The tRNA recognition mechanism of the minimalist SPOUT methyltransferase, TrmL

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Received February 22, 2013; Revised June 3, 2013; Accepted June 4, 2013

ABSTRACT
Unlike other transfer RNAs (tRNA)-modifying enzymes from the SPOUT methyltransferase superfamily, the tRNA (Um34/Cm34) methyltransferase TrmL lacks the usual extension domain for tRNA binding and consists only of a SPOUT domain. Both the catalytic and tRNA recognition mechanisms of this enzyme remain elusive. By using tRNAs purified from an Escherichia coli strain with the TrmL gene deleted, we found that TrmL can independently catalyze the methyl transfer from S-adenosyl-L-methionine to tRNALeuCAA and tRNALeuUAA isoacceptors without the involvement of other tRNA-binding proteins. We have solved the crystal structures of TrmL in apo form and in complex with S-adenosyl-homocysteine and identified the cofactor binding site and a possible active site. Methyltransferase activity and tRNA-binding affinity of TrmL mutants were measured to identify residues important for tRNA binding of TrmL. Our results suggest that TrmL functions as a homodimer by using the conserved C-terminal half of the SPOUT domain for catalysis, whereas residues from the less-conserved N-terminal half of the other subunit participate in tRNA recognition.

INTRODUCTION
Posttranscriptional modifications of transfer RNAs (tRNAs) are commonplace among the three kingdoms of life. Large amount of genes are involved in tRNA modification, between 1 and 10% of the genes in a given genome encode enzymes involved in tRNA modifications (1–6). The genes involved in tRNA modification outnumber the genes encoding the actual tRNAs, which indicates an extremely important role for these enzymes (1). The basic functions of tRNA modifications can be arranged into three classes (7). First, modifications in or around the anticodon loop improve the accuracy of decoding (8–10). Second, modifications in the main body affect the folding and stability of tRNAs (11–13). Third, various other modifications affect the tRNA identity (14–17). Besides directly affecting tRNA function, tRNA modifications have been shown to play regulatory roles such as responding to cellular stress, cancer or other diseases (18,19).

Most tRNA modifications were identified in the 1970s (1). Recently, however, nearly all the tRNA-modifying enzymes in model organisms such as Escherichia coli and Saccharomyces cerevisiae have been identified [RNA modification databases: http://rna-mdb.cas.albany.edu/RNAmods/ (20); http://modomics.genesilico.pl/ (21)]. The enzyme responsible for 2'-O-methylation at the 34th wobble position of the isoacceptors tRNACAA and tRNAUAA in E. coli was recently identified by mass spectrometry and designated TrmL (22). Deletion of TrmL in E. coli results in reduced efficiency of codon–wobble base interaction and affects recovery of cells from the stationary phase (22).

SPOUT represents a class of S-adenosyl-L-methionine (SAM)-dependent methyltransferases (MTases) (23,24). In 2002, Anantharaman et al. (23) first identified homology between the tRNA(Gm18) methyltransferase (SpoU, also named TrmH) and tRNA(m1G37) methyltransferase (TrmD) families and further defined the SPOUT (SpoU- TrmD) MTases superfamily. This was an important discovery owing to the low sequence similarity between SPOUT MTases, and crystal structures of SPOUT MTases were not available at that time.
However, several crystal structures of SPOUT MTases including TrmH (25) and TrmDs (26–28) have since been solved and have confirmed the homology between SPOUT MTases. All available structures of SPOUT MTases contain a common catalytic domain (SPOUT domain), which exhibits an unusual alpha/beta fold with a deep topological knot in the C-terminal half (25–29). Despite their ubiquitous nature, only a few SPOUT members have been functionally characterized (24). Of these, most are involved in posttranscriptional RNA modification by methylating the ribose or base moiety of tRNA or rRNA (24). It was thought that SPOUT MTases acted specifically on RNA; however, a recent study described a novel SPOUT MTase (Yor021c) that recognizes and methylates arginine residues on proteins (30).

Although the SPOUT domain contains a highly conserved structural fold, the amino acid sequences are not conserved throughout the SPOUT superfamily, and the specificity of substrate recognition cannot be predicted based on sequence or structural homology. Although the structures of multiple SPOUT MTases have been solved, there is currently no available structure of a SPOUT enzyme in complex with substrate, until recently, the crystal structure of TrmD dimer complex with a tRNA substrate was reported at the last tRNA Conference (31); therefore, the elucidation of the mechanism of substrate recognition results primarily from biochemical studies (32–37). Most SPOUT enzymes harbor C-terminal or N-terminal extensions, which serve to bind their RNA substrates (24,33). However, there is a group of minimalist SPOUT members, which contain only the catalytic SPOUT domain and lack the extension domains. This group includes the TrmL subfamily (22), RlmH subfamily for N3 methylpseudouridine at position 1915 in 23S ribosomal RNAs (38,39) and several other uncharacterized subfamilies (24). The enzymatic characterization and

Figure 1. Domain architectures of SPOUT tRNA MTases and the sequence alignment of TrmLs. (A) The common catalytic domain of SPOUT superfamily is represented as SPOUT; the extension domains are represented by secondary structures, and the amino acid length of the respective SPOUT MTase from E. coli are labeled. (B) Structure-based multiple amino acid sequence alignment of TrmLs from model organisms. Ec, E. coli; Hi, H. influenzae; Bs, Bacillus subtilis; Tt, Thermus thermophilus; Pm, Prochlorococcus marinus; Dv, Desulfovibrio vulgaris; Mg, Mycoplasma genitalium. The secondary structure elements of EcTrmL are labeled above the alignment. The basic amino acid residues on the protein surface that are manipulated in this study are marked with a star. The conserved Tyr142 is marked by a pentagon.
substrate recognition abilities of these smallest SPOUT MTases have not yet been investigated.

In *E. coli*, thus far, four tRNA modification enzymes, TrmH (40,41), TrmD (42–44), TrmJ [tRNA (Um32/Cm32) methyltransferase] (45) and TrmL [tRNA (Um34/Cm34) methyltransferase] (22) have been indentified as members of the SPOUT superfamily; TrmH, TrmJ and TrmL belong to the SpoU family, and TrmD belongs to the TrmD family. All four enzymes contain a conserved domain architecture of TrmD, TrmJ and TrmH from tRNA binding. The domain architectures of TrmL, extension sequences (from 70 to 100 amino acids) for TrmD and TrmJ, and TrmH from *E. coli* (EcTrmL, EcTrmD, EcTrmJ and EcTrmH) are shown in Figure 1A. The C-terminal extension of EcTrmD consists of four α-helices and two β-strands, whereas that of EcTrmJ conatis just three α-helices. In contrast, the extension domain of EcTrmL is formed by two α-helices at both the N- and C-terminal ends of the SpoU domain (Figure 1A). The structure of TrmL (also known as Yibk) from *Haemophilus influenza* confirmed that TrmL is composed only of the catalytic SPOUT domain (PDB: 1MXI and 1J8S, 29). TrmL enzymes are widely distributed throughout the bacterial kingdom, and their average length is ~150 amino acids, which suggests that the extension domains for tRNA binding are absent in all the TrmL enzymes. The sequence alignment of TrmLs from several model organisms are shown in Figure 1B.

Considering the absence of RNA binding domains in TrmLs, it was hypothesized that the methylation reaction requires a protein partner to support RNA binding (29). It was later demonstrated that EcTrmL failed to methylate tRNA\textsubscript{CAA} transcripts *in vitro* (45). More recently, *in vitro* methyltransferase activity of EcTrmL could be detected using a long RNA chimera (~170 nt) containing tRNA\textsubscript{Leu}\textsuperscript{CAA} purified from *E. coli* as a substrate (22).

Here, we demonstrate that EcTrmL alone can efficiently methylate both tRNA\textsubscript{CAA} and tRNA\textsubscript{Leu}\textsubscript{CAA} isoa losers by using *in vivo*-purified tRNA substrates with certain modifications. By solving the crystal stuctures of EcTrmL in apo form (2.0 Å) and in complex with S-adenosyl-homocysteine (AdoHcy, SAH) (2.0 Å), we identified the cofactor binding pocket and the potential active site. The catalytic activity and the tRNA recognition requirements of EcTrmL were further studied by structure-based site-directed mutagenesis combined with electrophoretic mobility shift assay and kinetic data. Our results demonstrate that the minimalist SPOUT Mtase EcTrmL functions as a homodimer by engaging residues from both subunits to bind tRNA. This work may help to elucidate the biochemical mechanism of other minimalist SPOUT MTases.

**MATERIALS AND METHODS**

**Materials**

[Methyl-\textsuperscript{3H}] SAM and [\textsuperscript{3H}] L-leucine were purchased from PerkinElmer Inc. (Waltham, MA, USA); SAM, SAH, L-leucine, dithiothreitol, NTPs, 5′-GMP, pyrophosphate, Tris-base, β-mercaptopoethanol (β-Me), MgCl\textsubscript{2}, NaCl and KCl, and the reagents used to optimize crystallization conditions were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). Crystallization kits were from Hampton research (Aliso Viejo, CA, USA). Primers for PCR were synthesized by Invitrogen (Shanghai, China); Nickel-nitrolotriacetic (Ni-NTA) Superflow was purchased from Qiagen Inc. (Germany). KOD-plus mutagenesis kit, Pyrobest DNA polymerase and the dNTP mixture were obtained from Takara (Japan). The pET30b vector was from MerckMillipore (Darmstadt, Germany). The *E. coli* JW3581-1 strain in which the gene of TrmL was deleted (46) was purchased from the *E. coli* genetic stock center (Yale University, New Haven, CT, USA). In all, 3 mm filter papers and Superdex\textsuperscript{TM} 75 column were from GE Healthcare.

**Preparation of tRNAs**

Unmodified tRNAs were made by *in vitro* transcription by T7 RNA polymerase as described previously (47). Transcripts were purified by urea denaturing 12% polyacrylamide gel electrophoresis (PAGE) followed by elution. Finally, tRNAs were refolded by fast heating and slow cooling down in the presence of 5 mM MgCl\textsubscript{2}. The transcript of the tRNA\textsubscript{Leu}\textsuperscript{CAA} was named Ts\textsubscript{EctRNA\textsubscript{CAA}} and Ts\textsubscript{EctRNA\textsubscript{UAA}}, respectively.

Purification of *in vivo* expressed tRNA\textsubscript{Leu}\textsuperscript{CAA} was performed as following. Plasmids expressing EctRNA\textsubscript{CAA} and EctRNA\textsubscript{UAA} were introduced into *E. coli* MT102 or JW3581-1, which lacks the TrmL gene. After induction with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside for 12 h, cells were harvested, and crude tRNA was isolated by the standard phenol extraction procedure (48). The resulting tRNA preparations exhibited a 5–10-fold higher leucine-accepting activity than the crude tRNAs isolated from control *E. coli* strain without tRNA\textsubscript{Leu}\textsuperscript{CAA} overexpression. The tRNA preparations were further purified by DEAE-Sepharose chromatography, urea denaturing PAGE and C18 reversed-phase high pressure (or high performance) liquid chromatography chromatography. At each step, denaturing PAGE, native PAGE and a leucine-accepting activity assay were used to monitor the purification of tRNA\textsubscript{Leu}\textsuperscript{CAA}. The tRNA\textsubscript{Leu} obtained from JW3581-1 was named dTrmL\textsubscript{EctRNA\textsubscript{CAA}} and dTrmL\textsubscript{EctRNA\textsubscript{UAA}}, respectively.

**Purification of EcTrmL and its mutants**

The gene encoding EcTrmL was amplified from the genome of *E. coli* MT102. Site-directed mutagenesis of EcTrmL was performed by the KOD-plus mutagenesis kit as previously described (48). The genes encoding wild-type and mutant EcTrmLs were cloned into vector pET30b and expressed in *E. coli* BL21 (DE3). The proteins were purified by affinity chromatography on a Ni-NTA Superflow resin, followed by gel filtration chromatography with a Superdex\textsuperscript{TM} 75 column.

**Methyltransferase activity assay**

The methyltransferase activity of wild-type and mutant EcTrmLs for EctRNA\textsubscript{CAA} and EctRNA\textsubscript{UAA} were measured in reactions containing 100 mM Tris–HCl
(pH 8.0), 150 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 7 μM EctRNA¹⁶¹eu and 20 μM [³H] SAM at 37°C. Reactions were initiated by adding wild-type or mutant EcTrmLs (0.5 μM). For measuring the total level of methylation, 1 μM enzymes were applied. At various time intervals, aliquots were quenched by spotting on filters and washed with 5% trichloroacetic acid. The amount of radioactive [³H]-methyl-tRNA was measured in a Beckman LS6500 scintillation counting apparatus.

RESULTS

To determine whether TrmL could independently catalyze the methylation of tRNA¹⁶¹⁰, we performed an in vitro methyltransferase assay. EcTrmL was purified as a dimer by analytical gel filtration chromatography. EcTrmL was eluted at 11.63 ml, corresponding to a calculated molecular mass of 37.8 KDa (Figure 2A), as compared with the theoretical molecular mass of EcTrmL (157 amino acids) plus linked histag (9 amino acids) of 18.8 KDa, suggesting that EcTrmL is a dimer in solution. All fractions from this dimer peak were collected together and concentrated for the following methylation assays. For the tRNA substrates, we prepared different forms of the two isoacceptors EctRNA¹⁶¹⁰ and EctRNA¹⁶¹³ in vitro.

Analytical gel filtration

Analytical gel filtration was performed using a Superdex™ 75 column (10/30; column volume, 23.6 ml) in AKTApurifier Chromatograph FPLC system (GE). The buffer used for gel filtration is 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl₂ and 5 mM β-Me. SAH titrated in an identical buffer was used as a control. Binding isotherms were fit by nonlinear regression using Origin Software version 7.0 (MicroCal Inc.). The ITC data were fit to a one-site binding model using software provided by MicroCal (56, 57).

Isothermal titration calorimetry measurements

Isothermal titration calorimetry (ITC) measurements were performed at 25°C, using an ITC200 Micro-calorimeter (MicroCal Inc.). Experiments included 20 injections of 2 μl of SAH (1 or 1.5 mM) into the sample cell containing 100 μM proteins. The SAH and protein samples were all kept in the same buffer system which contains 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl₂ and 5 mM β-Me. SAH titrated in an identical buffer was used as a control. Binding isotherms were fit by non-linear regression using Origin Software version 7.0 (MicroCal Inc.). The ITC data were fit to a one-site binding model using software provided by MicroCal (56, 57).

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TrmL alone catalyzes the methyl transfer from SAM to tRNA¹⁶¹² and tRNA¹⁶¹³ in vitro

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in *E. coli* JW3581-1 deleted TrmL gene. As a control, fully modified tRNAs were purified from *E. coli* MT102. The tRNA\textsubscript{Leu}\textsuperscript{CAAs} were purified to homogeneity as shown by denaturing and native gel analysis (Figure 2B). These tRNAs all exhibited a leucine-accepting activity of \( \sim 1600 \) pmol/A\textsubscript{260} with *E. coli* leucyl-tRNA synthetase.

The tRNA transcripts and the *in vivo* purified tRNAs were then used as substrates for the methyltransferase assay. As shown in Figure 2C, EcTrmL alone was able to transfer the methyl group from SAM to Ec\textit{tRNALeuCAAs} when using the partially modified Ec\textit{tRNALeuCAAs} substrate. However, there was no detectable methyltransferase activity when using the unmodified Ec\textit{tRNALeuCAAs} substrate (Figure 2C). Similar results were observed with the Ec\textit{tRNALeuCAAs} substrate (Figure 2D). As a control, we also performed the assay on the fully modified Ec\textit{tRNALeuCAAs} substrate. As expected, no additional methyl groups can be transferred by EcTrmL to these tRNAs (Figure 2C and D). Table 1 shows the steady-state kinetic parameters of EcTrmL for various tRNA\textsubscript{Leus} for the methyl transfer reaction.

| tRNAs                     | Ec\textit{tRNALeuCAAs} or Ec\textit{tRNALeuUAA} transcripts | dTrmL\_Ec\textit{tRNALeuCAAs} | dTrmL\_Ec\textit{tRNALeuUAA} |
|---------------------------|-------------------------------------------------------------|-------------------------------|-------------------------------|
| tRNAs                    | \( K_m \) (\( \mu M \))                                   | \( k_{cat} \) (min\(^{-1}\)) | \( k_{cat}/K_m \)             |
| Ec\textit{tRNALeuCAAs}   | N/A                                                         | 0.44 ± 0.07                   | 0.13                          |
| Ec\textit{tRNALeuUAA}    | 3.39 ± 0.33                                                 | 0.59 ± 0.07                   | 0.50                          |
| dTrmL\_Ec\textit{tRNALeuCAAs} | 1.17 ± 0.07                            |                                |                               |

N/A: non-available, the results are the average of three independent repeats with standard deviations indicated.

Ec\textit{tRNALeuCAAs} (1.17 \( \mu M \)); the \( k_{cat} \) values of EcTrmL for Ec\textit{tRNALeuCAAs} (0.44 min\(^{-1}\)) and Ec\textit{tRNALeuUAA} (0.59 min\(^{-1}\)) were similar. The reported steady state \( k_{cat} \) values of bacterial SPOUT tRNA MTases range from 1.0 to 15 min\(^{-1}\) (35, 36, 58). The \( k_{cat} \) value of EcTrmL (0.44 min\(^{-1}\) and 0.59 min\(^{-1}\)) is much lower than that of *Streptococcus pneumoniae* TrmD [15 min\(^{-1}\), (36)], but in a comparable level with EcTrmD [1.0 min\(^{-1}\), (35)] and *T. thermophilus* TrmH [1.15 min\(^{-1}\), (58)]. The total tRNA methylation levels by
EcTrmL were calculated by measuring the methylation plateau with known tRNA concentration. Both EcRNA₁₆₅⁰⁰ and EcRNA₁₆₅₅⁰⁰ were methylated to 80–90% in 60 min (Supplementary Figure S1), which suggest that the tRNA substrates are homogenous.

Therefore, EcTrmL alone can catalyze methyl transfer from SAM to partially modified EcRNA₁₆₅⁰⁰ and EcRNA₁₆₅₅⁰⁰, but not to unmodified tRNA.  

**EcTrmL forms a homodimer**

To understand the tRNA recognition mechanism of TrmL, we attempted to crystallize the EcTrmL complexed with tRNA; however, we only obtained crystals of EcTrmL alone or with a bound cofactor. We determined the tertiary structure of EcTrmL in the presence and absence of SAH, which is the product of methyl transfer reaction. The final models of apo EcTrmL (PDB ID: 4JAK) and the EcTrmL-SAH binary complex (PDB ID: 4JAL) were both refined to 2.0 Å. Data collection parameters and refinement statistics are summarized in Table 2. The space groups of apo EcTrmL and EcTrmL-SAH were P3₂ and P2₁, respectively. Both crystals contain two molecules in one asymmetric unit, forming a tightly packed dimer (Figure 3A). Dimer formation is consistent with the observed molecular mass during analytical gel filtration. Structurally, the dimer is formed in a ‘perpendicular’ mode, i.e. one subunit (green) is rotated by ~90 degrees with respect to the mirror molecule of the other subunit (cyan) (Figure 3A).

The overall structure of an EcTrmL monomer subunit is composed of six β-strands and six α-helices, in the order β₁-α₁-β₂-α₂-α₃-β₃-α₄-β₄-β₅-α₅-β₆-α₆ (Figure 3A).

**Table 2. Data collection and refinement statistics**

| Measurement          | EcTrmL | EcTrmL-SAH |
|----------------------|--------|------------|
| Data collection      |        |            |
| Wavelength (Å)       | 1.58   | 1.58       |
| Resolution range (Å) | 4.07–1.58 | 4.07–1.58 |
| No. of total reflections | 42,478  | 42,478     |
| No. of unique reflections | 19,381  | 19,381     |
| Completeness (%)     | 92.6   | 92.6       |
| Rmerge (%)           | 0.08   | 0.08       |
| Redundancy (%)       | 4.5    | 4.5        |
| Space group          | P3₂    | P2₁        |
| Unit cell dimensions | a, b, c (Å) | 84.95, 84.95, 78.89 | 84.95, 84.95, 78.89 |
| α, β, γ (deg)        | 90.00, 90.00, 120.00 | 90.00, 90.00, 120.00 |
| Refinement           |        |            |
| Resolution (Å)       | 1.58   | 1.58       |
| Rwork, Rfree (%)     | 0.08   | 0.08       |
| No. of reflections   | 40,388 | 42,478     |
| No. of atoms         | 2,450  | 2,462      |
| Protein atoms        |        |            |
| Water/other          | 214    | 49.2       |
| SAH                  | 0      | 2          |
| HEPES                | 0      | 1          |
| R.m.s. deviations    |        |            |
| Bond lengths (Å)     | 0.008  | 0.009      |
| Bond angles (deg)    | 1.143  | 1.24       |
| Average B factor (Å²)| 41.2   | 39.6       |
| Ramachandran statistics (%) | Most favored | 99.3 | 98 |
| Allowed              | 0.7    | 2          |

Values in parentheses are for highest-resolution shell.

The parallel six-stranded β-sheet is flanked by four α-helices on one side and by two α-helices on the other side (Figure 3A). The N-terminal half forms a Rossmann-like fold, which is common among SAM-dependent MTases, whereas the C-terminal half forms a deep trefoil knot (Figure 3A). The knot is formed by threading the C-terminus (residues 121–157) through a hoop composed of residues 78–86 (Figure 3A), which forms a hydrophobic core that stabilizes the polypeptide knot. The cofactor product SAH is bound at this knot region (Figure 3A). The overall structure of EcTrmL is similar to the crystal structure of HtYibk (PDB: 1MXI and 1J85, 29), only with variations in loop regions.

The folding of the catalytic domain (cyan) of EcTrmL is similar to other known SPOUT MTases involved in tRNA modification, including Thermoanaerobacter thermoaceticus TrmH (TtTrmH, PDB: 1V2X) and H. influenzae TrmD (HiTrmD, PDB: 1UAL) (Figure 3B). The structure of EcTrmL can be superimposed onto the catalytic domain of TtTrmH with a core rmsd of 2.02 Å (136 Cα, with 23.5% sequence identity) and to HiTrmD with a core rmsd of 2.54 Å (99 Cα), despite having a sequence identity of only 9.1%. TrmL, however, lacks the extension domains involved in tRNA binding found in TtTrmH and HiTrmD (Figure 3B, magenta).

EcTrmL contains many basic amino acid residues, comprising 13 arginines (8.3%), three lysines (1.9%) and five histidines (3.2%) in a total 157 residues, most of which are located on the protein surface (Figure 3C). A region of positively charged basic residues is located around the SAH binding pocket as well as other distal regions.

**Cofactor binding pocket and active site of EcTrmL**

SAH binding does not significantly affect the overall structure of EcTrmL (Figure 3A). Although both subunits have a bound SAH, the conformations of the two SAHs are strikingly different with respect to the orientation of the homocysteine groups (Figures 4A–C and Supplementary Figure S2). The adenine and ribose groups of SAH form a broad interaction network with the cofactor binding pocket formed primarily by residues from three loops (residues 78–80; 99–106; 120–124) (Figure 4A and B), and their conformations are exactly the same in both subunits. The SAH in subunit A is in the characteristic bent conformation (Figure 4A) already observed for SAH and SAM found in TtTrmH (25), HtYibk (29) and TrmDs (26,28). The homocysteine group of SAH points toward the entrance of the catalytic crevice, which does not have many interactions with EcTrmL (Figure 4A). Although in subunit B, the homocysteine group of SAH is located near to residues Asn14, Glu102 and 130–133 (Figure 4B). The amine group of homocysteine forms hydrogen bonds with the side chain of Ser130 and the carboxyl group of Met131. The carboxyl group of homocysteine forms hydrogen bonds with residues Asn14, Asn132 and Leu133. The Asn14 is conserved throughout the SpoU family and was reported to be involved in the release of SAH (58).

In subunit B, a HEPES molecule from the crystal growth buffer is observed on top of SAH in the potential active site and is well stabilized with high density.
occupancy (Supplementary Figure S2). From the chemical structure (Figure 4D), the HEPES molecule is similar to the ribose and the phosphate group, which suggest that HEPES may mimic the U34 or C34 of the tRNA substrate in this context. The HEPES molecule is involved in a complex interaction network with residues 79–81, 103–106, Ser130 and SAH (Figure 4E). The residues involved in HEPES binding are consistent with the putative active site for the SpoU family MTases (25). The HEPES molecule in subunit B is located in the same position as the homocysteine group of SAH in subunit A (Figure 4C). This suggests that in subunit B, the structure is in a state in which the SAH and the tRNA substrate are both bound. The wider interaction network for SAH binding in subunit B provides structural evidence that these residues comprise the active site.

Figure 3. Overall structure of EcTrmL. (A) Ribbon diagram showing the overall structure of EcTrmL in Apo form (left) and in complex with SAH (right). The structures are shown as dimers, with one subunit in green and the other one in cyan. (B) A subunit from EcTrmL, TtTrmH and HiTrmD are superimposed and represented from the same perspective. The common SPOUT domains are in cyan, the extensions are in magenta, SAH and SAM are shown as sticks. (C) Crystal structure of EcTrmL with the surface colored in light gray, the basic Arg, His and Lys residues are in blue and the SAH are shown as spheres.
From the crystal structures of EcTrmL with SAH and HEPES, the SAH binding pocket and the active site are primarily formed by residues from the C-terminal half of the subunit. Three loops (residues 78–80, 99–106 and 120–124) form the SAH binding pocket, and on tRNA substrate binding, loops comprising residues 79–81, 103–106 and 130–133 form the active site. Asn14 from the N-terminal half is also involved in binding with SAH in this state.

Glu scanning of basic amino acid residues on the protein surface

Our results show that EcTrmL can independently catalyze the methylation of tRNA\(^{\text{Leu}}\) despite lacking the usual extension domain for tRNA binding. However, the tRNA binding mechanism of EcTrmL remains unclear. From the tertiary structure, a high basic amino acid content was observed on the surface of EcTrmL to the negatively charged amino acid Glu. When selecting residues for mutagenesis, we tried to avoid residues that may be involved in SAH binding or in dimer formation. Finally, 14 residues (Arg20, 28, 43, 45, 46, 59, 64, 74, 104, 129; Lys42 and 81; His30 and 60) were selected. With the exception of EcTrmL-H30E and -H60E, all of the single mutants were soluble and exhibited a dimer structure in solution.

We first analyzed the affinities of the wild-type and mutant EcTrmLs for tRNA by the gel mobility shift assay (Figure 5A). In all, 80 nM dTrmL\(_{\text{Ec}}\)tRNAl\(_{\text{CAA}}\) or \(^{32}\)P-labeled dTrmL\(_{\text{Ec}}\)tRNAl\(_{\text{CAA}}\) was used in the system (Figure 5A and Supplementary Figure S3). For wild-type EcTrmL, a shift was observed starting at 0.75 \(\mu\)M enzyme representing the EcTrmL-tRNA complex (tRNA bound 1). A larger molecular mass complex or aggregate was represented by a supershift (tRNA bound 2) above 4 \(\mu\)M EcTrmL (Figure 5A and Supplementary Figure S3). A similar high-molecular mass complex was reported in a previous study performed on TtTrmH (58). The \(K_d\) value was calculated by quantifying the intensities of the lower
EcTrmL-tRNA complex band. Owing to the formation of the aggregate complex at high protein concentration, it was difficult to precisely determine the $K_d$ value, although it was estimated to be $\sim 1.9 \mu M$. The tRNA binding affinities of EcTrmLs are compared in Figure 5A. The mutants can be divided into three groups. Mutants in the first group (EcTrmL-R20E, -K42E, -R43E, -R45E, -R46E, -R59E and -R129E)
showed no detectable binding to tRNA (Figure 5A). Mutants in the second group (EcTrmL-R104E) exhibited decreased binding affinity to tRNA as compared with EcTrmL, and mutants in the last group (EcTrmL-R28E, -R64E, -R74E and -K81E) had a similar binding affinity for tRNA as wild-type EcTrmL (Figure 5A).

The methyltransferase activity of the wild-type and mutant EcTrmLs was measured, and the results are shown in Figure 5B and C. The majority of mutants display a methyltransferase activity that is consistent with their tRNA binding affinity. All of the mutants in the first group that did not bind tRNA showed no detectable methyltransferase activity (Figure 5B). Similarly, EcTrmL-R104E of the second group showed a reduction in tRNA-binding affinity, with an associated decreased in methyltransferase activity (Figure 5C). In the last group, EcTrmL-R28E, -R64E and -R74E had the same methyltransferase activity as EcTrmL, which was consistent with their tRNA-binding affinity (Figure 5C). The exception was EcTrmL-K81E, which efficiently bound tRNA showed greatly reduced catalytic activity. This mutant was selected for further study.

We then used ITC to measure the SAH-binding affinity of all the mutants with reduced catalytic activity (Figure 5D). The results showed that EcTrmL-R20E, -K42E, -R43E, -R45E, -R46E, -R104E and -R129E had dissociation constants (KD) of SAH comparable to EcTrmL, suggesting that the defects in methyltransferase activity were not caused by reduced SAH binding, but instead from reduced tRNA binding. Again, EcTrmL-K81E represents an exception, as the binding affinity of SAH was similar to EcTrmL (Figure 5D), but the enthalpy change on SAH binding was noticeably decreased compared with that of EcTrmL or the other mutants. As the methyltransferase activity of EcTrmL-K81E was too low to solve the kinetic parameters, we made an Ala mutation to analyze the function of K81. The kinetic parameters of EcTrmL-K81A are shown in Supplementary Table S1. Compared with EcTrmL, the Kₘ value for tRNA remains unchanged, but the k₉₅ value is 40% lower than EcTrmL, suggesting that K81 is involved in catalysis, but not in tRNA binding.

Ala scanning identifies residues required for tRNA recognition

To further understand the role of the basic residues involved in tRNA binding, we made Ala mutations of the residues described earlier in the text and further analyzed their properties. All of the mutants showed decreased (EcTrmL-R45A, -R59A, -R104A and -R129A) or not-detectable affinity to tRNA (EcTrmL-R20A, -K42A, -R43A and -R46A), as measured by the gel mobility shift assay (Figure 6A). All of the mutants showed reduced methyltransferase activity compared with EcTrmL, whereas EcTrmL-R20A, -R43A, -R46A and -R129A had no detectable catalytic activity (Figure 6B). The Kₘ values of EcTrmL-R104A, -R59A, -K42A and -R45A, for tRNA were 3–20-fold higher than that of EcTrmL while their k₉₅ values did not change.

Figure 6. Ala mutation of residues involved in tRNA binding. (A) The binding affinities of EcTrmLs for dTrmL-Ec tRNA⁵⁴⁴⁺ analyzed by the gel mobility shift assay. (B) The methyltransferase activities of the various EcTrmL mutants.
significantly (Table 3). These results suggest that the loss of catalytic activity of these mutants is due to their low affinity for tRNA, and this is consistent with the results from the gel mobility shift assay.

**EcTrmL dimer formation is essential for tRNA recognition**

The basic residues involved in tRNA binding are shown in Figure 7A (magenta). These residues appear to form a positively charged patch, which could represent the tRNA-binding site. The positively charged patch includes residues R104 and R129 near the active site from one subunit, and residues R20, K42, R43, R45, R46 and R59 from the other subunit (Figure 7A). It therefore appears that tRNA binding requires the cooperation of both subunits of the dimer. Based on our unpublished data, the nucleotide residues in the anticodon loop of the tRNA are important for recognition by EcTrmL. The proposed model of EcTrmL of cooperative tRNA binding by both dimer subunits is presented in Figure 7B. According to this model, residues R20, K42, R43, R45, R46 and R59 from the N-terminal half of the SPOUT domain appear to substitute for the extension domains present in TrmH and TrmD.

Based on the proposed model, the tRNA is bound by residues from both subunits. However, there still remains the possibility that the tRNA binds only one subunit, in which case the dissociation of the subunits should not affect tRNA binding. We analyzed the dimer interface (Supplementary Figure S4) and identified the most important interactions between the subunits. Ala substitutions of the residues at the dimer interface were performed to disrupt the interactions and cause dissociation of the dimer structure. Only the mutant EcTrmL-Y142A could affect the oligomeric structure as shown by analytical gel filtration. The elution volume of EcTrmL-Y142A through a Superdex-75 gel filtration column under the same conditions as EcTrmL (Figure 8A) was 13.13 ml, which corresponds to a calculated molecular mass of 19.8 KDa, indicating that EcTrmL-Y142A is a monomer in solution. The results showed that the residue Y142 is critical for maintaining the dimeric form of EcTrmL, which is consistent with its central position at the interface (Supplementary Figure S4). In support of this model, Y142 is strictly conserved in the TrmL subfamily (Figure 1B). EcTrmL-Y142A failed to form a complex with tRNA in the gel mobility shift assay, suggesting that the monomeric form of EcTrmL cannot form a stable complex with the tRNA (Figure 8B). In addition, the methyltransferase activity of EcTrmL-Y142A was completely abrogated (Figure 8C), despite the fact that the isolated subunit could bind SAH with comparable affinity to EcTrmL, as shown by ITC experiments (Figure 8D).

**DISCUSSION**

The purified EcTrmL alone can efficiently transfer a methyl group from SAM to both tRNA_{Leu}^{CAA} and tRNA_{Leu}^{UAA} isoacceptors, but only to tRNAs, which contain certain posttranscriptional modifications, which were purified from *E. coli* strain with TrmL gene

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**Table 3. tRNA-binding affinity and kinetic parameters of wild-type and mutant EcTrmL**

| EcTrmL variants | Affinity for tRNA by electrophoretic mobility shift assay | $K_m$ (μM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ (relative) |
|-----------------|---------------------------------------------------------|------------|------------------------|------------------------|
| Wild type      | ++                                                     | 3.39 ± 0.33 | 0.44 ± 0.07            | 1                      |
| R20A           | +                                                      | N/D        | N/D                    | N/D                    |
| K42A           | +                                                      | 26.9 ± 11.7 | 0.62 ± 0.18            | 0.17                   |
| R43A           | −                                                      | N/D        | N/D                    | N/D                    |
| R45A           | +                                                      | 58.5 ± 7.6  | 0.37 ± 0.04            | 0.05                   |
| R46A           | −                                                      | N/D        | N/D                    | N/D                    |
| R59A           | +                                                      | 11.17 ± 1.59 | 0.43 ± 0.13          | 0.29                   |
| R104A          | +                                                      | 9.78 ± 1.55 | 0.55 ± 0.21            | 0.42                   |
| R129A          | +                                                      | N/D        | N/D                    | N/D                    |

N/D: not detectable, the results are the average of three independent repeats with standard deviations indicated.

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**Figure 7. The proposed model of tRNA_{Leu}^{CAA} binding to EcTrmL.** (A) The EcTrmL basic amino acid surface residues (magenta) that are identified as being involved in tRNA binding, the dimer structure is shown in cartoon loop with same color used as in Figure 3. (B) The proposed model of EcTrmL bound with tRNA, the backbone of EcRNA_{Leu}^{CAA} is shown in brown.
deleted. Hence, *EcTrmL* only recognizes its tRNA substrates containing the natural modifications at other sites. These results are consistent with an earlier *in vivo* study (22), which showed that deletion of the MiaA gene, which is involved in ms2i6 modification at A37, caused loss of 2'-O-methylation of U34 and C34 of tRNA_{Leu}^{CAA} or tRNA_{Leu}^{UAA}, respectively (22). Therefore, the result suggests that ms2i6 modification at nucleotide A37 is one of the modifications required for *EcTrmL* to recognize its tRNA substrates. From the proposed model of *EcTrmL*
with tRNA, residues including A37 from the tRNA anti-

codon loop may have potential interactions with \( Ec\)TrmL;

the \( ms^\text{\textsuperscript{256}} \) modification that provides extra groups on A37

base may increase the chance of having direct interaction with \( Ec\)TrmL. To understand the role of this modification

in tRNA recognition, solving the structure of \( Ec\)TrmL/
tRNA complex will be ideal. Recent studies showed that a

lack of non-essential tRNA modifications can lead to a

rapid decay of tRNA (59). As the regulation of

posttranscriptional modifications of tRNA \textit{in vivo} is extremely complex, we suggest that TrmL may serve as a

checkpoint such that tRNAs lacking the necessary modi-
fications will be excluded from the process of tRNA

maturation.

The presence of a HEPES molecule in the \( Ec\)TrmL-

SAH complex serves to mimic the U34/C34 nucleotide

of the tRNA substrate and allows the opportunity to probe interactions between SAH and \( Ec\)TrmL and provide more information about the active site. The

SAH-binding pocket and the active site are formed pri-

marily by residues from the C-terminal half of \( Ec\)TrmL.

This is similar to all other reported SPOUT MTases, which may explain the higher sequence conservation in the C-terminal half than in the N-terminal half of SPOUT domains (24).

We have identified a series of basic amino acid residues (R20, K42, R43, R45, R46, R59, R104 and R129) on the protein surface that are involved in the initial binding of tRNA substrates. Residues R104 and R129 are located near the active site. R129 is conserved in all TrmLs and plays a role in catalysis, as mutating R129 to Glu or Ala led to a complete loss of methyltransferase activity. Residues K42, R43, R45 and R46 are all located on helix \( \alpha \text{2} \) in the N-terminal half of the SPOUT domain. Structurally, the side chains of these four basic residues are exposed on the surface of \( Ec\)TrmL, which provides a docking region for the tRNA substrate (Figure 7A). Sequence alignment of the TrmL subfamily shows that this region (K42–R46) is mainly composed of basic amino acids (Figure 1B), and that residue R46 is strictly conserved, which suggests that helix \( \alpha \text{2} \) plays a crucial role in binding the tRNA substrate in all TrmLs. Residues R20 and R59 are also located in the N-terminal half of the SPOUT domain. Residue R20 is a critically conserved residue in the SpoU family. The conservation of the residues involved in tRNA binding suggests that the tRNA recognition mechanism shown here for \( Ec\)TrmL should be conserved throughout the TrmL subfamily.

Dimerization of \( Ec\)TrmL is observed both in solution and in the crystal structure, suggesting that it functions as a homodimer. The tRNA recognition motifs from the protein surface suggest that the tRNA-binding site spans the two subunits of the dimer instead of being located in a single subunit. The dissociated monomer could efficiently bind the SAH cofactor, but did not retain the tRNA-binding capacity and catalytic activity of the homodimer. These results further support the assumption that the binding of tRNA requires the cooperation of both subunits in the dimer. Therefore, \( Ec\)TrmL can efficiently bind tRNA by recruiting residues from the SPOUT domain of the other subunit, in the absence of the tRNA-binding extension domain. From the structure, the residues K42, R43, R45, R46 and R59 are located far from the tRNA-binding site and are all from the less conserved N-terminal half of the SPOUT domain. We propose that these basic residues play the same role as the extension domains in the other SPOUT MTases. To date, all of the studied SPOUT members have been found to be functional dimers (24). TrmH dimerizes essentially like TrmL in a ‘perpendicular’ fashion, whereas TrmD dimerizes in an ‘antiparallel’ fashion (Figure 3 and Supplementary Figure S5). When analyzing the dimeric structures of \( Tt\)TrmH and \( H\)TrmD (Supplementary Figure S5), it appears that the extension domains responsible for tRNA binding are oriented toward the active site of the other subunit, which suggests that the tRNA-binding sites in these enzymes should also span both subunits as in \( Ec\)TrmL. However, the positions of the tRNA-binding domains of \( Tt\)TrmH or \( H\)TrmD and the tRNA-binding motifs of TrmL are different. This may be explained by the fact that these enzymes have to recognize and accommodate different tRNA structural elements to catalyze methylation at specific positions.

The SPOUT MTases can be divided into two classes, (i) the smallest SPOUT MTases that including TrmL, RlmH and other proteins with unknown functions and consist only of the SPOUT catalytic domain and (ii) the larger SPOUT proteins that all contain N- or C-terminal appended domains involved in substrate binding. As homodimer formation occurs in all of the known SPOUT MTases, we suggest that dimer formation also occurred in the Last Universal Common Ancestor of SPOUT members. The ability of \( Ec\)TrmL to recruit residues from the N-terminal half of the SPOUT domain to bind the tRNA substrate may be conserved in other small SPOUT MTases and the Last Universal Common Ancestor. As each SPOUT enzyme recognizes a specific substrate, this may explain the increased sequence divergence observed in the N-terminal half of the SPOUT domain as compared with the catalytic C-terminal half. Throughout the evolution of SPOUT MTases, they were required to recognize increasingly varied substrates, including tRNAs, rRNAs and even proteins. As a result, the SPOUT MTases evolved new extension domains to expand their substrate recognition capacity as in TrmH, TrmD and other larger SPOUT members.

During catalysis, whether one or two tRNA molecules bind to the \( Ec\)TrmL dimer remains unclear. As the two subunits of the homodimer are identical, they should be able to simultaneously bind one tRNA in each catalytic site. In the crystal structure, only one HEPES molecule is bound, where it potentially mimics the tRNA acceptor nucleotide. Even when the amount of HEPES was increased in the crystal growth conditions, only one HEPES molecule was bound. It is therefore possible that during catalysis, only one tRNA molecule binds per \( Ec\)TrmL dimer. Although several crystal structures of SPOUT MTases have been reported (25–29), there is no available structure of a SPOUT MTase in complex with an RNA substrate, and the details of the interactions between these enzymes and their substrates are poorly understood. Here, we present the tRNA substrate
acknowledgements
The authors gratefully acknowledge Prof. Jian-Ping Ding and Dr Tian-Long Zhang in their institute and Dr Ming-Zhu Wang (Institute of Biophysics, CAS) for their technical assistance. They also thank Dr Gilbert Eriani for carefully reading the manuscript and important discussion.

funding
Natural Science Foundation of China [31270775 and 31130064]; National Key Basic Research Foundation of China [2012CB911000]. Funding for open access charge: National Key Basic Research Foundation of China [2012CB911000].

Conflict of interest statement. None declared.

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