

Crystal structure and catalytic mechanism of the essential m¹G37 tRNA methyltransferase TrmD from *Pseudomonas aeruginosa*

JUTHAMAS JAROENSUK,^{1,2,11,13} YEE HWA WONG,^{3,4,13} WENHE ZHONG,^{2,4} CHONG WAI LIEW,⁴ SOMCHART MAENPUEN,⁵ ABBAS E. SAHILI,^{3,4} SOPAPAN ATICHARTPONGKUL,⁶ YOK HIAN CHIONH,^{2,12} QIANHUI NAH,² NARUMON THONGDEE,¹ MEGAN E. MCBEE,² ERIN G. PRESTWICH,⁷ MICHAEL S. DEMOTT,⁷ PIMCHAI CHAIYEN,⁸ SKORN MONGKOLSUK,^{6,9,10} PETER C. DEDON,^{2,7} JULIEN LESCAR,^{3,4} and MAYUREE FUANGTHONG^{1,6,10}

¹Applied Biological Sciences Program, Chulabhorn Graduate Institute, Chulabhorn Royal Academy, Bangkok 10210, Thailand

²Singapore-MIT Alliance for Research and Technology Antimicrobial Resistance and Infectious Disease Interdisciplinary Research Groups, 138602 Singapore

³School of Biological Sciences, Nanyang Technological University, 637551 Singapore

⁴NTU Institute of Structural Biology, Nanyang Technological University, 636921 Singapore

⁵Department of Biochemistry, Faculty of Science, Burapha University, Chonburi 20131, Thailand

⁶Laboratory of Biotechnology, Chulabhorn Research Institute, Bangkok 10210, Thailand

⁷Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

⁸School of Biomolecular Science and Engineering, Vidyasirimedhi Institute of Science and Technology (VISTEC), Rayong 21210, Thailand

⁹Department of Biotechnology, Faculty of Sciences, Mahidol University, Bangkok 10400, Thailand

¹⁰Center of Excellence on Environmental Health and Toxicology (EHT), Bangkok 10400, Thailand

ABSTRACT

The tRNA (m¹G37) methyltransferase TrmD catalyzes m¹G formation at position 37 in many tRNA isoacceptors and is essential in most bacteria, which positions it as a target for antibiotic development. In spite of its crucial role, little is known about TrmD in *Pseudomonas aeruginosa* (PaTrmD), an important human pathogen. Here we present detailed structural, substrate, and kinetic properties of PaTrmD. The mass spectrometric analysis confirmed the G36G37-containing tRNAs Leu(GAG), Leu(CAG), Leu(UAG), Pro(GGG), Pro(UGG), Pro(CGG), and His(GUG) as PaTrmD substrates. Analysis of steady-state kinetics with S-adenosyl-L-methionine (SAM) and tRNA^{Leu(GAG)} showed that PaTrmD catalyzes the two-substrate reaction by way of a ternary complex, while isothermal titration calorimetry revealed that SAM and tRNA^{Leu(GAG)} bind to PaTrmD independently, each with a dissociation constant of 14 ± 3 μ M. Inhibition by the SAM analog sinefungin was competitive with respect to SAM ($K_i = 0.41 \pm 0.07$ μ M) and uncompetitive for tRNA ($K_i = 6.4 \pm 0.8$ μ M). A set of crystal structures of the homodimeric PaTrmD protein bound to SAM and sinefungin provide the molecular basis for enzyme competitive inhibition and identify the location of the bound divalent ion. These results provide insights into PaTrmD as a potential target for the development of antibiotics.

Keywords: *Pseudomonas*; TrmD; tRNA modification; tRNA (m¹G37) methyltransferase

INTRODUCTION

Pseudomonas aeruginosa infection has become a serious global public health threat (Lucena et al. 2014; Hong

et al. 2015; Ventola 2015), due in part to increasing resistance to antibiotics such as carbapenems (National Nosocomial Infections Surveillance System 2004; Centers for Disease Control Ministry of Health and Welfare R.O.C. Taiwan 2010). This has fostered efforts to discover novel

¹¹**Present address:** School of Biomolecular Science and Engineering, Vidyasirimedhi Institute of Science and Technology (VISTEC), Rayong 21210, Thailand.

¹²**Present address:** Tychan Ltd., 117604 Singapore.

¹³These authors contributed equally to this work.

Corresponding authors: mayuree@cri.or.th, julien@ntu.edu.sg, pcdedon@mit.edu

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targets for antibiotic development, including tRNA-modifying enzymes. In all organisms, tRNA is transcribed as a pre-tRNA that is processed to maturity by a variety of editing steps such as CCA addition and by extensive posttranscriptional chemical modification of the four canonical ribonucleotides (El Yacoubi et al. 2012). While these modifications are widely distributed throughout the tRNA molecule and affect ~10% of the ~80–90 tRNA nucleotides, modifications at positions 32, 34, and 37 are recognized for their role in regulating cellular stress responses (Begley et al. 2007; Chan et al. 2012; Chionh et al. 2016; Jaroensuk et al. 2016) and promoting antibiotic resistance (Taylor et al. 1998). In this regard, the tRNA (m^1G37) methyltransferase TrmD (Byström and Björk 1982; Hjalmarsson et al. 1983; Hou et al. 2017), which is essential in most bacterial species (Hagervall et al. 1993; Forsyth et al. 2002; Björk and Nilsson 2003; O'Dwyer et al. 2004; de Berardinis et al. 2008; Winsor et al. 2011), has emerged as an important target for drug development (Lahoud et al. 2011; Hill et al. 2013; Baumgartner and Camacho 2016). TrmD uses S-adenosylmethionine (SAM) as a methyl donor and catalyzes the methylation of G37 to form 1-methylguanosine (m^1G) mainly in tRNAs containing a G36G37 motif, thus reading codons that start with C, including those for leucine (CUN), proline (CCN), and arginine (CCG) (Björk et al. 2001; Sprinzl and Vassilenko 2005). Only TrmD from *Aquifex aeolicus* is known to recognize tRNAs with A36G37 (Takeda et al. 2006). With a general requirement for Mg^{2+} to promote proton abstraction from N1 of G37 and stabilize the negative charge on O⁶ of G37 (Sakaguchi et al. 2014; Ito et al. 2015), structural analysis of TrmD from *Escherichia coli* and *Haemophilus influenzae* revealed a homodimer that uses a deep trefoil knot structure at its amino-terminal domain for SAM binding (Elkins et al. 2003; Ito et al. 2015), in contrast to the human homolog, Trm5, which is active in a monomeric form and uses a Rossmann fold for SAM (Goto-Ito et al. 2008). The presence of m^1G37 has been shown to be essential for maintaining the ribosome reading-frame and fidelity of protein synthesis (Björk et al. 1989), with its loss leading to increased +1 frameshifting events, premature termination of protein translation, and cellular toxicity (Björk et al. 1989; Hagervall et al. 1993; Li and Björk 1995). While well studied in a variety of bacteria, the structure and properties of TrmD in *P. aeruginosa* have not been reported.

Here we establish that the *PA14_15990* gene in *P. aeruginosa* PA14 is a TrmD m^1G37 -forming tRNA methyltransferase, and characterize its structure, kinetics and substrate properties. The results revealed that PaTrmD catalyzes the formation of m^1G37 in tRNA by a ternary complex mechanism in which tRNA and SAM can bind the protein independently. The unique and shared features of PaTrmD presented here expand the opportunities for developing TrmD as an antibiotic target.

RESULTS AND DISCUSSION

PA14_15990 (trmD) is essential for growth in P. aeruginosa PA14

Attempts to construct a *PA14_15990/trmD* mutant in *P. aeruginosa* PA14 using homologous recombination were not successful, which is consistent with reports that *trmD* is essential in *E. coli*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* (O'Dwyer et al. 2004; Chaudhuri et al. 2009). We therefore tested the essentiality of *PA14_15990/trmD* in *P. aeruginosa* PA14 using a temperature-sensitive allele of a *Pseudomonas* replicon, *mSFts¹* (Silo-Suh et al. 2009), to regulate expression of extra chromosomal *trmD*. Wild-type (WT) *P. aeruginosa* PA14 containing *pBBR-trmD-mSFts¹* and the *trmD* conditional knockout strain (*trmD::Gm/pBBR-trmD-mSFts¹*) were both grown at a permissive temperature to maintain the stability of the plasmid (28°C and 37°C) or at a nonpermissive temperature to cause plasmid loss (46°C). As demonstrated in Figure 1, the growth of the *trmD* conditional knockout strain at the permissive temperature was similar to WT PA14 (Fig. 1A,B), while growth of the knockout strain was no longer observed at the nonpermissive temperature (Fig. 1C). These results suggest that PaTrmD is essential in *P. aeruginosa* PA14.

PA14_15990 encodes a tRNA (m^1G37) methyltransferase homolog

Homology analyses (BLASTp) using *E. coli* TrmD (EcTrmD) to search against the *P. aeruginosa* PA14 genome resulted in one hit with *PA14_15990* sharing 66% identity to EcTrmD. As shown in Figure 2A, a protein sequence alignment of *PA14_15990* and TrmD homologs with known structures from *Haemophilus influenza* (HiTrmD) (Ahn et al. 2003), *Aquifex aeolicus* (AaTrmD) (Liu et al. 2003), and *E. coli* (Elkins et al. 2003), reveals that PaTrmD has 64% amino acid identity with the HiTrmD and 41% amino acid identity with the AaTrmD. PaTrmD also shares functionally important amino acid residues involved in cofactor binding (Ser93–Gly96, Gly118, Ile123, Ser137, Gly145), tRNA binding (Gly60, Gly64, Ser203–His206), and catalytic activity (Asp54, Arg159, and Asp174). These results support the idea that *PA14_15990* encodes the tRNA (m^1G37) methyltransferase (TrmD) in *P. aeruginosa* PA14. Subsequent structural, biochemical and kinetic data support this conclusion.

The overall structure of PaTrmD

The structure of the PaTrmD protein bound to the copurified methyl donor SAM was determined at a resolution of 2.16 Å. A schematic representation of the topology of the PaTrmD monomer and of its fold is shown in Figure 2B,C.

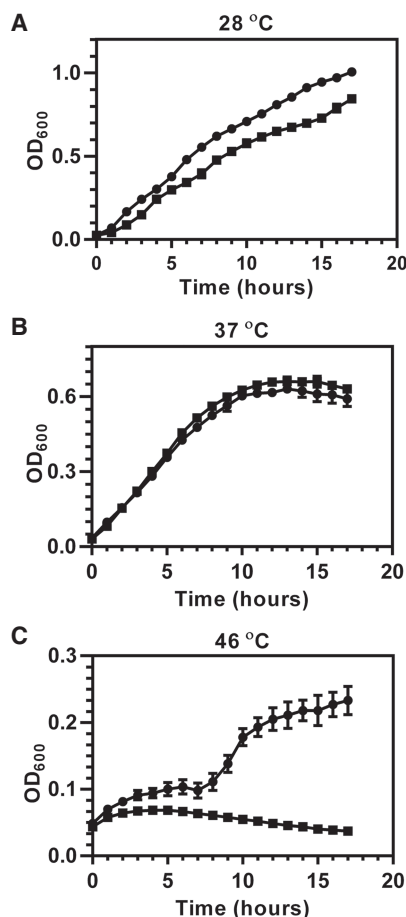


FIGURE 1. Growth of wild-type *P. aeruginosa* PA14 and the *trmD* conditional knockout strain establishes TrmD as an essential enzyme in PA14. Wild-type PA14 (●) and the *trmD* conditional knockout strain (■) were grown in LB broth in 96-well plates, and growth was monitored for 18 h at temperatures that maintain stability of the plasmid carrying *trmD* gene (A, 28°C; B, 37°C) or cause loss of the plasmid (C, 46°C).

A second crystal structure of PaTrmD bound to the SAM-competitive inhibitor sinefungin (SFG) was refined at a resolution of 2.45 Å. In order to unambiguously locate the divalent ion, a third structure where crystals of PaTrmD were soaked with Mn²⁺ and SFG was determined (Table 1). For these three structures, the refined model comprises a homodimer in the asymmetric unit with the two monomers (Fig. 2B,C; Supplemental Fig. S1) related by a non-crystallographic dyad (Fig. 2D). Pairwise comparisons with published TrmD structures from *E. coli* (PDB code 1P9P) (Elkins et al. 2003), *H. influenzae* (PDB code 1UAK) (Ahn et al. 2003), and *A. aeolicus* (PDB code 1OY5) (Liu et al. 2003) return deviation values of 0.9, 1.0, and 2.4 Å for 235, 236, and 205 superimposed α -carbons, respectively. A structure-based sequence alignment of TrmD from nine bacteria is displayed in Supplemental Figure S2. The active site is located in a pocket of the trefoil knot at the dimer interface (Supplemental Fig. S1). The

PaTrmD monomer is formed by an amino-terminal domain (NTD) that adopts an α/β fold (residues 5–165) and a mostly helical carboxy-terminal domain (CTD) spanning residues 178–250 (Fig. 2B,C). These two domains are connected by a linker spanning residues 166–177 that acts as a lid covering up the active site (Fig. 2; Supplemental Fig. S1). The lid was reported to become ordered only upon tRNA binding, which is accompanied by folding into an α -helix (Ito et al. 2015). Here, the lid appears rather flexible in the two molecules present in the asymmetric unit of the SAM complex and more ordered in the complexes with SFG. Thus, in the SAM-bound structure, residues 166–173 are missing from the model and for the SFG complexes, residues 167–172 are missing in molecule A while the loop could be built entirely for molecule B (Fig. 3A,B; Supplemental Fig. S4).

Features of PaTrmD bound to SAM and SFG

Clear electron density corresponding to two bound SAM molecules per PaTrmD dimer was visible (Fig. 3B; Supplemental Fig. S4). SAM adopts a bent conformation with the adenine moiety in an *anti* conformation and the ribose pucker in C3'-endo (Fig. 3B). Since no SAM was added during purification and crystallization, it presumably originates from the *E. coli* culture medium and has copurified with the protein. The adenine base is stacked between Pro94 and Pro149. Its N⁶ atom hydrogen bonds with carbonyl oxygen atoms of Gly139 and Tyr141, while the 2'-hydroxyl group of the ribose hydrogen bonds with the carbonyl oxygen of Leu92 and the amide group of Gly118 (Fig. 3B; Supplemental Fig. S4). The 3'-hydroxyl group of the ribose forms another hydrogen bond with the side chain of Tyr91. The amino acid tail of SAM is stabilized by two sets of polar interactions: (i) the side chains of Gln95 and His185* (residues from chain B are marked by an asterisk) make contact with the carboxylic group of SAM and (ii) the amino tail makes a salt bridge with the carboxylic group of residues Asp182* (Fig. 3B; Supplemental Fig. S4).

SFG is an isosteric analog of SAM, in which the methyl group of SAM is replaced by an amino group and the sulfur by a carbon atom. The protein components of the SFG- and SAM-bound structures are closely superimposable, with an RMS deviation of 0.58 Å for all 252 C α atoms. However, a close-up view of the superposed active sites reveals conformational changes at the level of the amino acid tails of the ligands (Fig. 3A; Supplemental Fig. S5). The carboxylic group of SFG is reoriented toward the inter-domain linker, forming a close hydrogen bond with Ser175*, while its amino group hydrogen bonds the side chain of Asp182*. These additional contacts account for the lid ordering in the SFG complex compared to the SAM complex.

Given the need for an Mg²⁺ ion for catalysis, our search of the electron density map revealed a possible location for

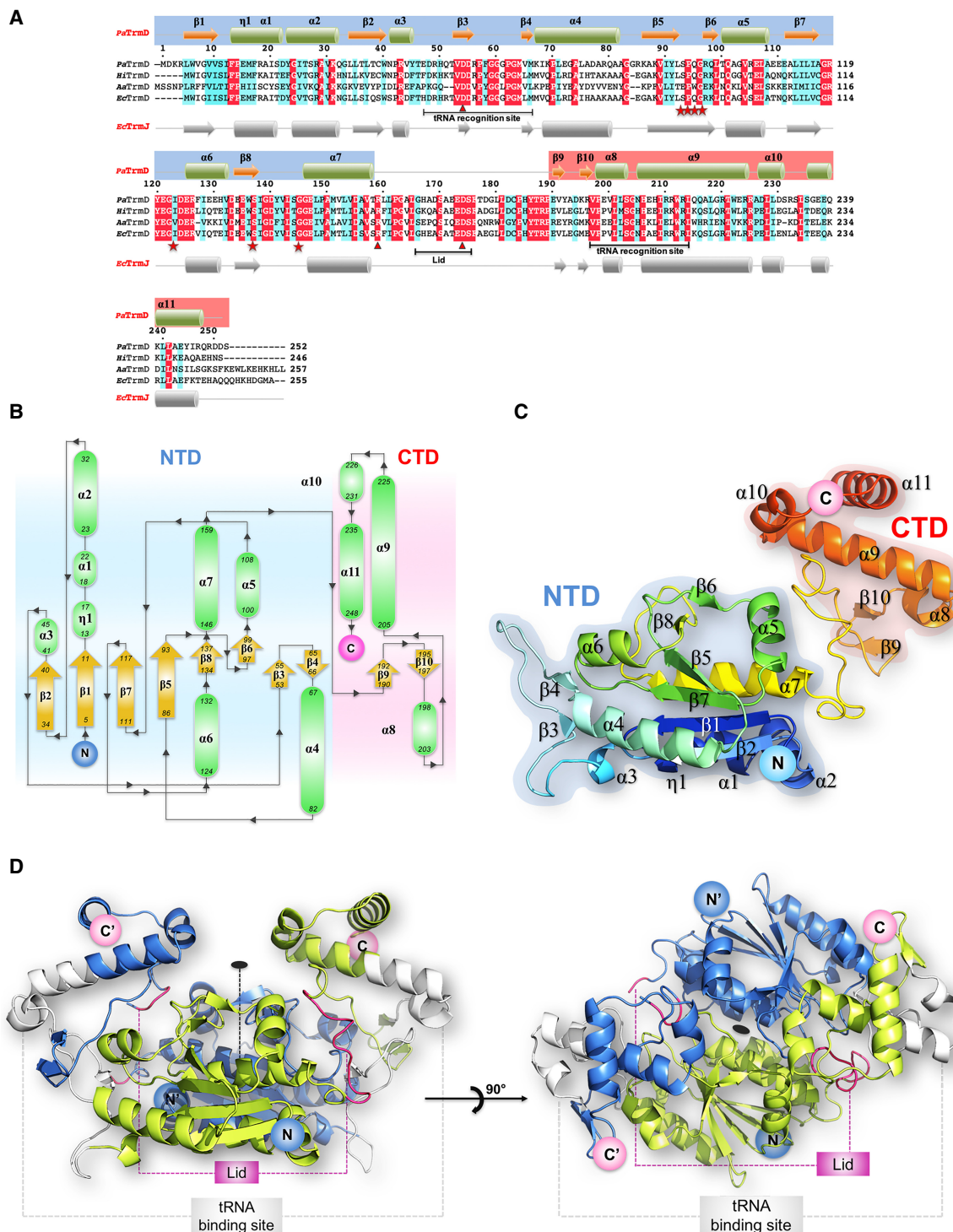


FIGURE 2. The structure of PaTrmD. (A) Structure-based sequence alignment showing the amino acid sequences of TrmD from *P. aeruginosa* PA14 (PaTrmD, this work), *H. influenzae* (HiTrmD), *A. aeolicus* (AaTrmD), and *E. coli* (EcTrmD). The amino acids involved in cofactor binding are indicated by stars in red, and amino acids involved in the catalytic activity are indicated by triangles. (B) Schematic representation of the PaTrmD monomer topology (with the NTD shaded in blue and the CTD in pink) indicating the arrangement of secondary structure elements (α -helices are represented as green ovals and β -strands as yellow arrows) with the residues numbered. The amino and carboxyl termini are indicated. (C) Model representation of the PaTrmD monomer 3D structure colored from dark blue (amino terminus) to red (carboxyl terminus). (D) Overall view of the PaTrmD homodimer present in the asymmetric unit (one monomer is colored blue and the other light green). The dyad in the plane of the figure is represented by a dashed line and by an oval (perpendicular to the plane of the figure) in the right panel. Main functional elements are indicated: lid (or interdomain linker region) and the possible tRNA binding sites (one being used at a time yielding a 2:1 stoichiometry) (Ito et al. 2015).

TABLE 1. Crystallographic data and refinement statistics

PDB code	PaTrmD-SAM 5WYQ	PaTrmD-sinefungin 5WYR	PaTrmD- sinefungin-Mn 6JKI
Data collection			
Resolution range (Å)	28.12–2.16 (2.29–2.16)	42.65–2.45 (2.56–2.45)	50.00–2.60 (2.59–2.60)
Beamline	X06DA, SLS	X06DA, SLS	Proxima-2A, SOLEIL
Space group	P3 ₂ 21	P3 ₂ 21	P3 ₂ 21
Dimers per asymmetric unit	1	1	1
Cell parameters: <i>a, b, c</i> (Å); α, β, γ (°)	85.90, 85.90, 147.84; 90, 90, 120	85.29, 85.29, 147.84; 90, 90, 120	85.68, 85.68, 147.61; 90, 90, 120
No. of measured reflections	120,767 (10,006)	123,092 (14,245)	739,353 (116,722)
No. of unique reflections	34,505 (2,943)	23,225 (2,638)	37,629 (6,010)
Completeness (%)	99.9 (99.9)	99.3 (99.4)	99.8 (98.9)
Multiplicity	3.5	5.3	19.6
R_{merge}^a	0.057 (0.910)	0.088 (0.741)	0.187 (1.374)
$CC_{1/2}^b$	0.99 (0.88)	0.99 (0.91)	0.99 (0.88)
Average $I/\sigma(I)$	15.9 (1.7)	16.9 (3.1)	14.38 (1.85)
Refinement			
Resolution range (Å)	28.12–2.16 (2.23–2.16)	40.96–2.45 (2.56–2.45)	26.16–2.59 (2.73–2.59)
R factor ($R_{\text{work}}/R_{\text{free}}^c$) (%)	20.7/24.9	19.1/23.1	20.7/24.5
Model contents			
Protein atoms	3804	3875	3848
Water molecules	180	161	121
Ligand atoms	54 (2 × SAM)	54 (2 × SFG)	54 (2 × SFG) 14 (2 × PEG) 30 (5 × GOL) (2 × Na) (3 × Mn)
Root mean square deviation			
Distances (Å)	0.010	0.010	0.010
Bond angles (°)	1.02	1.08	1.18
Clash score	3.69	6.99	3.95
Rotamer outliers (%)	0.75	0.25	0.75
Ramachandran plot			
Favored (%)	96.8	96.7	97.91
Allowed (%)	3.2	3.3	2.09
Outlier (%)	0	0	0

Values in parentheses indicate values in the highest resolution shell.
^a $R_{\text{merge}} = \sum |I_i - \langle I \rangle| / \sum I_i$, where I_i is the intensity of an individual reflection and $\langle I \rangle$ is the average intensity of that reflection.
^b $CC_{1/2}$ = percentage of correlation between intensities from random half-data set.
^c $R_{\text{work}} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where F_o denotes the observed structure factor amplitude, and F_c the structure factor amplitude calculated from the model.
^d R_{free} is as for R_{work} but calculated with 5% of randomly chosen reflections omitted from the refinement.

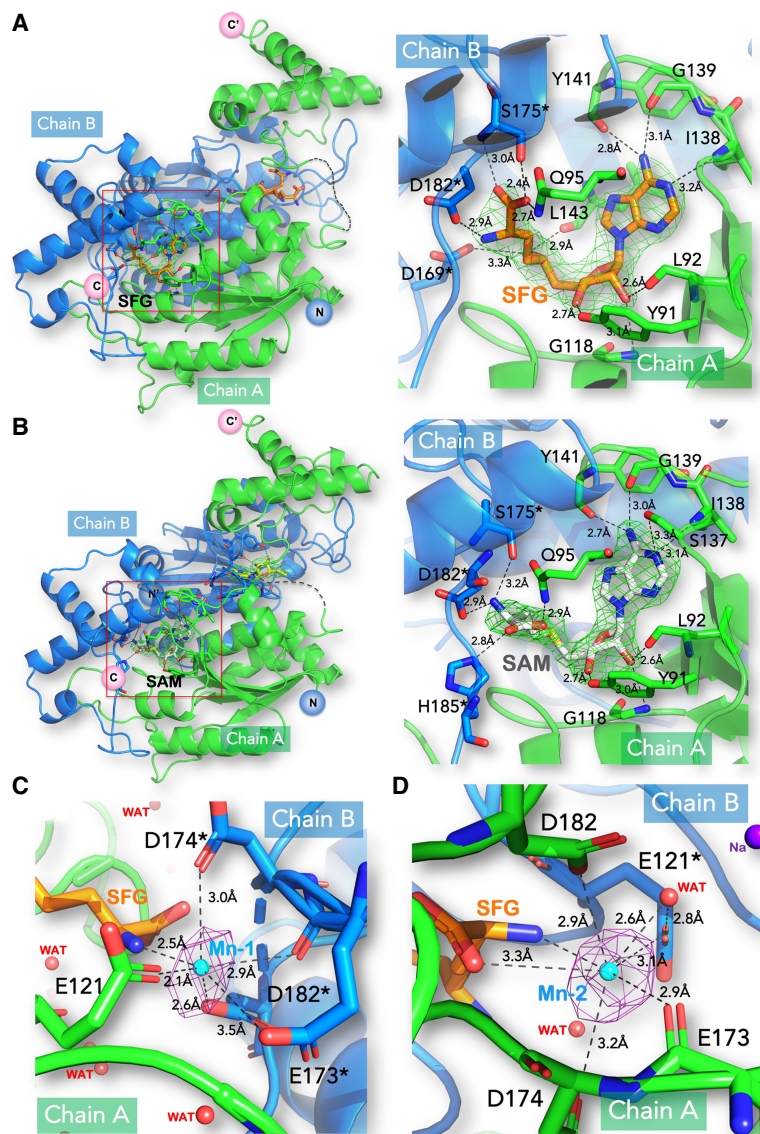


FIGURE 3. Structures of the PaTrmD-SFG, PaTrmD-SAM, and PaTrmD-SFG-Mn complexes. (A,B) Overall (left panels) and magnified views (right panels) for the two binary complexes of TrmD with sinefungin (SFG) (A) and SAM (B). Subunits A and B of the TrmD homodimer are colored with green and blue, respectively. Residues from chain B are marked by an asterisk. The SFG ligand is depicted as orange sticks and SAM as white sticks. Polar contacts are represented by dashed lines with interacting residues labeled and ligand-protein distances in Å. Difference Fourier electron density maps where the bound ligands were omitted from the model for phase calculation are overlaid and contoured at a level of 3σ ; simulated annealing is shown in Supplemental Figure S3. (C,D) The PaTrmD-SFG-Mn complex. The anomalous electron density map allowing unambiguous positioning of the bound manganese in the active site (Mn) in the presence of SFG (see Supplemental Fig. S8 for the measurement of the K absorption edge of Mn in the crystal). The anomalous map (colored in purple) was calculated from 40 to 5 Å and contoured at a level of 4.6σ . Mn is coordinated by a side-chain carbonyl group of E173*, carboxylic groups of E121, D174*, and D182*, and the nitrogen atom of the SFG tail. These interacting residues and the ligand SFG are shown as sticks. Mn is shown as a cyan sphere.

such a divalent ion. We found hints for the presence of an Mg^{2+} ion in the active site near the carboxylic tail of SAM. However, the octahedral coordination shell was incomplete and the putative position did not match the location

of Mn^{2+} (for the SFG- Mn^{2+} complex) that we could confidently assign based on a clear anomalous signal (see below). Next, we measured the Mg^{2+} content in the PaTrmD protein and found that the molar ratios of PaTrmD to Mg^{2+} ion of the Mg^{2+} -reconstituted PaTrmD was 1:0.97 (Table 2). These data imply that one PaTrmD monomer can bind to one Mg^{2+} ion. Conversely, the molar ratio of PaTrmD to Mg^{2+} ion for the purified enzyme without the Mg^{2+} reconstitution was 1:0.02 indicating that Mg^{2+} ion does not bind tightly to PaTrmD. This is consistent with the reported weak Mg^{2+} binding affinity for *E. coli* TrmD of 0.7 mM, (Sakaguchi et al. 2014). We then investigated the presence of a bound divalent metal at the active site: We soaked PaTrmD crystals with manganese (Mn^{2+}) and sinefungin (SFG) and collected these data at the Mn^{2+} K edge to maximize the anomalous signal (see Materials and Methods). In Figure 3C,D, two peaks in the anomalous Fourier map were readily apparent at $>5\sigma$ level. One Mn^{2+} near each of the bound SFG could be built. Mn^{2+} is coordinated by a side-chain carbonyl group of E173*, carboxylic groups of E121, D174*, and D182*, and the nitrogen atom of the SFG tail (Fig. 3C,D). Although the catalytic mechanism that was proposed for the majority of SAM-dependent methyl transferases requires no divalent metal ions, Sakaguchi et al. reported that TrmD-catalyzed methyl transfer from SAM to the N^1 atom of G37 from the tRNA is strongly dependent on the presence of divalent metal ions, with Mg^{2+} as the most physiologically relevant (Sakaguchi et al. 2014; Hou et al. 2017). Based on the pH-activity profile, they proposed a catalytic mechanism in which Mg^{2+} increases the nucleophilicity of N^1 of G37 and stabilizes the developing negative charge on O^6 during an attack on the methyl

sulfonium of the SAM donor. Moreover, an Mg^{2+} ion was also found near the active site in contact with the N^7 atom of G37 in a ternary complex between TrmD from *H. influenzae*, tRNA and SAM (Ito et al. 2015), lending further

TABLE 2. Measurement of Mg^{2+} using ICP-OES

Sample	Concentration of PaTrmD	Concentration of Mg^{2+}	TrmD: Mg^{2+} ratio
Reagent blank	Not applicable	0.730 ± 0.0257 μM	Not applicable
Purified PaTrmD	20 μM	0.402 ± 0.0054 μM	1: 0.02
Purified PaTrmD preincubated with 1 mM Mg_2Cl	20 μM	19.34 ± 0.114 μM	1: 0.97

The data represent mean $\pm$ SD for three independent determinations.

support for a positive contribution of Mg^{2+} ion to the catalytic mechanism. Interestingly, a requirement for a bent conformation of the methyl donor was proposed based on molecular dynamics simulations (Christian et al. 2016).

Conformational changes are required to form a ternary complex with tRNA

A TrmD enzymatic mechanism involving deprotonation of G37 by a catalytic base followed by a nucleophilic attack on the methyl group of SAM by the N1 atom has been proposed (Ahn et al. 2003; Elkins et al. 2003; Ito et al. 2015). The side chains of Arg159*, Leu165*, and Ser170* of PaTrmD (Fig. 4) are well-positioned to form hydrogen bonds or to stack with G37, and Asp174* is the likely catalytic base (Figs. 2A, 4). Interestingly, Asp174* makes a salt bridge with Arg159*, in a conformation that might mimic a preactivated state in which the linker/lid is brought into proximity with the rest of the active site upon tRNA binding.

PaTrmD catalyzes only the m^1G modification in PA14 tRNAs that possess a G36G37 motif

TrmD from most bacteria catalyzes the methylation m^1G37 in tRNAs containing a G36G37 motif (Björk et al. 2001; Sprinzl and Vassilenko 2005). Many conserved residues of PaTrmD interact with G37 as mentioned above. In addition, the carboxylic group of an evolutionarily conserved Asp residue (Asp54 in PaTrmD) interacts with the H atoms at positions N^1 and N^2 of G36 (Fig. 4C). A study in *E. coli* showed that EcTrmD recognizes G36 as well as A36 with comparable binding affinity as the carboxylic groups of Asp54 interact with the hydrogen at position N^6 of A36 (Ito et al. 2015). However, the A36G37 sequence is not observed in bacterial tRNA. Here we tested whether the G36G37 motif is a substrate of PaTrmD by defining the activity of PaTrmD as a tRNA (m^1G37) methyltransferase by quantifying m^1G following in vitro reactions with synthetic PA14 tRNAs possessing a G36G37 motif, which are tRNA^{Leu(GAG)},

tRNA^{Leu(CAG)}, tRNA^{Leu(UAG)}, tRNA^{His(GUG)}, tRNA^{Pro(GGG)}, tRNA^{Pro(CGG)}, and tRNA^{Pro(UGG)}. tRNA^{Gln(UUG)} with a G36A37 dinucleotide served as a negative control. Analysis of the 23 ribonucleosides shown in Supplemental Table S2 by HPLC-coupled tandem quadrupole mass spectrometry (LC-MS/MS) relative to standards revealed only a single modification, m^1G , in all G36G37-containing tRNAs (Fig. 5A). This is illustrated in the extracted ion chromatogram for tRNA^{Pro(GGG)} shown in Figure 5B. As expected, m^1G was not detected in the G36A37-containing tRNA^{Gln(UUG)}. The fact that PaTrmD catalyzes m^1G formation in synthetic tRNA substrates indicates that PaTrmD can use G36G37-containing tRNAs without other modifications as substrates. Initially, the TrmD-mediated m^1G37 modification was shown for a subset of tRNA^{Leu}, tRNA^{Pro}, and tRNA^{Arg} in bacteria (Li and Björk 1999; Ito et al. 2015). With genome sequencing analyses additional tRNA substrates for TrmD are predicted (Subramanian et al. 2014; Chan and Lowe 2016). In this study, our data expand the substrate specificity of TrmD from tRNA^{Leu}, tRNA^{Pro}, and tRNA^{Arg} to tRNA^{His} in bacteria. The substrate specificity is driven by the presence of G36G37 in each species.

PaTrmD catalyzes m^1G at position 37 in the tRNA anticodon loop

Having established the m^1G product of PaTrmD activity, we next defined the position of the m^1G in tRNAs by mass spectrometry-based RNA fragment analysis. Methylated tRNA^{Leu(GAG)} from an in vitro reaction with PaTrmD was digested with RNase A to give a signature oligoribonucleotide, GAG m^1GU , carrying m^1G at position 37. As shown in Figure 5C,D, the doubly charged negative ion (m/z 810.1356) of fragment GAG m^1GU was observed at an HPLC retention time of 18.9 min. To determine the position of m^1G , the doubly charged ion m/z 810.1356 was analyzed by CID, with product ions shown in Figure 5E. The resulting CID mass spectra were analyzed with Simple Oligonucleotide Sequencer (SOS) (Rozenki and McCloskey 2002), with the ribonucleotide sequence determined based on the a-B-, w-, and y-ions. This analysis revealed that PaTrmD catalyzes methyl transfer to G37 in the tRNA anticodon loop. This is entirely consistent with previous studies of EcTrmD and HtTrmD, in which TrmD was found to recognize and methylate tRNA substrates by making a contact with the D-arm, anticodon arm, and the variable region of tRNA (Ahn et al. 2003; Ito et al. 2015). The study of HtTrmD demonstrated that TrmD uses the following conserved amino acid residues, Ser198, Gly199, His200/Asp200, and His201 of carboxy-terminal domain (CTD) to make contact with G37 in the substrate tRNAs for methylation (Ito et al. 2015). The structural and biochemical studies to this point prove that PaTrmD catalyzes m^1G formation at position 37 in tRNAs

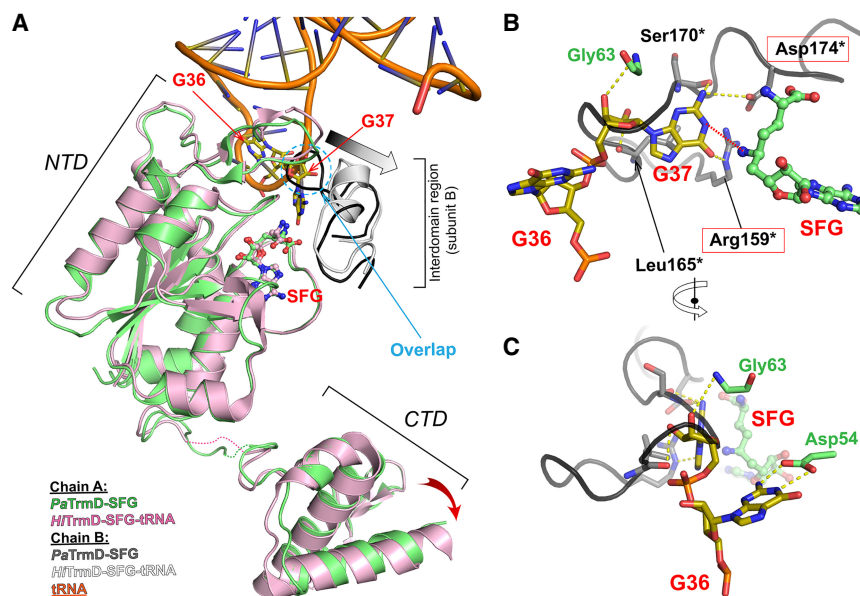


FIGURE 4. Comparison between the binary complex PaTrmD-SFG and the ternary complex HiTrmD-SFG/tRNA. Models of SFG-bound PaTrmD (this work) and SFG/tRNA-bound *H. influenzae* TrmD (PDB ID: 4YVI) (Ito et al. 2015). The two complexes were superimposed based on the C α atoms of NTD of subunit A. The interdomain region of subunit B is also displayed colored in dark gray (SFG-bound) or white (SFG/tRNA-bound), respectively. Ligands and key contacting residues are shown as sticks. Panel A highlights conformational differences; the interdomain region (subunit B, dark gray) of PaTrmD-SFG would sterically collide with G37 of the bound tRNA in the ternary complex (dashed circle). Thus, a loop rearrangement is induced upon tRNA binding. The induced movement of the interdomain region (subunit B) is indicated by a gray arrow. The accompanying movement in CTD domain (subunit A) is shown by a red arrow. The poorly structured interdomain loops of subunit A are shown as dotted lines. (B,C) Orthogonal views of superposed structures showing the putative tRNA binding mode with PaTrmD based on the experimental HiTrmD-SFG/tRNA complex (Ito et al. 2015), highlighting the conservation of residues interacting with G37 between PaTrmD and HiTrmD. G37-interacting catalytic residues are in red boxes. The transfer of the methyl group to G37 is indicated by a red dotted line. Residues from chain B are marked by an asterisk. HiTrmD structure was omitted for clarity.

containing a G36G37 motif. However, these studies do not provide insights into the enzymatic mechanism governing PaTrmD activity. This was addressed next.

Steady-state kinetics of PaTrmD

To gain insight into the kinetics and thermodynamics of PaTrmD activity, we performed a series of enzymological and biophysical studies, starting with a detailed analysis of PaTrmD kinetics. The linear range of the initial velocity of the methyltransferase reaction was first established by plotting initial velocities as a function of PaTrmD concentration, which revealed linearity up to 36 nM (Supplemental Fig. S6). Working in this linear range, we investigated the steady-state kinetics of PaTrmD by varying the concentrations of SAM and tRNA^{Leu(GAG)} in reactions containing 23 nM PaTrmD. The initial velocities were calculated and fit to the ternary complex Equation 1. A summary of steady-state kinetic parameters for PaTrmD is shown in Table 3. The K_m of PaTrmD for tRNA^{Leu(GAG)} of 0.8 ± 0.2

μM is ~ 3 - to 10-times smaller than that for EcTrmD with tRNA^{Pro(UAG)} ($2.8 \pm 0.1 \mu\text{M}$) (Christian and Hou 2007), *Streptococcus pneumoniae* TrmD (SpTrmD) with tRNA^{Leu(CAG)} ($4.3 \pm 1.7 \mu\text{M}$) (O'Dwyer et al. 2004), and HiTrmD with tRNA^{Leu(CAG)} ($8.0 \pm 1.1 \mu\text{M}$) (Ito et al. 2015). The K_m of PaTrmD for SAM was found to be $3.0 \pm 0.8 \mu\text{M}$, which is similar to data reported for EcTrmD ($5.0 \pm 0.8 \mu\text{M}$) (Lahoud et al. 2011) and HiTrmD ($3.0 \mu\text{M}$) (Ito et al. 2015). PaTrmD catalyzed the formation of m¹G with a K_{cat} of $13.0 \pm 1.4 \text{ min}^{-1}$, which was comparable to values reported for HiTrmD ($19 \pm 0.01 \text{ min}^{-1}$) (Ito et al. 2015) and SpTrmD ($15 \pm 2 \text{ min}^{-1}$) (O'Dwyer et al. 2004). Overall, the kinetic properties of PaTrmD quantified using the MTase-Glo assay are similar to other bacterial TrmD proteins previously characterized with a radiometric assay (Redlak et al. 1997; Brulé et al. 2004; O'Dwyer et al. 2004; Ito et al. 2015). When a double-reciprocal plot of $1/v_i$ versus $1/[\text{SAM}]$ at different fixed concentrations of the tRNA Leu^{GAG} was performed, the lines intersect (Fig. 6), which suggests that PaTrmD operates by forming a ternary complex. This is consistent with previous analyses performed with TrmD from *S. pneumoniae* (O'Dwyer et al. 2004), *E. coli* (Redlak et al. 1997; Brulé et al. 2004) and *H.*

influenzae (Ito et al. 2015; Christian et al. 2016). These results are also consistent with the structural data that revealed two separate binding pockets for tRNA and SAM (Figs. 2, 3) and with the ligand-binding analyses performed next.

SAM and tRNA bind to PaTrmD independently

Since the kinetics studies suggested that methylation of G37 by PaTrmD occurs in a ternary complex mechanism, we next investigated the interdependence of SAM and tRNA binding to PaTrmD. Here we used isothermal titration calorimetry (ITC) to quantify binding of SAM and tRNA. tRNA^{Leu(GAG)} was chosen as the substrate given its activity in the kinetics studies and the established position of the PaTrmD-induced m¹G. Refolded PaTrmD protein (Supplemental Fig. S7) was used here to eliminate the effects of copurified SAM.

As shown in Figure 7, our results suggest that SAM and tRNA can independently bind PaTrmD to form the binary complexes PaTrmD:SAM and PaTrmD:tRNA. This is

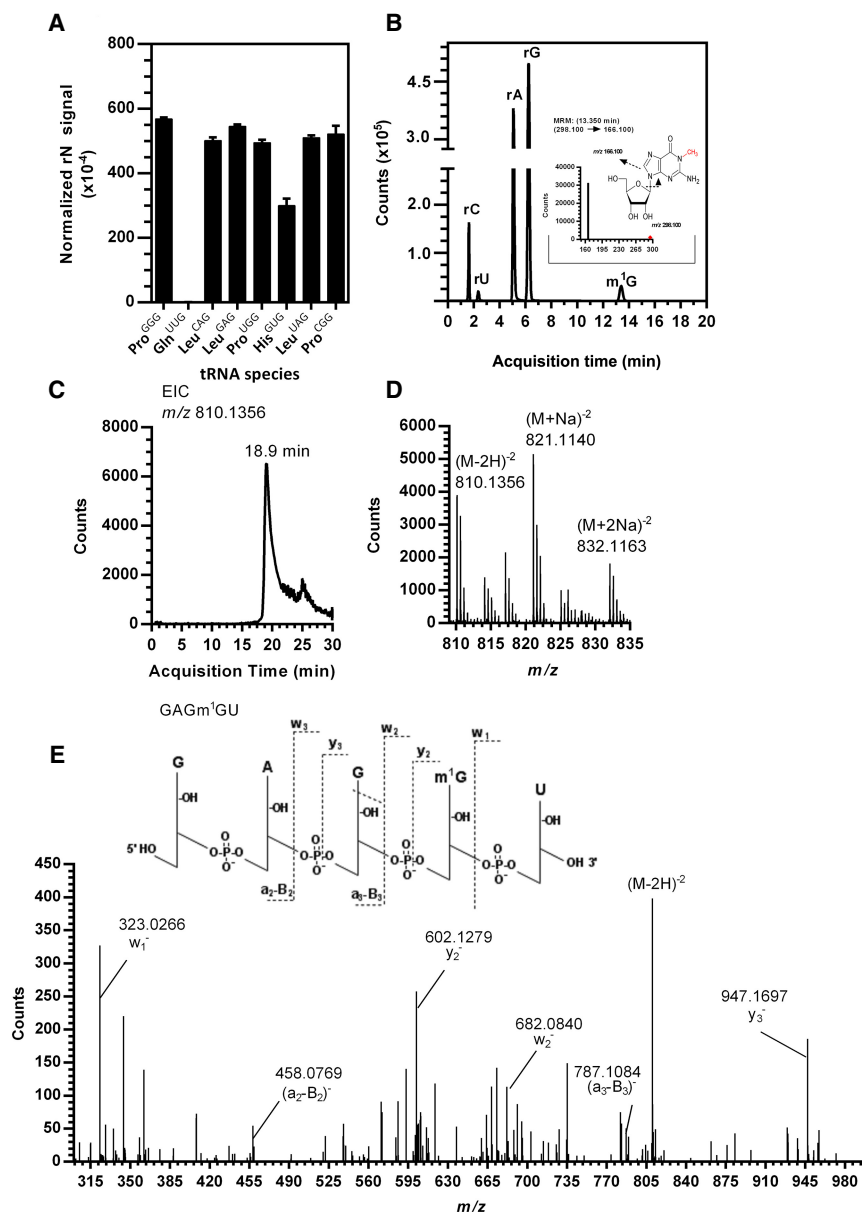


FIGURE 5. Quantification and mapping of m¹G catalyzed by *PaTrmD* in synthetic tRNA^{Leu(GAG)}. (A) The level of m¹G in tRNAs reacted with *PaTrmD*, and SAM was measured by HPLC-coupled triple-quadrupole mass spectrometry in tRNAs possessing G36G37 motifs and tRNA^{Gln(UUG)} with a G36A37 motif. Values represent mean $\pm$ SD for three biological replicates. All ribonucleosides analyzed are shown in Supplemental Table S2. (B) Extracted ion chromatograms of ribonucleosides detected in *PaTrmD*-methylated tRNA by LC-MS/MS in MRM mode. The inset shows the product ions arising from CID of the *m/z* 298.1 for parent m¹G. (C) Extracted ion chromatogram for *m/z* 810.1356 released from tRNA^{Leu(GAG)} by RNase A and eluting at 18.9 min. (D) The mass spectrum for the tRNA^{Leu(GAG)} oligo shows an $[M-2H]^{-2}$ ion with *m/z* 810.1356 and sodium adducts at *m/z* 821.1140 and 832.1163. (E) CID of *m/z* 810.1356 yields products consistent with GAGm¹GU.

consistent with previous analyses performed with TrmD from *S. pneumonia*, *E. coli* (Redlak et al. 1997; Brulé et al. 2004) and *H. influenzae* (Ito et al. 2015; Christian et al. 2016). Fitting the data for SAM and tRNA^{Leu(GAG)} to a single-site binding model using MicroCal PEAQ-ITC analysis software, we determined that SAM bound to *PaTrmD*

with a K_d of 14.4 ± 3.1 μ M and tRNA^{Leu(GAG)} bound to *PaTrmD* with a similar K_d of 13.6 ± 3.0 μ M (Fig. 7). Interestingly the stoichiometry of the *PaTrmD*:tRNA^{Leu(GAG)} complex was 2:1, which is in line with a published structural study showing that only one of the two possible tRNA binding sites is used at a time on the dimeric TrmD protein (Sakaguchi et al. 2014). The observed binding affinity for SAM and tRNA can be interpreted in light of the estimated concentrations of these ligands in vivo. For example, the intracellular concentration of SAM in *E. coli* is estimated to be ~ 180 μ M (Bennett et al. 2009), while the intracellular concentration of total tRNA in *E. coli* is ~ 290 μ M (Zucconi and Beatty 1988). If each tRNA is assumed to be present at the same concentration, then *PaTrmD* substrates (nine of 61 tRNAs in PA14 as there are two copies of tRNA^{Leu(GAG)} and tRNA^{His(GUG)}) represent $\sim 15\%$ of total tRNA or ~ 43 μ M. Based on this information, it is anticipated that *PaTrmD* is at least partially bound with tRNA and probably fully bound with SAM (Bennett et al. 2009). However, care must be taken in interpreting these binding results since we have not accounted for confounders such as accessory proteins that might modulate the affinity of *PaTrmD* binding to specific tRNAs and the effects of other modified ribonucleosides on the affinity of tRNA for *PaTrmD*.

Sinefungin inhibition and the order of substrate binding to *PaTrmD*

Results from the steady-state kinetics analyses indicated that both SAM and tRNA must form a ternary complex of *PaTrmD*:SAM:tRNA in order for the reaction to proceed, while

the thermodynamics studies using ITC revealed a similar affinity for tRNA and SAM. However, these results did not give information about the binding order of SAM and tRNA to *PaTrmD*. To address this problem, we used SFG as a dead-end inhibitor to explore the *PaTrmD* kinetic mechanism (Fig. 8). The results showed that SFG inhibited

TABLE 3. Steady-state kinetic parameters of PaTrmD

Substrate	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m ($\text{min}^{-1} \mu\text{M}^{-1}$)
SAM	3.0 ± 0.8	13.0 ± 1.4	5.3 ± 1.3
tRNA ^{Leu(GAG)}	0.8 ± 0.2	13.0 ± 1.4	16.0 ± 3.0

The data represent mean $\pm$ SD for three independent determinations.

PaTrmD competitively with respect to SAM (Fig. 8A) and uncompetitively with respect to tRNA^{Leu(GAG)} (Fig. 8B). The K_i of SFG with respect to SAM and tRNA were determined as $0.41 \pm 0.07 \mu\text{M}$ and $6.4 \pm 0.8 \mu\text{M}$, respectively. Based on these results, there are two models—random and ordered—that explain a ternary-complex mechanism for PaTrmD. Interestingly, the binding order of substrates appears to vary among PaTrmD, EcTrmD (Redlak et al. 1997; Brulé et al. 2004), and HlTrmD (Ito et al. 2015; Christian et al. 2016). Studies using product inhibition analysis suggested that EcTrmD uses a random-order binding mechanism, whereas structural studies of HlTrmD suggested a compulsory-order binding mechanism in which SAM binds first followed by tRNA (Ito et al. 2015; Christian et al. 2016). We cannot completely rule out the possibility that these variations may arise from methodological differences. Here a simple comparison of the putative cellular concentrations of SAM 180 μM (Bennett et al. 2009) with the dissociation constant of the complex (14.4 μM , Fig. 7A) indicate that most of PaTrmD molecules in vivo are preloaded with SAM, thus favoring a sequential model where tRNA binds to this preformed binary complex, leading to further structural rearrangements, as suggested in Figure 4 and proposed by Ito et al. (2015). The results of our studies with PaTrmD reveal novel features of this tRNA-modifying enzyme that may explain its unique sensitivity to SAM-competitive inhibitors (Hill et al. 2013).

In summary, we present structural, biochemical, and enzymatic analyses of PaTrmD, which is an essential enzyme for the growth of *P. aeruginosa*. These data give insight into the catalytic mechanism of PaTrmD and should inform antibiotic discovery against this microorganism. The structure of PaTrmD is well conserved with the EcTrmD and HlTrmD further supporting TrmD as a good candidate for the development of broad-spectrum antibiotics targeting microorganisms possessing TrmD. To a large extent, the catalytic mechanism of most SAM-dependent methyltransferases requires no metal as a cofactor. However, bacterial TrmD proteins require Mg^{2+} ions for promoting the methyl transfer reaction. Here we show that an Mg^{2+} ion binds PaTrmD at 1:1 ratio lending further support for the requirement of Mg^{2+} ions for TrmD catalytic activity. Our kinetics data indicate a formation of the ternary complex of PaTrmD:SAM:tRNA in order for the reaction to proceed. The kinetic study using a dead-end inhibitor SFG together

with the known cellular concentration of SAM and the fact that PaTrmD was preloaded with SAM in vivo support the binding order of SAM and then tRNA. Our structural data also show the conformational change in the interdomain region, which indicates that the binding of tRNA (likely after SAM binding) induces this change before catalysis can proceed. With regards to substrates of TrmD, G36G37-containing tRNA^{His} is shown in this study as a new tRNA substrate of TrmD in addition to the known substrates tRNA^{Leu}, tRNA^{Pro}, and tRNA^{Arg}.

MATERIALS AND METHODS

Chemicals and reagents

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich Corporation and used as provided. Single-stranded oligonucleotides and PCR primers were purchased from Integrated DNA Technologies (IDT). Solutions of UltraPure 1 M Tris-HCl, pH 7.5, and molecular biology grade MgCl_2 (1 M) for enzyme assays were purchased from ThermoFisher Scientific and Invitrogen, respectively. Methyltransferase-Glo assay kits with S-adenosyl-L-methionine (SAM) and S-adenosyl-L-homocysteine (SAH) were purchased from Promega.

Construction of TrmD conditional knockout strain (PA1415990::Gm/pBBR-trmD-mSF^{ts1})

The forward and reverse primers (BT4298/BT4299) (Supplemental Table S1) were designed and used to amplify the full-length *trmD* gene (PA14_15990) from PA14 genomic

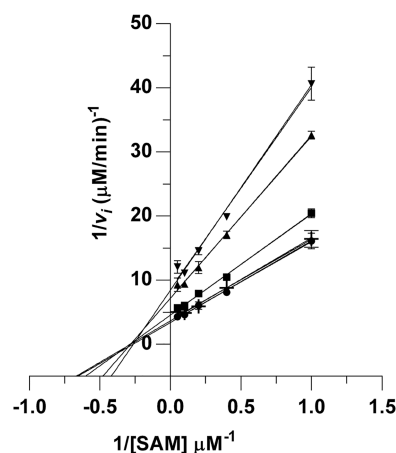


FIGURE 6. Analysis of steady-state kinetics of PaTrmD reveals a ternary complex mechanism. The in vitro PaTrmD assay was used to determine initial velocity (v_i) as a function of SAM and tRNA concentrations. The data were then subjected to a double-reciprocal plot for v_i determined at SAM concentrations ranging over 1–20 μM with different concentrations of tRNA^{Leu(GAG)}: 0.4 μM ($\blacktriangle$), 0.8 μM ($\blacktriangle$), 1.6 μM ($\blacksquare$), 3.2 μM ($\bullet$), or 5 μM ($\circ$). Data points represent mean $\pm$ SD for $n = 3$. The intersecting lines suggest that a ternary complex is required for the enzymatic reaction.

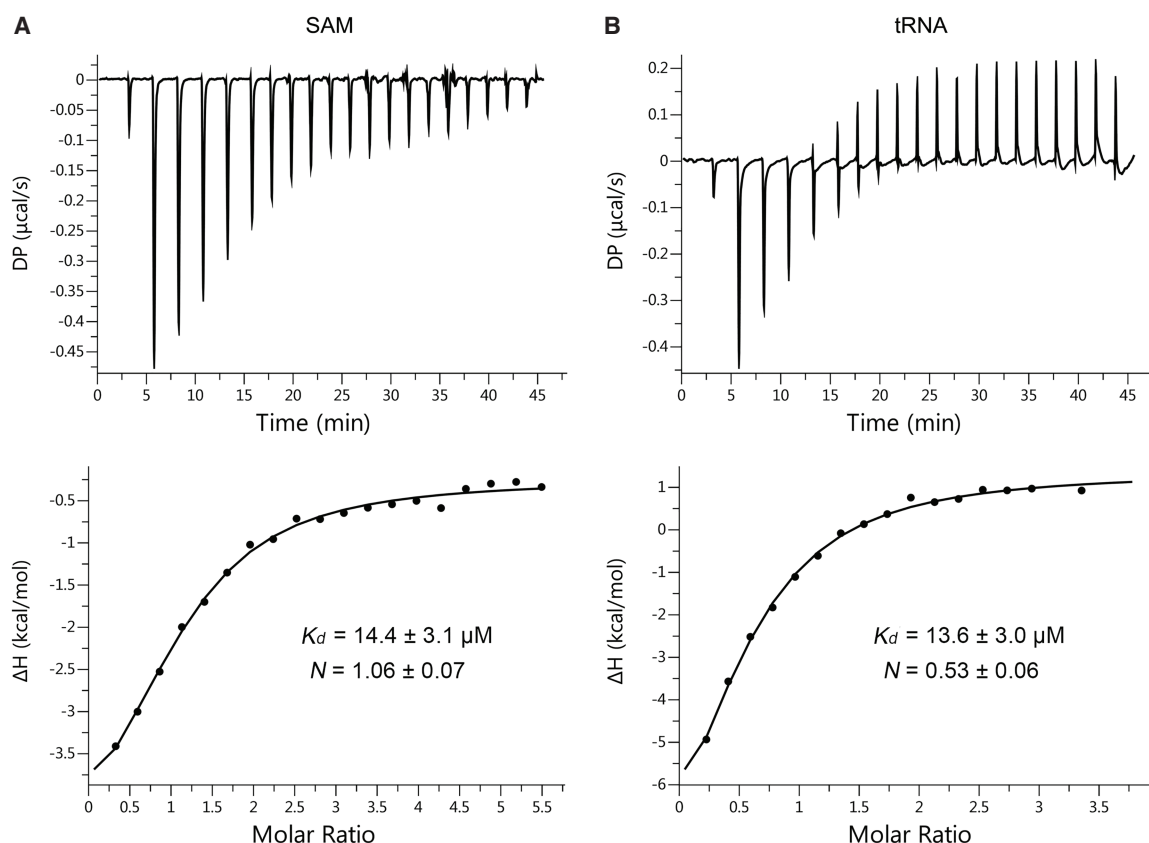


FIGURE 7. Analysis of binding thermodynamics reveals independent binding of SAM and tRNA to PaTrmD. The binding of SAM (A) or tRNA^{Leu(GAG)} (B) to PaTrmD was quantified by changes in the heat. Data were fit to a single-site fitting model using MicroCal PEAQ-ITC analysis software to determine binding constants (K_d).

DNA and the resulting PCR product was cloned into pBBR1-MCS4 (Kovach et al. 1995) at the SmaI site, yielding the complementation plasmid designated pBBR-*trmD*. To create a temperature-sensitive *trmD* expression plasmid (pBBR-*trmD*-mSF^{ts1}), the fragment containing a temperature-sensitive allele of a *Pseudomonas* replicon, mSF^{ts1}, was obtained from plasmid pSS255 (Silo-Suh et al. 2009) by enzymatic digestion with BamHI, with the resulting digestion fragment cloned into the pBBR-*trmD* plasmid at the BamHI site. The pBBR-*trmD*-mSF^{ts1} plasmid was introduced into *P. aeruginosa* PA14 wild-type strain by electroporation. *P. aeruginosa* PA14 wild-type containing pBBR-*trmD*-mSF^{ts1} was selected on a lysogeny agar plate containing 200 $\mu\text{g mL}^{-1}$ carbenicillin, with the presence of pBBR-*trmD*-mSF^{ts1} verified by PCR analysis. This strain was used as an intermediate in the construction of a *trmD* conditional knockout strain. The upstream and downstream regions of PA14_15990 were PCR-amplified with the primer pairs BT4316/BT4317 and BT4318/BT4319, respectively, and then cloned into pKNOCK-Ap, a suicide plasmid (Alexeyev 1999) containing a Gm cassette. A pKNOCK-Ap plasmid containing a Gm cassette flanked with upstream (500 bp) and downstream (500 bp) regions of PA14_15990 (*trmD*) was then transferred into the intermediate strain by conjugation. The transconjugants harboring the allelic exchange of chromosomal *trmD* with a Gm cassette were selected on a lysogeny agar plate containing 75 $\mu\text{g L}^{-1}$ gentamycin. The PaTrmD conditional knockout strain (*trmD*::Gm/pBBR-*trmD*-

mSF^{ts1}) was subsequently screened by colony PCR, and confirmed by Southern blot analysis.

Protein expression, purification, crystallization, and data collection

PaTrmD (construct VC008, obtained from NTU Protein Production Platform) spanning residues Leu5 to Asp250, with amino-terminal hexa-histidine tag followed by a TEV cleavage site, was used for crystallization, enzymatic, and kinetics analysis. *E. coli* BL21 (DE3) Rosetta T1R cells harboring PaTrmD plasmid, were cultivated at 37°C to OD₆₀₀ ~ 1. The proteins were overexpressed with 0.5 mM IPTG at 18°C for 18 h. Cells were pelleted and stored at -80°C. With all steps performed at 4°C, protein purification was achieved by resuspending thawed pellets in 30 mL of lysis buffer (20 mM Na-HEPES, pH 7.5, 0.3 M NaCl, 10% glycerol, 0.5 mM TCEP), sonicating the pellets in the presence of protease inhibitor cocktail (product 539134, Calbiochem), and clearing the lysates by centrifugation at 58,000g for 30 min. The proteins were first purified by affinity chromatography (Ni-NTA agarose, ThermoFisher Scientific), followed by size exclusion chromatography (HiLoad 16/60 Superdex 200, GE Healthcare). The PaTrmD proteins (30,296 Da monomer) eluted as dimers during size exclusion purification. PaTrmD was concentrated to 20 mg mL⁻¹ in 20 mM Na-HEPES, pH 7.5, 0.3 M NaCl, 5% (v/v)

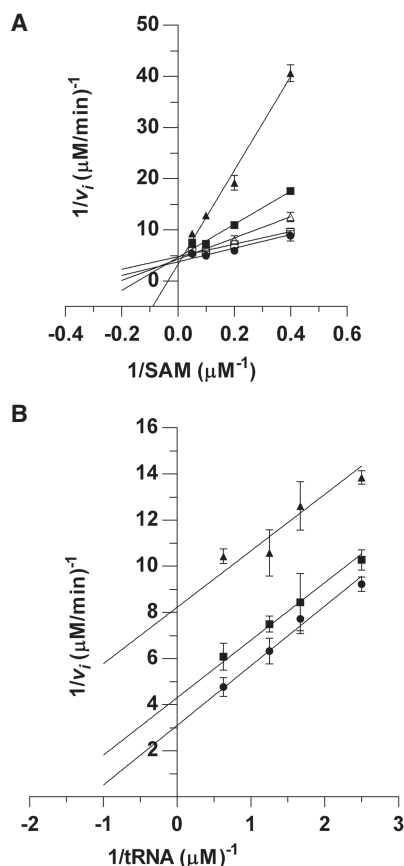


FIGURE 8. Sinefungin inhibition kinetics is consistent with initial binding by tRNA. Dead-end inhibition by SFG was used to assess the order of binding of tRNA and SAM from double-reciprocal plots for the inhibition of methylation by PaTrmD. (A) Inhibition by SFG at 0 μM ($\bullet$), 0.1 μM ($\blacksquare$), 0.3 μM ($\blacktriangle$), 1 μM ($\blacklozenge$), or 4 μM ($\blacktriangleleft$) as a function of SAM concentrations ranging from 2.5 to 20 μM . tRNA^{Leu(GAG)} was fixed at 5 μM . (B) Inhibition by SFG at 0 μM ($\bullet$), 4 μM ($\blacksquare$), or 8 μM ($\blacktriangle$) as a function of tRNA^{Leu(GAG)} concentrations ranging from 0.4 to 1.6 μM . The SAM concentration was fixed at 20 μM . Data points represent mean $\pm$ SD ($n = 3$).

glycerol, 0.5 mM TCEP. Aliquots were flash-frozen in liquid nitrogen and stored at $-80^{\circ}C$.

An automated initial crystallization screening was performed using the Phoenix crystallization robot (Art Robbins Instruments) at $20^{\circ}C$. Three precipitant: protein ratios (1:1, 1:2 and 2:1) were tested using the vapor diffusion method and commercial crystallization screens (Molecular Dimensions) in Intelli 96-3 wells sitting drop plates. Optimized elongated PaTrmD-SAM crystals were obtained by mixing equal volumes of protein and a precipitant solution containing 0.1 M Tris-HCl, pH 8.4, 12.5% (v/v) MPD, 12.5% (w/v) PEG 1000, 12.5% (w/v) PEG 3350, and 5% (w/v) PEG 200. Prior to data collection, crystals were cryo-protected by a brief soak in the precipitating solution supplemented with 20% (v/v) glycerol and rapidly frozen in liquid nitrogen. The PaTrmD-sinefungin crystals were obtained by soaking the PaTrmD-SAM crystals in the precipitating solution supplemented with 2 mM sinefungin at $20^{\circ}C$ for 2 h. For the complex with SFG and Mn^{2+} , one crystal of PaTrmD-SAM was soaked in the precipitating solution supplemented with 10% (v/v) glycerol, 2 mM sinefungin, and

5 mM $MnCl_2$ at $20^{\circ}C$ for 24 h. The crystal was then rapidly frozen in liquid nitrogen and data were collected at the K absorption edge of Mn^{2+} (Supplemental Fig. S8) at the Soleil synchrotron. Data collection details are given in Table 1.

For the binding assay, the expression of PaTrmD was as described above. However, the thawed pellet was resuspended in 30 mL of denaturing lysis buffer (20 mM Na-HEPES, pH 7.5, 0.5 M NaCl, 10% (v/v) glycerol, 0.5 mM TCEP, 8 M urea). The supernatant was loaded onto a 5 mL IMAC column (resins packed in Econo-Pac chromatography column, precharged with Ni^{2+}). The column was extensively washed with 30 CV of lysis buffer. After washing, the PaTrmD-bound beads were transferred to a dialysis bag, and dialyzed overnight at $4^{\circ}C$ against 1 L of dialysis buffer without urea (20 mM Na-HEPES, pH 7.5, 0.5 M NaCl, 10% glycerol, 0.5 mM TCEP). The overnight dialyzed sample was further dialyzed against a new batch of 1 L dialysis buffer for another 8 h at $4^{\circ}C$. The dialyzed sample was then reloaded onto the IMAC Econo-Pac chromatography column and washed with 5 CV of dialysis buffer. Bound PaTrmD protein was eluted by applying 5 CV of elution buffer (20 mM Na-HEPES, pH 7.5, 500 mM NaCl, 500 mM imidazole, 10% glycerol, 0.5 mM TCEP). The purity of each fraction was checked on SDS-PAGE and fractions containing target protein were pooled and concentrated to 5 mL. Concentrated protein was further purified by size exclusion chromatography and the purified protein was then stored as described above.

Structure determination and refinement

Diffraction intensities were integrated with XDS (Kabsch 2010), scaled, merged and truncated with SCALA/TRUNCATE (Collaborative Computational Project 1994). The structures were determined by molecular replacement using BALBES (Collaborative Computational Project 1994) with the structure from *E. coli* (PDB:1P9P) as a search probe (Elkins et al. 2003). A model for the 3D structure of PaTrmD was built iteratively by using COOT (Emsley and Cowtan 2004) and refined using Autobuster (Smart et al. 2012). The geometrical parameters for sinefungin were generated using coordinates 4R8S from the PDB (www.rcsb.org) and program PRODRG (Schüttelkopf and van Aalten 2004). Structure comparison was performed with the DALI server (echidna.biocenter.helsinki.fi/dali_server/start). The quality of the structures was assessed using the MOLPROBITY server (molprobity.biochem.duke.edu) and figures were generated using the Pymol software (Schrodinger). Data collection and structure refinement parameters are summarized in Table 1. The atomic coordinates and structure factors are deposited with the Protein Data Bank under accession codes 5WYQ (PaTrmD-SAM complex), 5WYR (PaTrmD-sinefungin complex), and 6JKI (PaTrmD-sinefungin-Mn complex).

Magnesium-bound TrmD analysis

The level of Mg^{2+} bound to PaTrmD was measured in the purified PaTrmD and the Mg^{2+} -reconstituted PaTrmD. To prepare the Mg^{2+} -reconstituted PaTrmD, 1 mL solution of 100 μM PaTrmD in 20 mM Na-HEPES, pH 7.5 was incubated with 1 mM $MgCl_2$ for 2 h at $25^{\circ}C$. The unbound Mg^{2+} ion was then removed with a PD-10 desalting column, and the protein was eluted with 20 mM Na-HEPES, pH 7.5. The concentration of the protein was

determined using the Bradford assay. The concentration of Mg^{2+} ion in samples was determined using inductively coupled plasma-optical emission spectrometry (ICP-OES). A calibration curve of Mg^{2+} ion (1000 ppm stock in 2% HNO_3) (Perkin-Elmer) at concentration ranges of 0.025 to 2 ppm for ICP-OES was used to calculate the mole of magnesium ion in samples.

Circular dichroism (CD) experiments

To verify the success of the protein refolding, we compared the CD spectra of native PaTrmD and refolded PaTrmD. Proteins were buffer exchanged to the CD buffer containing 20 mM HEPES pH 7.5, 50 mM NaCl, and concentrated to 0.5 mg ml^{-1} . The spectra were recorded in a cell width of 0.2-mm pathlength (121.QS, Hellma) from 195 to 260 nm at 20°C using a spectropolarimeter (Jasco J-810) equipped with a water-cooled Peltier unit. The spectrum from buffer was first recorded for baseline correction. Data were recorded in mdeg, converted as molar epsilon (Θ), and the spectra were superimposed using Prism.

Preparation of tRNA substrates by in vitro transcription

Single-stranded synthetic DNAs coding for *P. aeruginosa* PA14 tRNA^{Leu(GAG)}, tRNA^{Leu(CAG)}, tRNA^{Leu(UAG)}, tRNA^{His(GUG)}, tRNA^{Pro(GGG)}, tRNA^{Pro(CGG)}, tRNA^{Pro(UGG)}, and tRNA^{Glu(UUG)} containing a T7 promoter sequence at 5'-end were PCR-amplified with Phusion High-Fidelity DNA polymerase (Thermo Scientific) using primer pairs listed in Supplemental Table S1. PCR products were then used as templates for in vitro transcription with Mega short script (Thermo, Ambion). At the end of the reaction, the DNA template was removed by DNase digestion and the tRNA transcript was purified and quantified as described previously (Jaroensuk et al. 2016).

In vitro tRNA methylation by PaTrmD

The in vitro transcribed tRNA substrates tRNA^{Leu(GAG)}, tRNA^{Leu(CAG)}, tRNA^{Leu(UAG)}, tRNA^{His(GUG)}, tRNA^{Pro(GGG)}, tRNA^{Pro(CGG)}, tRNA^{Pro(UGG)}, and tRNA^{Glu(UUG)} were subjected to methylation reactions by incubating the tRNA with 50 μM of SAM and 100 nM PaTrmD in a buffer containing 50 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$ at 37°C for 1 h. Reaction products were analyzed for m¹G content by HPLC-coupled mass spectrometry. These enzyme reaction conditions were not optimized for kinetics assays as described below.

Analysis of PaTrmD methylation products by HPLC-coupled tandem quadrupole mass spectrometry

The tRNA transcripts (2 μg) from the in vitro methylation reactions were enzymatically hydrolyzed to ribonucleosides as described previously (Chan et al. 2011; Su et al. 2014; Jaroensuk et al. 2016). The resulting hydrolysate was analyzed by HPLC-coupled mass spectrometry using a Thermo Hypersil GOLD aQ HPLC column (100 $\times$ 2.1 mm, 1.9- μm particle size) operated at 25°C at a flow rate of 0.3 mL min^{-1} with the gradient mixture of mobile phase B (0.1% formic acid in acetonitrile) in mobile phase A

(0.1% formic acid in water) as follows: 0–15.3 min, 0%; 15.3–18.7 min, 1%; 18.7–20 min, 6%; 20–27.3 min, 100%; 27.3–41 min, 0%. The HPLC column was directly coupled to an Agilent 6460 triple quadrupole mass spectrometer (LC-MS/MS) with an electrospray ionization source operated in a positive ion mode and the following parameters: gas temperature, 300°C; gas flow, 5 L min^{-1} ; nebulizer, 40 psi; sheath gas flow, 7 L min^{-1} ; capillary voltage, 3500 V; and dwell time, 100 msec. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode to detect and quantify a variety of potential methylation products based on retention time, m/z of the transmitted parent ion, m/z of the monitored product, fragmentor voltages, and collision energy as noted in Supplemental Table S2. The identities of methylation products were confirmed by comparison with the synthetic standards.

Mapping PaTrmD products in tRNA by HPLC-coupled mass spectrometry

In vitro methylated tRNA^{Leu(GAG)} transcript (5 μg) were digested with 5 U of RNase A and dephosphorylated with 10 U of bacterial alkaline phosphatase in 10 mM ammonium acetate buffer (pH 7.0) at 37°C for 4 h in the presence of deaminase inhibitors and anti-oxidants as described previously (Chan et al. 2011; Su et al. 2014; Jaroensuk et al. 2016). The RNA fragments were then resolved by HPLC using an Amide-HILIC TSK-gel Amide-80 column (2.0 mm ID $\times$ 150 mm, 3 μm particle size) coupled to a quadrupole time-of-flight mass spectrometer (Agilent 6520) with an electrospray ionization source operated in a negative ion mode as described previously (Chionh et al. 2016). Collision-induced dissociation (CID) analysis was performed to obtain sequence information from the RNase A-digested products. Collision energies were varied from 25 to 40 V and products were scanned from m/z 100 to 2000, with RNA sequences determined using a-B, w and y ions.

Developing an in vitro PaTrmD assay for kinetics studies

For the kinetics assays, we developed an in vitro assay for PaTrmD activity based on the MTase-Glo coupled bioluminescent assay. In this assay, the SAH that is a product of the PaTrmD methylation reaction with SAM is converted to ADP by coupling enzymes in the MTase-Glo reagent. ADP is then converted to ATP using MTase-Glo detection solution generating a luminescent signal, which is proportional to the TrmD methyltransferase activity. We optimized the PaTrmD concentration by varying it from 0 to 100 nM. Reactions were prepared in 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 4 μM synthetic tRNA^{Leu(GAG)}, 50 μM SAM, and 1 $\times$ MTase-Glo reagent in a final volume of 55 μL . The reaction was incubated at 37°C for 5 min and a 10 μL aliquot was removed for the zero time point (T_0). PaTrmD (1 μL) was then added to initiate the reaction. During the 8-min time course at 37°C, 10 μL aliquots were collected every 2 min and quenched with 10 μL of MTase-Glo detection solution prepared in a 384-well plate (Grenier Item No. 784904; white color). The plate was incubated at ambient temperature for 30 min and the luminescence signal was quantified using a luminometer plate reader (Thermo Scientific). The resulting luminescent signal was then converted to SAH concentration using the SAH standard curve. A reaction

without PaTrmD was performed as a control. The initial velocities (v_i) were determined by plotting the SAM concentration as a function of time and the data were fitted by linear regression with GraphPad Prism version 6 (GraphPad Software, Inc.).

Determination of two-substrate PaTrmD steady-state kinetics

The steady-state kinetics parameters for PaTrmD were determined using the MTase-Glo assay (Hsiao et al. 2016) described earlier with varying concentrations of tRNA^{Leu(GAG)} (0.4–5.0 μ M) or SAM (1–20 μ M) and the PaTrmD concentration fixed at 23 nM. Reactions were initiated by adding PaTrmD and monitored using a luminometer plate reader (Thermo Scientific). The initial velocities (v) were measured as described above. The methylation kinetic parameters of the PaTrmD reaction were determined by plotting initial velocities versus substrate concentrations as described by a ternary-complex rate Equation 1, where A is SAM, B is tRNA^{Leu(GAG)}, K_a and K_b are the Michaelis constants for substrate A and B, respectively, and K'_a is the dissociation constant for enzyme-SAM complex. Data were fitted to the equation using GraphPad Prism version 6 (GraphPad Software, Inc.) and GraFit version 7 (Erithacus Software, Ltd.) for global analysis.

$$v = \frac{V_{\max}[A][B]}{K'_a K_b + K_b[A] + K_a[B] + [A][B]} \quad (1)$$

Isothermal titration calorimetry (ITC) assay

Assessment of SAM and tRNA binding to refolded PaTrmD was performed with a MicroCal PEAQ-ITC (Malvern Instruments Limited). The refolded PaTrmD and tRNA used for the experiments were dialyzed extensively against a buffer comprised of 50 mM Na-HEPES, pH7.5, 5 mM MgCl₂, and the dialysate was used to prepare fresh working solutions of SAM. Titrations were performed at 25°C and consisted of a single initial injection of 0.5 μ L, followed by 19 injections of 2 μ L of either 800 μ M SAM or 550 μ M of tRNA into the sample cell containing 30 μ M refolded PaTrmD. Two consecutive injections were separated by 3 min to allow the binding to come to equilibrium. Thermodynamic data were analyzed with a single-site fitting model using MicroCal PEAQ-ITC analysis software provided by the manufacturer.

Sinefungin (SFG) inhibition study

Inhibition of PaTrmD by the SAM substrate analog sinefungin (SFG) was investigated by measuring reaction velocities at fixed concentrations of SAM and tRNA with varying concentrations of sinefungin (0, 0.1, 0.3, 1, or 4 μ M). Reactions were performed in 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 1 $\times$ MTase-Glo reagent, a saturating concentration of tRNA^{Leu(GAG)} (5 μ M), PaTrmD at 23 nM and various concentrations of SAM (2.5–20 μ M). Assays were initiated by adding PaTrmD and the residual activity of PaTrmD in the presence of varying SFG concentrations was measured. The double-reciprocal plot of initial velocities (v) and SAM at various concentrations of SFG was plotted and the inhibition constant (K_i) for sinefungin with respect to SAM was determined by a competitive inhibition equation (Equation 2), where $V_{\max}$ is the maximum velocity of enzyme reaction, $[S]$ is the SAM concen-

tration, K_m is the Michaelis constant for SAM, $[I]$ is the SFG concentration and K_i is the inhibition constant for competitive inhibition. Data were fitted to Equation 2 using a GraphPad Prism version 6 (GraphPad Software, Inc.).

$$v = \frac{V_{\max}[S]}{K_m(1 + [I]/K_i) + [S]} \quad (2)$$

Analysis of SFG inhibition with respect to tRNA concentration was also performed and carried out as described above, except that the SAM concentration was kept constant at 20 μ M while the tRNA concentrations were varied from 0.4 to 1.6 μ M. PaTrmD activity was measured in the presence of SFG concentrations of 0, 4 and 8 μ M. The double-reciprocal plot of the initial velocities and tRNA concentrations was analyzed and the K_i for SFG with respect to tRNA was determined using an uncompetitive inhibition equation (Equation 3), where $V_{\max}$ is the maximum velocity of enzyme reaction, $[S]$ is a tRNA concentration, K_m is the Michaelis constant for tRNA, $[I]$ is an SFG concentration, and K_i is the inhibition constant for uncompetitive inhibition. Data were fitted to Equation 3 using GraphPad Prism version 6 (GraphPad Software, Inc.).

$$v = \frac{V_{\max}[S]/(1 + [I]/K_i)}{K_m(1 + [I]/K_i) + [S]} \quad (3)$$

DATA DEPOSITION

Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data bank under accession numbers 5WYQ, 5WYR, and 6JKI.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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