Biochemical and structural characterization of oxygen-sensitive 2-thiouridine synthesis catalyzed by an iron-sulfur protein TtuA

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Two-thiouridine (s^2U) at position 54 of transfer RNA (tRNA) is a posttranscriptional modification that enables thermophilic bacteria to survive in high-temperature environments. s^2U is produced by the combined action of two proteins, 2-thiouridine synthetase TtuA and 2-thiouridine synthesis sulfur carrier protein TtuB, which act as a sulfur (S) transfer enzyme and a ubiquitin-like S donor, respectively. Despite the accumulation of biochemical data in vivo, the enzymatic activity by TtuA/TtuB has rarely been observed in vitro, which has hindered examination of the molecular mechanism of S transfer. Here we demonstrate by spectroscopic, biochemical, and crystal structure analyses that TtuA requires oxygen-labile [4Fe-4S]-type iron (Fe)-S clusters for its enzymatic activity, which explains the previously observed inactivation of this enzyme in vitro. The [4Fe-4S] cluster was coordinated by three highly conserved cysteine residues, and one of the Fe atoms was exposed to the active site. Furthermore, the crystal structure of the TtuA-TtuB complex was determined at a resolution of 2.5 Å, which clearly shows the S transfer of TtuB to tRNA using its C-terminal thiocarboxylate group. The active site of TtuA is connected to the outside by two channels, one occupied by TtuB and the other used for tRNA binding. Based on these observations, we propose a molecular mechanism of S transfer by TtuA using the ubiquitin-like S donor and the [4Fe-4S] cluster.

Fe-S cluster | sulfur transfer | tRNA modification | 2-thiouridine | crystal structure

Thiolation of nucleotides is a posttranscriptional modification of RNA widely conserved in three domains of life. To date, 22 types of thionucleosides have been identified (1), classified into four major groups: 2-thiouridine (s^2U), 4-thiouridine (s^4U), 2-thiocytidine (s^2C), and 2-methylthio-*N*6-alkyladenosine (2). Most of these modifications are found in transfer RNA (tRNA), where they have various important cellular roles, such as controlling the fidelity of translation (3–5), recognizing aminoacyl-tRNA synthetase (6), sensing UV radiation stress (7), and stabilizing the ternary structure of tRNA for growth at high temperatures (8–10).

The sulfurtransferases of s^2U can be divided into two major groups: tRNA-specific 2-thiouridylase MnmA type and cytoplasmic tRNA 2-thiolation protein Ncs6/2-thiouridine synthetase TtuA type (10). The MnmA type is a well-studied prototype of tRNA thiolation enzymes. Bacterial MnmA is responsible for 5-methylaminomethyl-2-thiouridine at position 34 of several tRNAs (11). The thiolation reaction of this type of enzyme consists of two steps: a first activation step by ATP to form an adenylated intermediate of the target base, and a second nucleophilic substitution step of the sulfur (S) atom for the adenyl group (Fig. S1*A*) (12). This reaction is conducted by the *N*-type ATP-pyrophosphatase domain of the enzyme. A PP-loop motif (SGGXDS) located in this domain contributes to the hydrolysis of ATP in the first step. In the second step, a persulfide (R-SSH) S formed on modification enzymes nucleophilically attacks the adenylated intermediate, resulting in formation of the thiocarboxylated base. R-SSH is first generated from cysteine (Cys) by a Cys desulfurase, IscS (13, 14), then transferred directly or via S-carrier proteins to the catalytic Cys residue of MnmA, and finally used as an S donor (Fig. S1*A*).

Another prototype of thiolation enzymes, the Ncs6/TtuA type, is found in eukaryotic cytoplasm and thermophilic bacteria. The Ncs6/Ncs2 heterocomplex (15, 16) and TtuA (17) are sulfurtransferases responsible for 5-methoxycarbonylmethyl-2-thiouridine at position 34 of several cytosolic tRNAs ($tRNA^{Glu/Gln/Lys}$) in eukaryotes and for 5-methyl-2-thiouridine (m^5s^2U, or s^2T) (Fig. 1) at position 54 of almost all tRNA species in *Thermus thermophilus*, respectively (Fig. 1 and Fig. S1*B*). These proteins share the same catalytic domain (*N*-type ATP-pyrophosphatase domain) with MnmA-type enzymes, but have more conserved Cys residues (Figs. S1*B* and S2). Although a previous structural study has shown that

Significance

One of the posttranscriptional modifications of tRNA, 2-thiouridine (s^2U), enhances thermostability. Although extensive studies have been conducted to understand the mechanism behind this modification, many ill-defined points remain, because the S-transfer enzyme 2-thiouridine synthetase TtuA has shown very low activity in previous in vitro experiments. Here we demonstrate that TtuA requires oxygen-labile [4Fe-4S] clusters for its activity. Furthermore, we determine the crystal structure of TtuA in complex with the Fe-S cluster and ATP analog and also with its S-donor protein, 2-thiouridine synthesis sulfur carrier protein (TtuB). The combined actions of TtuA and TtuB using the Fe-S cluster aid the S-transfer mechanism.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 5B4E, 5B4F, and 5GHA).

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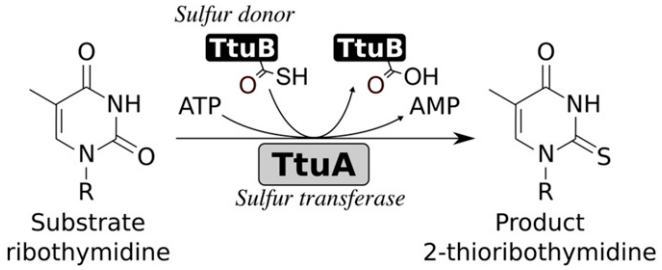

Fig. 1. Sulfur transfer reaction catalyzed by TtuA and C-terminal thiocarboxylated TtuB.

3 of 10 conserved Cys residues accumulate in the catalytic center, their functional roles remain unclear (18). It should be noted that the full in vitro enzymatic activity could not be confirmed for either Ncs6 or TtuA, and that quite weak activity was detected only after a cell extract was added, which hinders investigation of the molecular basis of S transfer by these types of enzymes. Based on these observations, it has been suggested that additional factor(s) may be required for the enzymatic activity of Ncs6 and TtuA (19, 20). More recently, TtcA, which is a homolog of Ncs6/TtuA but catalyzes s^2C synthesis at position 32 (21) of bacterial tRNA, was reported to require an Fe-S cluster for its activity (22). This finding suggests that Ncs6/TtuA also has an Fe-S cluster.

Distinct from the MnmA-type enzymes, Ncs6/Ncs2 and TtuA have another prominent characteristic on the S donor. The S atom is supplied as a thiocarboxylate moiety (R-COSH) on the C terminus of an ubiquitin-like S-carrier protein, i.e., Urm1 in eukaryotes (15–17, 23–25) and 2-thiouridine synthesis sulfur carrier protein TtuB in thermophiles (17, 20) (Fig. 1 and Fig. S1*B*). How Urm1/TtuB transfers the activated S atom to Ncs6/TtuA, which is necessary to elucidate the S-transfer mechanism catalyzed by Ncs6/TtuA-type enzymes, remains unclear, however.

In the present study, we characterize the sulfurtransferase TtuA from *T. thermophilus* from biochemical, spectroscopic, and structural viewpoints. We demonstrate that a [4Fe-4S] type of an Fe-S cluster is essential for the activity of TtuA. Furthermore, we determine the crystal structures of TtuA in complex with the [4Fe-4S] cluster and ATP analog (AMPPNP) and the TtuA-TtuB complex. Based on these results, we reveal a molecular basis of the s^2U biosynthesis mechanism involving the Fe-S protein and ubiquitin-like S-donor protein.

Results

TtuA Is an Oxygen-Labile Iron-Sulfur Protein. We first investigated *T. thermophilus* TtuA from a spectroscopic standpoint. TtuA expressed in *Escherichia coli* and purified under aerobic conditions is almost colorless and exhibits no UV-visible (UV-VIS) spectrum suggestive of an Fe-S cluster (17). Because a component of Fe-S protein is known to be sensitive to oxygen, we attempted anaerobic reconstitution of the TtuA Fe-S cluster. When the aerobically purified TtuA (apo-TtuA) was incubated with Fe and sulfide in anaerobic conditions, a brown solution of the TtuA protein was obtained that exhibited a UV-VIS spectrum with a shoulder at ~410 nm (Fig. 2*A*), which is characteristic of Fe-S proteins, namely the [4Fe-4S] form. The Fe content of reconstituted TtuA was determined to be 3.84 ± 0.08 mol per mol of TtuA, which also suggests the presence of a [4Fe-4S]-type Fe-S cluster.

To clarify the properties of the Fe-S center of TtuA in detail, we analyzed reconstituted TtuA by electron paramagnetic resonance (EPR) spectroscopy. Upon reduction by excess dithionite, the absorbance at ~410 nm was decreased (Fig. 2*A*). Reduced TtuA exhibited an EPR spectrum with principal *g* values of 2.06, 1.94, and 1.86 at 10 K (Fig. 2*B*, *a*), characteristic of $[4Fe\text{-}4S]^{1+}$ centers in proteins such as aconitase and fumarate and in nitrate reduction transcriptional regulator (FNR) (26, 27). We next analyzed TtuA treated with excess ferricyanide. On oxidation, the absorbance at around 410 nm was diminished (Fig. 2*A*). Oxidized TtuA exhibited an EPR spectrum with principal *g* values of 2.02, 2.01, and 2.00 at 10 K (Fig. 2*B*, *b*, *Upper*). At 40 K, the

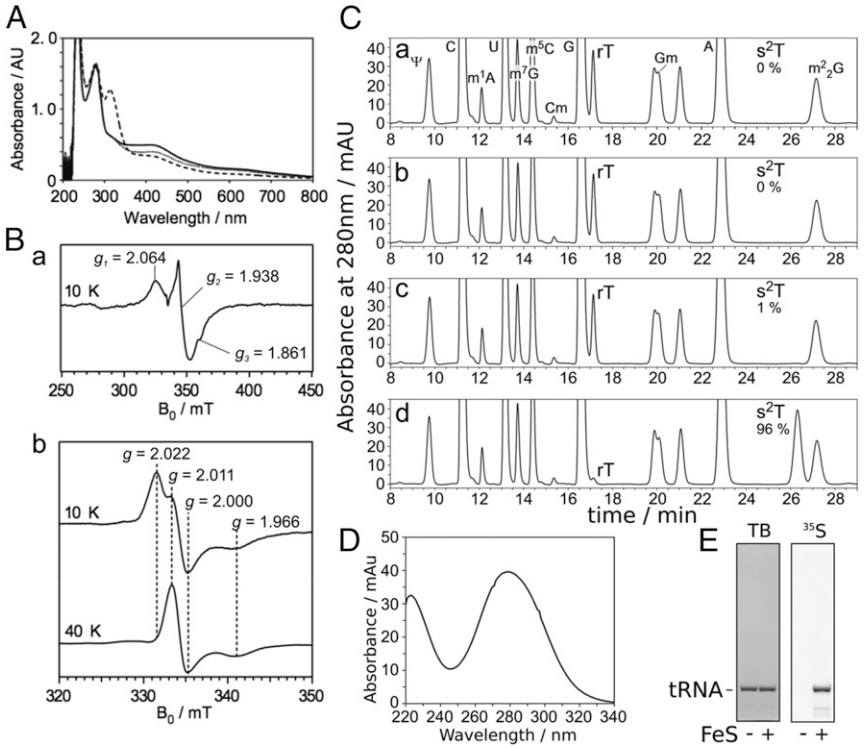

Fig. 2. The iron-sulfur cluster on TtuA is required for s^2T synthesis in vitro. (*A*) UV-VIS spectrum of TtuA. Reconstituted TtuA (solid line), TtuA treated with dithionite (dashed line), and TtuA treated with ferricyanide (dotted line) are shown. TtuA was diluted to 1.0 mg/mL in buffer A. The peak at around 310 nm in the reduced sample was derived from excess dithionite. (*B*) EPR spectrum of TtuA treated with dithionite at 10 K (*a*), and EPR spectra of TtuA treated with ferricyanide at 10 K (*b*, *Upper*) and 40 K (*b*, *Lower*). Principal *g* values are shown. (*C*) Nucleoside analysis of yeast $tRNA^{Phe}$ reacted in vitro. Apo-TtuA was treated with DTT (*a*), DTT and $FeCl_3$ (*b*), DTT and Na_2S (*c*), or DTT, $FeCl_3$, and Na_2S (*d*). Using these TtuA samples, tRNA (450 pmol) was reacted at 60 °C for 1 h with 75 pmol TtuA, TtuB, TtuC, TtuD, and SufS in the presence of 0.1 mM Cys and 2.5 mM ATP. The modified nucleosides of reacted RNA were analyzed by HPLC. The amounts of s^2T formed are indicated; complete conversion of rT to s^2T was set to 100%. (*D*) UV spectrum of s^2T detected at 26.3 min in *C*, *d*. (*E*) Incorporation of ^{35}S-sulfur to s^2T from [^{35}S]Cys. Yeast $tRNA^{Phe}$ (450 pmol) was reacted at 60 °C for 1 h with 75 pmol apo- or holo-TtuA, TtuB, TtuC, TtuD, and SufS in the presence of 0.1 mM [^{35}S]Cys and 2.5 mM ATP. The reacted RNA was separated by 10% (wt/vol) denaturing PAGE and then stained with toluidine blue (TB; *Left*), after which ^{35}S radioactivity was visualized (*Right*).

signal with $g = 2.01$ was the most intense (Fig. 2*B*, *b*, *Lower*). The signals with $g = 2.02$ and 2.00 obtained at 10 K are characteristic of the $[3Fe\text{-}4S]^{1+}$ center in proteins such as aconitase and RumA (28, 29). The signal with $g = 2.01$, which can be detected even at high temperatures (40 K), seemed to be an "unidentified radical" species commonly observed when [4Fe-4S] protein is oxidated by ferricyanide (29, 30). Both the $[3Fe\text{-}4S]^{1+}$ center and the radical are thought to be derived from the oxidative degradation of the $[4Fe\text{-}4S]^{2+}$ center (29, 30). These results suggest the existence of a $[4Fe\text{-}4S]^{2+}$ center in reconstituted TtuA. It should be noted that the EPR properties of TtuA are distinct from those of 4-thiouridine synthetase ThiI and *O*-phospho-L-seryl-tRNA:Cys-tRNA synthase SepCysS, which were recently reported to possess [3Fe-4S]-type clusters (*SI Discussion*) (31). When reconstituted TtuA was exposed to air, the brown color of the solution quickly disappeared and a white precipitate formed (Fig. S3), demonstrating the oxygen sensitivity of the Fe-S cluster of TtuA. Therefore, we conducted the subsequent biochemical experiments under anaerobic conditions.

The Iron-Sulfur Cluster of TtuA Is Required for Sulfurtransferase Activity. Because TtuA can bind to the oxygen-labile $[4Fe\text{-}4S]^{2+}$ cluster (see above), we investigated the requirement of the Fe-S cluster for the sulfurtransferase activity of TtuA in vitro. Here, under anaerobic conditions, we performed the 2-thiolation reaction of yeast $tRNA^{Phe}$, which possesses rT54, in the presence of recombinant proteins involved in s^2T biosynthesis: TtuA, TtuB, TtuC [an enzyme responsible for generating thiocarboxylated TtuB (20)], TtuD [a homolog of yeast Tum1 (19), an S carrier recently identified to enhance the reaction efficiency (32)], SufS [Cys desulfurase (17)]), 0.1 mM Cys, and 2.5 mM ATP. Several preparations of TtuA samples were used in this experiment (Fig. 2*C*). Nucleoside analysis of the reacted tRNAs by HPLC indicated that highly efficient s^2T formation occurred only after the addition of TtuA treated with $FeCl_3$ and Na_2S (Fig. 2*C*, *d*). Concomitant with the formation of s^2T (retention time, 26.3 min; the UV-VIS spectrum is also shown in Fig. 2*D*), the amount of substrate rT (retention time, 17.2 min) was reduced. Using ^{35}S-Cys as an S donor, the incorporation of ^{35}S-S in tRNA was readily detected using holo-TtuA (Fig. 2*E*). Taken together, these data demonstrate that the Fe-S cluster of TtuA is required for its sulfurtransferase activity.

In our previous work, we observed the in vitro formation of s^2T in tRNAs under aerobic conditions in quite low yields (~0.1%) only when the desalted cell extract of *T. thermophilus* was doped in (20). It is plausible that very small amounts of holo-TtuA in the reaction mixture catalyze the slow 2-thiolation reaction.

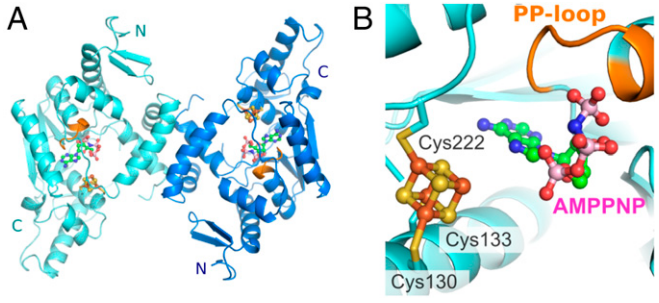

Fig. 3. Structures of TtuA in complex with the 4Fe-4S cluster and AMPPNP. (*A*) Overall structure of the Z-shaped TtuA dimer. Two protomers are shown in cyan and blue. The PP-loop is colored orange. The bound [4Fe-4S] cluster and AMPPNP are shown as balls and sticks (yellow indicates sulfate; brown, iron; green, carbon; blue, nitrogen; red, oxygen; pink, phosphorous). (*B*) Close-up view of the catalytic center of TtuA. The Fe-S cluster and AMPPNP are shown as balls and sticks. The PP-loop binding with AMPPNP is colored orange.

Crystal Structures of *T. thermophilus* TtuA Possessing the Fe-S Cluster and AMPPNP. The crystal structures of holo-TtuA from *T. thermophilus* and its complex with AMPPNP (hereinafter, the *Tth*TtuA-Fe-S-AMPPNP complex) were determined (Fig. 3*A*). In both crystals, TtuA forms a Z-shaped dimer, as has been observed for apo-TtuA (18). A difference Fourier map of the holo-TtuA crystal showed a clear cube-shaped peak in the vicinity of the three conserved Cys residues (Fig. S4*A*). Based on this observation, along with the spectroscopic and biochemical studies, we concluded that *Tth*TtuA contains a [4Fe-4S]-type Fe-S cluster. Three of four Fe atoms in the [4Fe-4S] cluster were coordinated by three conserved Cys residues (Cys130, Cys133, and Cys222), and the remaining Fe atom was chelated by three inorganic S atoms of Fe-S without any coordination by main chain or side chain groups of TtuA (Fig. 3*B*).

In the difference Fourier map of the AMPPNP-soaked crystal, we observed a distinct electron density corresponding to the AMPPNP around the PP-loop motif. The γ-phosphate of AMPPNP was coordinated by the PP-loop (Fig. 3*B*). A similar AMPPNP-binding mode was observed in *Mycobacterium tuberculosis* NAD^+ synthase (33) [Protein Data Bank (PDB) ID code 3SEQ], a member of the *N*-type ATP phosphatase group, suggesting that a common ATP recognition manner is adopted in this family. Furthermore, the bound ATP and the PP-loop were located close to the [4Fe-4S] cluster (Fig. S4*B*), suggesting that this region is a catalytic center of TtuA. The adenine moiety of AMPPNP was bound in a pocket of the catalytic center. No significant conformational change in TtuA was observed on AMPPNP binding.

In Vitro Characterization of Conserved Cys Residues of TtuA. Our structural study revealed that a [4Fe-4S] cluster is coordinated by three conserved Cys residues: Cys130, Cys133, and Cys222 (Fig. 3). To evaluate the importance of these Cys residues, as well as that of the [4Fe-4S] cluster, we reconstituted TtuA with the Fe-S cluster using a series of Ser-substituted mutants of these Cys residues, and then determined their Fe contents and enzymatic activities (Table S1). Fe content was decreased in each of these mutants (Table S1), and the shoulder peaking at ~410 nm in the UV-VIS spectrum was also diminished (Fig. S5), suggesting a reduced amount of the Fe-S cluster in these mutants. Concomitant with the decrease in quantities of Fe, 2-thiolation activities were also decreased for single mutants or completely abolished for double and triple mutants (Table S1). These data confirm that the Fe-S cluster chelated by the three Cys residues is essential for 2-thiolation activity.

In Vitro Characterization of s^2T Synthesis. We measured the 2-thiolation reaction of yeast $tRNA^{Phe}$ using various S donors. With use of the thiocarboxylated TtuB (TtuB-COSH), almost all TtuB-COSH added to the reaction mixture was converted to s^2T in tRNA. In contrast, almost no s^2T was detected when using TtuB-COOH (Fig. 4*A*). The initial velocity of the 2-thiolation reaction was measured without the addition of DTT (Fig. 4*B*). The reaction exhibited strong dependency on SufS, TtuB, or TtuC, consistent with in vivo data indicating that *ΔttuB* and *ΔttuC* mutants lack s^2T (17, 20). When ATP was omitted from the reaction mixture, the 2-thiolation reaction did not occur. We also measured the initial velocity of s^2T formation in the presence of 0.1 mM DTT (Fig. 4*C*). When sulfur was supplied by Cys/SufS, a twofold enhancement of the reaction was detected in the presence of DTT (assays 1 and 5), most likely because bisulfide (S^{2-}) was released from SufS-SSH and both S^{2-} and TtuB-COSH were used by TtuA. Indeed, S^{2-} itself could be engaged by TtuA (assays 4 and 8). Thus, the minimal components for reconstitution of the 2-thiolation reaction in vitro are holo-TtuA, Na_2S, and ATP. The reaction kinetics were approximately threefold faster in the assay conditions with 0.1 mM S^{2-} (assay 3) compared with in the conditions with 0.1 mM Cys/SufS (assay 1). The addition of TtuBCD enhanced the reaction by approximately fourfold (assays 3 and 4, or 7 and 8), most likely because TtuB-COSH also may be formed from S^{2-}.

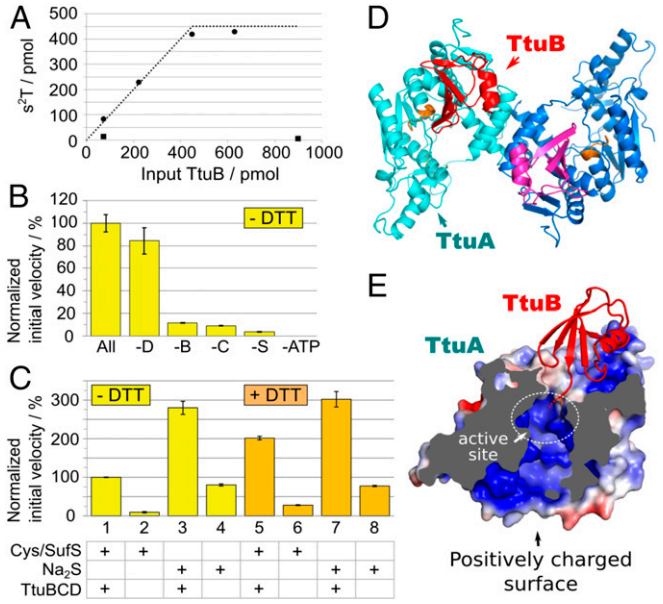

Fig. 4. (*A–C*) In vitro characterization of s^2T synthesis. The 2-thiolation reaction of yeast $tRNA^{Phe}$ (450 pmol; 7.5 pmol/μL) was examined at 60 °C with various combinations of the recombinant proteins, ATP, and sulfur donors. (*A*) The 2-thiolation reaction using TtuB-COSH as a sulfur donor. The reaction was performed in the presence of 75 pmol (1.25 pmol/μL) holo-TtuA, 2.5 mM ATP, and various amounts of either TtuB-COSH (closed circle) or TtuB-COOH (closed square) for 1 h. The *x*- and *y*-axes represent the amounts of input TtuB and s^2T formed, respectively. The dotted line represents the maximum possible values of s^2T had all of the sulfur atoms of input TtuB-COSH been incorporated into s^2T. The value plateaus at 450 pmol because that was the amount of tRNA present. (*B*) The reaction was performed in the presence of all protein factors (holo-TtuA, TtuB, TtuC, TtuD, and SufS), 0.1 mM Cys, and 2.5 mM ATP in the absence of DTT (All). –D, –B, –C, –S, and –ATP represent the absence of TtuD, TtuB-COOH, TtuC, SufS, and ATP in each reaction mixture, respectively. Holo-TtuA (9.4–75 pmol; 0.16–1.25 pmol/μL) and other proteins (75 pmol; 1.25 pmol/μL) were included in reaction mixtures. The initial velocity of s^2T formation is presented relative to that of the complete mixture (0.349 ± 0.027 pmol s^2T/pmol TtuA/min). The experiment was performed in triplicate, and the data are presented with SD values. (*C*) The initial velocity of s^2T formation was measured with 0.1 mM cysteine and 75 pmol (1.25 pmol/μL) SufS (assays 1, 2, 5, and 6), or 0.1 mM Na_2S (3, 4, 7, and 8) as sulfur donors. 0.1 mM DTT was included in assays 5–8. Holo-TtuA (9.4–75 pmol; 0.16–1.25 pmol/μL) was included in all of the reaction mixtures. The values are presented relative to assay 1 (0.356 ± 0.002 pmol s^2T/pmol TtuA/min). TtuB-COOH, TtuC, and TtuD (75 pmol; 1.25 pmol/μL) were included in assays 1, 3, 5, and 7. The experiment was performed in triplicate, and the data are presented with SD values. (*D* and *E*) Crystal structure of *Tth*TtuA-TtuB (G65C) complex. (*D*) Overall structure of the TtuA-TtuB complex. Two protomers of TtuA dimer are colored in cyan and blue, and two molecules of TtuB are in red and magenta. The PP-loop is shown in orange. (*E*) Sectional view of the TtuA-TtuB complex. The surface of TtuA is colored according to the electrostatic potential (red, –4 kT/e; blue, +4kT/e) calculated with APBS (48, 49). The position of the catalytic site is indicated by a white dotted circle.

Crystal Structure of the TtuA-TtuB Complex. In the s^2T54 synthesis pathway of *T. thermophilus*, the S is transferred from the C-terminal thiocarboxylate of TtuB (TtuB-COSH) to the tRNA by the catalytic activity of TtuA. To elucidate the details of S delivery by TtuB-COSH, we determined the crystal structure of the TtuA-TtuB complex in the absence of the Fe-S cluster at a resolution of 2.5 Å, using a TtuB G65C mutant (34). Two copies of the Z-shaped TtuA dimer were present as an asymmetric unit, in which each protomer of TtuA independently captured TtuB (Fig. 4*D* and Table S2). No significant structural diversity among the four TtuA-TtuB complex copies was observed (rmsd <0.5 Å). On binding with TtuB, the conformation of a loop containing Cys222 of TtuA was fixed, which covers the active site. Consequently, the active site of TtuA connected to the exterior through two tunnels (Fig. 4*E*). TtuB was bound on the exit of one of the tunnels by its C-terminal loop inserted deep into the tunnel. Although two residues of the C terminus were invisible owing to disorder, the orientation reasonably accounts for the manner of the S transfer of TtuB to TtuA. The absence of the Fe-S cluster is a plausible cause of the disorder of the C terminus of TtuB (Discussion). The other tunnel connects the active site to the positively charged surface, which may allow it to interact with tRNA (18). These structural characteristics strongly suggest that TtuA interacts simultaneously with both tRNA and TtuB during the S-transfer reaction (Discussion).

Discussion

In Vitro Reconstitution of 2-Thiouridine Synthesis in *T. thermophilus*. We were able to characterize s^2T54 synthesis in vitro using TtuA reconstituted with the Fe-S cluster under anaerobic conditions (Figs. 2 and 4). Our results indicate that TtuA has a [4Fe-4S] cluster (Fig. 3), and that this cluster is required for in vitro s^2T54 synthesis (Fig. 2 *C* and *E*).

Distinct from the previously reported in vitro s^2T synthesis reaction using doped cell-free extract (20), the present in vitro system used only recombinant proteins, and the efficiency of the reaction was very high, which allowed us to establish the detailed characterization of s^2T synthesis in vitro. The S atoms of TtuB-COSH (Fig. 4*A*) and Cys (Fig. 4*B*) were effectively incorporated into s^2T, and turnover of the S-transfer reaction was observed. Furthermore, holo-TtuA did not form s^2T in the absence of the S source (TtuB-COSH or Na_2S). These observations demonstrate that the S atom of the [4Fe-4S] cluster is not incorporated into s^2T, and thus the Fe-S cluster does not act as S reservoir for the reaction, but must play another important role in the enzymatic reaction.

Structural Implication of the Iron-Sulfur Cluster in TtuA. Our crystal structure analysis revealed that the [4Fe-4S] cluster was coordinated by three conserved Cys residues: Cys130, Cys133, and Cys222 (Fig. 3). Mutation analysis demonstrated that the substitution of these conserved Cys residues of TtuA caused a significant decrease in enzymatic activity (Table S1). This finding is consistent with the results of a previous mutation analysis in vivo (18); therefore, we concluded that the absence of these residues led to the loss of the Fe-S cluster, which results in the inactivation of TtuA. The [4Fe-4S] cluster reportedly has various roles, such as in stabilization of the protein structure, as an electron donor, for substrate binding, and for enzymatic reactions (35). Most of the Fe-S clusters chelated by the four Cys residues are responsible for structure stabilization and electron transfer. Because *Pyrococcus horikoshiii* TtuA, which lacks Fe-S clusters, maintained a similar structure as our [4Fe-4S] cluster-bound form even after incubation at 85 °C (rmsd = 0.862 for 217 Cα atoms), its function in TtuA likely extends beyond just structure stabilization. In contrast, the Fe with free coordination sites of Fe-S clusters is often used for substrate binding and in enzymatic reactions. Given that the [4Fe-4S] cluster of TtuA was chelated by three Cys residues, the Fe with a free coordination site of the Fe-S cluster of TtuA may contribute to an enzymatic role.

Structural Insight into the TtuA-TtuB-tRNA Ternary Complex. One of the most important benefits of focusing on the structural study of TtuA is that its S donor, TtuB, has been identified. We have elucidated its structural basis in this study. Because TtuA transfers the S atom from the thiocarboxylated C terminus of TtuB to tRNA, TtuA should bind these two macromolecules during the reaction. Our crystal structure of the TtuA-TtuB complex demonstrates that TtuB delivers the S atom by inserting its C terminus into one of the tunnels leading to the active site (Fig. 4*E*). Furthermore, the structure suggests that the positively charged surface located opposite the TtuB binding site is used for binding with tRNA. This

hypothesis is also supported by the superposition of our TtuA structure onto the *Geobacillus kaustophilus* TilS-tRNA complex (PDB ID code 3A2K). TilS is a tRNA modification enzyme similar to TtuA in terms of function (3) and structure (36, 37); that is, both enzymes adenylate uridine bases using the PP-loop domain. In the superposed model, the substrate tRNA was located within the tunnel connecting the active site and the positive surface (Fig. S6*A*).

Considering the foregoing observations together, we propose a TtuA-TtuB-tRNA ternary complex structure model in which TtuB and tRNA simultaneously access the active site through two different tunnels (Fig. S6*A*). This manner of binding allows all of the participants (i.e., the Fe-S cluster, ATP, the thiocarboxylated C terminus of TtuB, and the substrate uridine base) to enter the narrow catalytic site of TtuA. In particular, the Fe with a free coordination site of the Fe-S cluster faces the C terminus of TtuB, ATP, and the uridine base (Fig. S6*B*), which reasonably suggests an enzymatic role of the Fe-S cluster in s^2T generation. Through formation of the TtuA-TtuB-tRNA ternary complex, accurate S transfer between two macromolecules (i.e., from the C terminus of TtuB to tRNA) could be achieved.

Role of the Iron-Sulfur Cluster of TtuA. s^2T synthesis comprises two reactions, adenylation and thiolation (Fig. S1). An initial adenylation reaction is commonly observed for many other *N*-type ATP-pyrophosphatases, such as MnmA and TilS. In these enzymes, ATP is bound close to the nucleobase, followed by transfer of the adenyl group from ATP to the nucleobase by the inherent enzymatic activity of the PP-loop domain (38). To our knowledge, no PP-loop domain is reported to catalyze its adenylation reaction in a Fe-S cluster-dependent manner. Furthermore, MnmA does not possess the Fe-S cluster, even though it catalyzes the adenylation of tRNA using the PP-loop domain. Based on the foregoing observations, we can conclude that the Fe-S cluster in TtuA does not contribute to the initial adenylation reaction.

Thiolation makes use of the S atom supplied by the ubiquitin-like protein TtuB. Our in vitro experiments demonstrate the importance of TtuB for the enzymatic activity of TtuA (Fig. 4). Furthermore, a previous report showed that the generation of s^2T was completely obstructed in the *ttuB* gene-deficient strain (17). Such an S-transfer pathway of s^2T biosynthesis is reminiscent of thiamin and molybdenum cofactor biosynthesis (39) (40). Ubiquitin-like proteins ThiS and molybdopterin synthase sulfur carrier subunit (MoaD) also serve as S donors in those pathways, in which the S atom of the C-terminal thiocarboxylate directly attacks the substrate nucleophilically. Analogous to these homologous proteins, the S on the C-terminal thiocarboxylate of TtuB nucleophilically attacks the adenylated nucleobase, completing the thiolation reaction (Fig. S7 *A*, *d* and *B*, *iii-1* and *iii-2*). Of note, the C-terminal loop of TtuB appeared quite flexible (Fig. S4*C*). Although the C terminus Gly (G65) was substituted for Cys so as to mimic the thiocarboxylated state, the two terminal residues of TtuB were completely disordered in all four copies of the determined TtuA-TtuB complex structure. In contrast, the C terminus loops of ThiS and MoaD were rigid in the catalytic sites of their corresponding thiotransfer enzymes, ThiG and MoaE, respectively (PDB ID codes 1TYG and 2QIE). The Fe with a free coordination site at the Fe-S cluster is anticipated to be located close enough to interact with the C terminus of TtuB (Fig. S6). The Fe-S cluster may coordinate and constrain the flexible C terminus of TtuB in an appropriate orientation for the direct attack (Fig. S7 *A*, *d* and S7 *B*, *iii-1*), which would then enhance the rapidity and fidelity of a series of the S-transfer reactions of TtuA shown in Fig. S7*B*, *iii-1*, *iii-2*, and *v*), including nucleophilic substitution by an S atom for AMP, an elimination-addition reaction of the thiocarboxylated C terminus of TtuB, double-bond formation between the released S atom and nucleobase, and protonation.

Another possible scenario is an indirect S-transfer mechanism in which the S atom supplied by TtuB is first extracted from the C-terminal thiocarboxylate and then used as the substrate for TtuA (Fig. S7 *A*, *e-1* and *2* and *B*, *iv-1*–*iv-3*). Our in vitro experiment shows that TtuA can use Na_2S as the S source (Fig. 4*C*), indicating that a bare sulfide ion is sufficient for the reaction in vitro. Furthermore, some organisms possessing TtuA do not have TtuB-like S carriers. These observations suggest that thiocarboxylate is not used in its native form, but rather is used after decomposition to a sulfide ion and carboxylate group. In this mechanism, the Fe-S cluster might receive the S atom liberated from TtuB-COSH on its Fe with a free coordination site owing to the inherent affinity for sulfur, as has been observed for tRNA-2-methylthio-*N*6-dimethylallyladenosine synthase (MiaB), another sulfur transfer enzyme using Fe with a free coordination site as a receptor of sulfide ions (41).

Materials and Methods

Analysis of tRNA Modification. Hydrolysates of tRNAs from *T. thermophilus* and the in vitro assay were prepared and analyzed by HPLC with a photodiode array detector (GL Sciences) as described previously (20). The amount of s^2T was quantified using pseudouridine as a standard.

Recombinant Proteins for Biochemical Assays. N-terminal His_6-tagged *T. thermophilus* TtuA and TtuD were expressed in *E. coli* and purified as described in *SI Materials and Methods*. The N-terminal His_6-tagged versions of *T. thermophilus* TtuB, TtuC, and SufS were purified as described previously (17, 20). Protein concentrations were determined using a Bio-Rad protein assay kit with a BSA standard. The C-terminal carboxylate and thiocarboxylate versions of TtuB were prepared using the intein fusion technique (IMPACT Kit; New England BioLabs) (42). The fusion protein was cleaved in the presence of DTT and $(NH_4)_2S$ for the preparation of TtuB-COOH and TtuB-COSH, respectively. The production of TtuB-COSH was confirmed by SDS/PAGE with the gels containing [(*N*-acryloylamino)phenyl]mercuric chloride (APM) (43, 44) and LC/MS (Fig. S8). The APM gel showed that ∼70% of the sample cleaved by sulfide was TtuB-COSH. Detailed procedures for the preparation and confirmation of TtuB-COSH are described in *SI Materials and Methods*.

Reconstitution of the Iron-Sulfur Cluster of TtuA. Apo-TtuA was purified under aerobic conditions as described in *SI Materials and Methods*. All manipulations were performed under strictly anaerobic conditions in an anaerobic glove box (Coy Laboratory Equipment) in an atmosphere of 95% N_2 and 5% H_2. Solutions were made anoxic by bubbling for a minimum of 3 h. TtuA (0.032 mM) was incubated for 3 h at 25 °C in buffer A [50 mM Hepes-KOH pH 7.6, 200 mM $(NH_4)_2SO_4$, 50 mM NH_4OAc, 5 mM $MgCl_2$, 10% (vol/vol) glycerol, and 7 mM β-mercapto-ethanol (β-ME)] with 1.5 mM DTT, 0.3 mM $FeCl_3$, and 0.3 mM Na_2S. Excess $FeCl_3$ and Na_2S were removed using a NAP 5 gel filtration column (GE Healthcare) with buffer A, after which reconstituted TtuA was concentrated using 10-kDa cutoff Amicon Ultra filters (Merck Millipore). Protein concentrations were determined using a Bio-Rad protein assay kit, and the Fe concentration of protein samples was measured by a colorimetric method with $FeSO_4$ as a standard (Bio-Chain). All samples were stored at 20 °C and kept anoxic in a glove box.

EPR Spectroscopy. Anaerobically reconstituted TtuA was concentrated to ∼0.9 mM, then reduced or oxidized by 10 eq of sodium dithionite or 5 eq of potassium ferricyanide, respectively. EPR spectra were obtained with a Bruker ELEXSYS E580 spectrometer in continuous-wave mode operating at 9.38 GHz. An Oxford liquid helium flow cryostat was used for cryogenic measurements. The microwave frequency was monitored by a frequency counter (Bruker SuperX-FT bridge), and the magnetic flux density was measured with a Teslameter (Bruker ER 036TM). The microwave power was 1 mW.

In Vitro s^2T Formation with Holo-TtuA. s^2T formation on substrate tRNA was assayed in strictly anaerobic conditions in a glove box. The standard assay was performed at 60 °C for the indicated times in 60 μL of buffer H (50 mM Hepes-KOH pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, and 0.1 mM DTT) containing 2.5 mM ATP, 0.1 mM Cys, 20 μM pyridoxal phosphate, 450 pmol yeast $tRNA^{Phe}$ (Sigma-Aldrich), and 75 pmol of each recombinant protein (holo-TtuA, TtuB, TtuC, TtuD, and SufS). After the reactions, RNA was recovered using an acid-guanidinium thiocyanate-phenol-chloroform reagent (Isogen; Wako Chemicals) and precipitated with ethanol. tRNA modifications were analyzed by HPLC and quantified. For the experiment depicted in Fig. 4 *B* and *C*, β-ME in the enzyme stock solutions was removed by NAP 5 gel filtration columns (GE Healthcare).

The reaction with ^{35}S-Cys as an S donor was performed using 0.1 mM ^{35}S-Cys (6 μCi). RNA was separated by PAGE on 10% (wt/vol) gels containing 7 M urea, and the gels were stained with 0.025% (wt/vol) toluidine blue. Finally, the gels were dried, exposed on an imaging plate, and analyzed using the BAS 2500 system (Fuji Photo Systems).

Crystal Structure Analysis. The holo-TtuA used for crystallization was prepared as described in *SI Materials and Methods*. Crystals of holo-TtuA were obtained from a reservoir solution consisting of 0.1 M sodium acetate (pH 3.8–5.6), and 30–60% (vol/vol) 1,2-propanediol at 25 °C. For the AMPPNP complex, the holo-TtuA crystals were soaked in a crystallization buffer containing 5 mM of AMPPNP for 30 s. Diffraction data for the TtuA-Fe-S-AMPPNP crystal complex were collected using beam line BL17A of the Photon Factory. Data were indexed, integrated, and scaled with XDS (45). The crystal structure was determined by the molecular replacement method with Phase-MR (46) using a polyalanine model of apo-*P. horikoshii* TtuA (18) as a search probe. Structure refinement was carried out with phenix.refine (47). Finally, the crystallographic R_{work} and R_{free} factors converged to 21.9% and 25.7%, respectively, for holo-TtuA and to 21.6% and 23.7%, respectively, for the AMPPNP complex. Refinement statistics are summarized in Table S2.

Crystallization, data collection, and initial phase determination of the TtuA-TtuB complex were performed as reported previously (34). Finally, the crystallographic R_{work} and R_{free} factors converged to 19.6% and 24.7%, respectively.

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