigated the requirement of the Fe–S cluster for its sulfurtransferase activity in vitro. We reconstituted the 2-thiolation reaction of the transcribed tRNALys under anaerobic conditions in the presence of holo-MnmA, 0.1 mM sodium sulfide, and 2.5 mM ATP (Fig. 2C). Nucleoside analysis of the tRNA reaction products by HPLC indicated the formation of s<sup>2</sup>U (~18.5 min) (a), which was confirmed by observing it had the same retention time (b) and UV spectrum as authentic s<sup>2</sup>U (Fig. 2D). Under these conditions, MnmA catalyzed the s<sup>2</sup>U formation reaction with multiple turnovers. The initial velocity of this reaction was 0.537 ± 0.052 pmol s<sup>2</sup>U/pmol MnmA/min (n = 3). A tRNA<sup>Lys</sup> U34A mutant was not a MnmA substrate (Fig. 2C[c]), supporting the formation of s<sup>2</sup>U at position 34 in wild-type tRNALys. When we used apo-MnmA (d), no s<sup>2</sup>U formation was detected. The reaction also required ATP (e) and sodium sulfide (f) in the reaction mixture. We also observed s<sup>2</sup>U formation in transcribed tRNA<sup>Gln</sup> (Fig. 2E). The initial velocity was 0.421 ± 0.013 pmol s<sup>2</sup>U/pmol MnmA/min (n = 3), which is comparable (~80%) to that for tRNA<sup>Lys</sup>. In order to examine the incorporation of exogenous sulfur into s<sup>2</sup>U, a coupled reaction with [<sup>35</sup>S]-cysteine and cysteine desulfurase with holo-MnmA was performed, and the incorporation of [<sup>35</sup>S]-sulfur in tRNA was readily detected (Fig. 2F). The [<sup>35</sup>S]-sulfide formed on the catalytic Cys residue (R–S[<sup>35</sup>S]H) in the cysteine desulfurases was released as [<sup>35</sup>S]-sulfide by dithiothreitol (DTT) in the reaction mixture (Behshad et al. 2004), and the released [<sup>35</sup>S]-sulfide could be further utilized and incorporated into tRNA by holo-MnmA. Taken together, these data demonstrate that the MnmA Fe–S cluster is required for s<sup>2</sup>U34 synthesis in tRNAs. These results also suggest that exogenous sulfur (i.e., bisulfide), rather than the sulfur atom of the Fe–S cluster, is incorporated into s<sup>2</sup>U. In addition, TtMnmA does not require CS-modification of U34 for s<sup>2</sup>U synthesis, because the transcript tRNAs were good substrates for TtMnmA.
Mutational analysis of conserved residues in TtMnmA

A comparison of the structure of the TtMnmA model with that of TtTtuA (Fig. 1B) suggested that the TtMnmA [4Fe–4S] cluster is coordinated by three conserved Cys residues: Cys105, Cys108, and Cys200. To evaluate the importance of these Cys residues, we reconstituted Fe–S clusters in a series of Ala-substituted TtMnmA mutants (Supplemental Fig. S5A) and measured their Fe content and enzymatic activities (Table 1). As expected, the Fe content was...
loss of a binding site for the genuine Fe in the triple mutant relative to the wild-type protein may be diminished (Supplemental Fig. S5B), especially in the wild-type. Consistent with these observations, the 2-thiolation activity was completely abolished in the triple mutant, indicating that it is essential for 2-thiolation activity.

In vitro mutational analysis of TtMnmA

| Table 1. In vitro mutational analysis of TtMnmA |
|-----------------------------------------------|
| Amount of Fe | Enzyme activity |
| (pmol Fe/ pmol MnmA) | (pmol s²U/pmol MnmA/min) | %b |
| Wild-type | 3.72 ± 0.03 | 0.567 ± 0.078 | 100.0 |
| C105A | 3.32 ± 0.21 | 0.007 ± 0.000 | 1.3 |
| C108A | 3.02 ± 0.12 | Not detected | 0 |
| C200A | 3.49 ± 0.15 | 0.195 ± 0.013 | 34.4 |
| C105A, C108A, C200A | 1.96 ± 0.08 | Not detected | 0 |
| D13A | 3.73 ± 0.05 | Not detected | 0 |
| C55A | 3.65 ± 0.25 | 0.614 ± 0.009 | 108.3 |
| C56A | 3.83 ± 0.12 | 0.957 ± 0.048 | 168.7 |
| C55A, C56A | 3.57 ± 0.22 | 0.609 ± 0.021 | 107.4 |

*Experiments were repeated in triplicate and the data are shown with s.d. values. 
*The wild-type value was set to 100.

Cysteine desulfurases can supply sulfur to TtMnmA

In E. coli, the sulfur atom of cysteine is first activated by the IscS cysteine desulfurase to form a protein-persulfide (IscS-SSH) derivative (Flint 1996; Ikeuchi et al. 2006). The persulfide group is relayed by the TusA, TusBCD, and TusE carrier proteins and is passed onto the catalytic Cys199 residue in MnmA; finally, the sulfur atom is incorporated into s²U34. In B. subtilis (Black and Dos Santos 2015), the BsrYvo cysteine desulfurase and BsMnmA are necessary and sufficient for s²U34 synthesis. Although cysteine desulfurases, TusA, and MnmA are conserved in many organisms, TusBCD and TusE are not conserved (Koter et al. 2010), implying that variations exist in the upstream sulfur-transfer pathways for s²U synthesis.

Two cysteine desulfurases, IscS and SuF, are encoded by the T. thermophilus genome (Shigi et al. 2006a), and no homologs of the Tus proteins have been identified, suggesting direct transfer of sulfur from the cysteine desulfurases to MnmA, although indirect sulfur transfer via unidentified sulfur-carrier protein(s) could exist. Therefore, we investigated s²U34 formation in vitro by cysteine
desulfurases with cysteine as the sulfur donor. In this assay, the reductants included in the enzyme stock solutions were eliminated by gel filtration to prevent possible sulfide release into the reaction mixture. In the absence of reductant, s²U was readily formed by the cysteine desulfurases using cysteine as the sulfur donor. The initial velocities were 0.136 ± 0.010 and 0.329 ± 0.013 (pmol s²U/pmol MnmA/min, n = 3) for 1.25 µM IscS and SufS with 0.1 mM cysteine, respectively. These results suggest direct persulfide transfer from the cysteine desulfurases to MnmA during s²U formation.

Concluding remarks

For s²U₃₄ formation in tRNAs, there are many C-type MnmA homologs (Figs. 1C,D, 3) that likely possess Fe–S clusters similar to that of TtMnmA, in addition to the previously characterized DxxC—C-type (D-type, non-FeS) MnmAs. As C-type MnmAs are found in many thermophilic bacteria (Fig. 1C,D), which are generally considered to be closely related to the common ancestor of life (Akanuma et al. 2013), C-type MnmA seems to be an ancient member of the MnmA family. Figure 3 summarizes the distribution of the s²U thiouridylases in the three domains of life. In the cytoplasm of eukaryotes and archaea, s²U₃₄ is formed by the Ncs6 and NcsA Fe–S proteins (Liu et al. 2016). Furthermore, in T. thermophilus, s²U₅₄ is synthesized by the TtuA Fe–S protein. Ncs6/NcsA/TtuA possess catalytic centers similar to that of C-type MnmA, although they may bind tRNAs differently by distinct RNA binding domains (Fig. 1C). Many archaea carry two ORFs related to NcsA/TtuA (Shigi et al. 2006a; Chavarria et al. 2014), suggesting that they may be involved in the biosynthesis of s²U₃₄ and s²U₅₄; however, experimental validation is needed. Another important issue that remains to be addressed is whether a sulfide group bound to an iron atom in a free coordination site of the [4Fe–4S] cluster is truly incorporated into s²U.

In some cases of the PP-loop ATP pyrophosphatases family, the Fe–S-dependent and Fe–S-independent enzymes possess the same enzymatic activities. Although most Thi homologs responsible for 4-thiouridine synthesis at position 8 of tRNAs are Fe–S cluster-independent enzymes, some archaeal (e.g., Methanogenic archaea and Thermococcales) Thi homologs have conserved CxxC—C motifs (Liu et al. 2012), and are essential for their [3Fe–4S] cluster binding and enzymatic activity (Liu et al. 2016). It is quite intriguing that loss or acquisition of Fe–S clusters may occur via changes in one or two Cys residues and that these changes seem to have occurred independently in several enzyme groups and even in orthologous enzymes in closely related species, suggesting a general evolutionary strategy for enzymes with several sulfur-transfer activities. The oxygen sensitivity of Fe–S proteins may be compensated for by universally conserved iron–sulfur cluster repair systems in the cells (Zheng and Dos Santos 2018), which support wide-spread utilization of these oxygen-sensitive sulfurtransferases and no definite correlation with habitat environments (e.g., anaerobic or aerobic). Because s²U₃₄ moieties in tRNA have quite important roles for translation and defects in s²U₃₄ synthesis lead to severe growth retardation or death (Kambampati and Lauhon 2003; Ikeuchi et al. 2006; Bjork et al. 2007; Dewez et al. 2008; Chavarria et al. 2014; Black and Dos Santos 2015), s²U₃₄ biosynthesis is a good target for antibiotic development. The different architectures in their catalytic centers and domain structures of s²U₃₄ thiouridylases could provide a basis for the development of novel antibiotics, as specific inhibitors of the C-type MnmAs of pathogens are unlikely to inhibit the eukaryotic counterparts.

MATERIALS AND METHODS

Recombinant proteins

The expression plasmid for MnmA was pET22b-TtMnmA. The gene encoding T. thermophilus HB27 MnmA (Tc1727) was cloned into the NdeI and HindIII sites of pET22b (Novagen), resulting in the generation of a carboxy-terminally His₁₀-tagged fusion protein. The expression plasmid for the Ala variants was constructed by site-directed mutagenesis (QuickChange II Mutagenesis Kit, Stratagene). The sequences of the plasmids were confirmed by sequencing. The primers used are listed in Supplemental Table S1. The wild-type and variants of the His₁₀-tagged TtMnmA were expressed in the Rosetta2 DE3 E. coli strain (Novagen) and purified under

![FIGURE 3. Sulfur transferases involved in s²U synthesis in the three domains of life. In this study, it was found that a C-type TtMnmA is a Fe–S protein. See main text for details.](image-url)
aerobic conditions. Cultures were grown to an OD600 of approximately 0.6, induced with 0.5 mM isopropyl-β-D-galactopyranoside, and then incubated for an additional 6 h at 37°C. Cells were lysed for ~30 min at 25°C in A buffer [50 mM Hepes-KOH (pH 7.6), 200 mM (NH4)2SO4, 50 mM NH4OAe, 5 mM MgCl2, 10% glycerol, and 7 mM β-mercapto-ethanol (βME)] containing 1 x bugbuster reagent (Novagen) and 0.2 mM phenylmethylsulfonyl fluoride. The cell debris were then removed by centrifugation. The TtMnmA was concentrated using 30 kDa-cutoff Amicon Ultra filters (Merck Healthcare) with A buffer, and the reconstituted TtMnmA was purified under aerobic conditions. Cultures were grown to an OD600 of approximately 0.6, induced with 0.5 mM isopropyl-β-D-galactopyranoside, and then incubated for an additional 6 h at 37°C. Cells were lysed for ~30 min at 25°C in A buffer [50 mM Hepes-KOH (pH 7.6), 200 mM (NH4)2SO4, 50 mM NH4OAe, 5 mM MgCl2, 10% glycerol, and 7 mM β-mercapto-ethanol (βME)] containing 1 x bugbuster reagent (Novagen) and 0.2 mM phenylmethylsulfonyl fluoride. The cell debris were then removed by centrifugation. The TtMnmA was concentrated using 30 kDa-cutoff Amicon Ultra filters (Merck Healthcare) with A buffer. The TtMnmA samples purified under aerobic conditions were further purified using a Bio-Rad protein assay kit with bovine serum albumin as a standard. Amino-terminal His6-tagged versions of T. thermophilus IscS and SuS were purified as described previously (Shigi et al. 2006a).

**Reconstitution of the TtMnmA iron–sulfur cluster**

All manipulations were performed under strictly anaerobic conditions (<2 ppm O2) in an anaerobic glove box (CO2) under an atmosphere of 95% N2 and 5% H2 with a palladium catalyst. Solutions were made anoxic by bubbling for a minimum of 3 h. Apo-TtMnmA (0.03 mM) purified under aerobic conditions was incubated for 3 h at 25°C in A buffer containing 1.5 mM DTT, 0.3 mM FeCl3, and 0.3 mM Na2S. Excess FeCl3 and Na2S were removed using NAP 5 gel filtration columns (GE Healthcare) with A buffer. The reconstituted TtMnmA was concentrated using 30 kDa-cutoff Amicon Ultra filters (Merck–Millipore). Protein concentrations were determined using a Bio-Rad protein assay kit, and the Fe concentrations of the protein samples were determined by a colorimetric method with FeSO4 as a standard (BioChain) (Hoppe et al. 2003). The UV–VIS spectra of the protein samples were recorded using a UV-1800 spectrophotometer (Shimadzu). All of the samples were stored at 20°C and kept anoxic in a glove box.

**EPR spectroscopy**

Anaerobically reconstituted TtMnmA was concentrated to ~0.7 mM and then reduced or oxidized via the addition of 10 eq of sodium dithionite or potassium ferricyanide, respectively. Continuous-wave X-band EPR spectra were collected using a Bruker ELEXSYS E580 spectrometer operating at ~9.59 GHz, equipped with an Oxford Instruments ESR 910 continuous helium flow cryostat (Analytical Research Center for Experimental Sciences, Saga University). Other experimental parameters are indicated in the figure legends. Simulation of EPR spectra was performed using the EasySpin 5.2.20 program (Stoll and Schweiger 2006) in the MATLAB environment.

**In vitro tRNA transcription**

tRNAlys and tRNA<sub>Gln</sub> (with a U1G mutation for efficient transcription) were prepared by in vitro transcription using T7 RNA polymerase (Milligan and Uhlenbeck 1989). Templates for the in vitro transcription were constructed by PCR using synthetic oligonucleotide DNAs carrying the tRNA genes under the control of the T7 promoter sequence. The PCR products were cloned into the EcoRI and HindIII sites of pUC18. Plasmids for the U34A mutants were constructed by site-directed mutagenesis (QuikChange II Mutagenesis Kit, Stratagene). For the in vitro transcription reactions, primer pairs were used to PCR-amplify the template plasmid. The primers used are listed in Supplemental Table S1. The transcripts of the tRNA genes were prepared at 37°C for 3 h in a reaction mixture containing 40 mM HEPES-KOH (pH 7.6), 100 mM KCl, 10 mM MgCl2, and 0.2 mM phenylmethylsulfonyl fluoride. The RNA was extracted from the reaction mixture by the acid-quinadium thiocyanate-phenol-chloroform reagent (Isogen, Wako) and precipitated with 2-propanol. Next, the reaction for CCA addition was performed as described previously (Tomari et al. 2000). The transcripts of the tRNA genes were treated with E. coli CCA-adding enzyme at 37°C for 30 min in a reaction mixture containing 50 mM HEPES-KOH (pH 7.6), 100 mM KCl, 10 mM MgCl2, 2 mM DTT, 1 mM ATP, and 0.2 mM CTP. The RNA was recovered using Isogen, precipitated by 2-propanol, and further purified using denaturing 10% PAGE.

**In vitro s<sup>2</sup>U formation with holo-TtMnmA**

s<sup>2</sup>U formation on substrate tRNA was assayed under strictly anaerobic conditions in a glove box. The standard assay was performed at 60°C for 5 – 30 min in 60 µL of H buffer (50 mM Hepes-KOH [pH 7.6], 100 mM KCl, 10 mM MgCl2, and 0.1 mM DTT) containing 2.5 mM ATP, 0.1 mM Na2S, 450 pmol of tRNA, and 9.4–75 pmol of holo-MnmA. For the cysteine desulfurase coupled reactions, 0.1 mM Cys, 20 µM pyridoxal phosphate, and 75 pmol of IscS or SuS were included in the reaction mixture. For the experiment under nonreducing conditions, βME was removed from the enzyme stock solutions using NAP 5 gel filtration columns (GE Healthcare), and H buffer without DTT was used. To determine the initial velocities, the reactions were analyzed in triplicate with tRNA<sup>34s</sup>U (Jena Bioscience) as standard. Details of the determination of initial velocities are presented in Supplemental Figure S4.

The reaction using [35S]-Cys as the S donor was performed using 20 µM [35S]-Cys (6 µCi, PerkinElmer), 75 pmol of cysteine desulfurases, and 19 pmol of holo-MnmA in the presence of 20 µM pyridoxal phosphate, 2.5 mM ATP, and 0.1 mM DTT. After the completion of the reactions, the recovered RNA was separated by PAGE in 10% gels containing 7 M urea, and the gels were stained with 0.025% toluidine blue. Finally, the gels were dried, exposed on an imaging plate, and analyzed using the BAS 2500 system (Fuji Photo Systems).

**Analysis of tRNA modifications by LC/MS**

For the HILIC/ESI-MS analyses, a ZIC-cHILIC column (3 µm particle size, 2.1 x 150 mm, Merck–Millipore) was used on a Q Exactive hybrid Quadrupole-Orbitrap mass spectrometer.
(Thermo Fisher Scientific) equipped with an ESI source and an Ultimate 3000 liquid chromatography system (Dionex) (Sakaguchi et al. 2015). The mobile phase consisted of 5 mM ammonium acetate (pH 5.3) (solvent A) and acetonitrile (solvent B). Total nucleosides were prepared by digesting 2–4 μg of a tRNA mixture from T. thermophilus HB87 or total RNA from E. coli BW25113 with 0.05 U nuclelease P1 (Wako) and 0.04 U dialyzed bacterial alkaline phosphatase (C75, Wako) in 20 mM trimethylamine-acetate buffer (pH 5.3) at 37°C for 1 h. Nucleosides dissolved in 90% acetonitrile were injected and chromatographed at a flow rate of 100 μL/min over a multistep linear gradient: 90%–80% B from 0 to 10 min, 80%–30% B from 10 to 30 min, 30% B for 10 min, and then 90% B. The proton adducts of the nucleosides were scanned in a positive polarity mode (m/z 110–900).

SUPPLEMENTAL MATERIAL
Supplemental material is available for this article.

ACKNOWLEDGMENTS
We would like to thank Dr. Toshifumi Ueda (AIST) for constructing the plasmids encoding the MnmA mutants. We would also like to thank Dr. Koh Takeuchi (AIST) for comments on this study. This study was supported in part by Grants-in-Aid for Scientific Research (16K07311 to N.S., 17H06955 and 18H02412 to M.H.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, the Precise Measurement Technology Promotion Foundation (to N.S.), the Takeda Science Foundation (to N.S.), and Sumitomo Electric Group CSR Foundation (to N.S.).

Received September 6, 2019; accepted December 1, 2019.

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