Conservation of structure and mechanism by Trm5 enzymes

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ABSTRACT

Enzymes of the Trm5 family catalyze methyl transfer from S-adenosyl methionine (AdoMet) to the N1 of G37 to synthesize m1G37-tRNA as a critical determinant to prevent ribosome frameshift errors. Trm5 is specific to eukaryotes and archaea, and it is unrelated in evolution from the bacterial counterpart TrmD, which is a leading anti-bacterial target. The successful targeting of TrmD requires detailed information on Trm5 to avoid cross-species inhibition. However, most information on Trm5 is derived from studies of the archaeal enzyme Methanococcus jannaschii (MjTrm5), whereas little information is available for eukaryotic enzymes. Here we use human Trm5 (Homo sapiens; HsTrm5) as an example of eukaryotic enzymes and demonstrate that it has retained key features of catalytic properties of the archaeal MjTrm5, including the involvement of a general base to mediate one proton transfer. We also address the protease sensitivity of the human enzyme upon expression in bacteria. Using the tRNA-bound crystal structure of the archaeal enzyme as a model, we have identified a single substitution in the human enzyme that improves resistance to proteolysis. These results establish conservation in both the catalytic mechanism and overall structure of Trm5 between evolutionarily distant eukaryotic and archaeal species and validate the crystal structure of the archaeal enzyme as a useful model for studies of the human enzyme.

Keywords: burst kinetics; S-adenosyl methionine; m1G37-tRNA; pH-activity profile; structure-guided mutagenesis

INTRODUCTION

The extensive base and backbone modifications present in natural tRNAs serve to promote translational fidelity and efficiency (Bjork et al. 1999; Motorin and Helm 2010; Yi and Pan 2011). Of the more than 100 modifications identified to date, the m1G37 base modification is strictly conserved in evolution for tRNAs specific for leucine (CUN codons, N being one of the four natural nucleotides), proline (CCN codons), and one of the arginine isoacceptors (the CGG codon) (Bjork et al. 2001). This modification is also present in mitochondria and chloroplasts and even in Mycoplasma spp., the latter of which has one of the smallest sequenced genomes known to date. The m1G37 modification replaces the imino proton of N1 of guanosine with a methyl group, thus preventing base-pairing on the 3′ side of the anticodon in +1 frameshifts (Fig. 1A,B). Indeed, m1G37 is essential for cell viability; its elimination leads to accumulation of frameshifts and delayed entry of charged tRNA to the ribosome (Bjork et al. 1989; Hagervall et al. 1993; Li and Bjork 1995). In yeast, elimination of m1G37 also promotes misacylation of tRNAArg with arginine (Putz et al. 1994). For eukaryotic tRNA^Phe specifically, m1G37 is the substrate for conversion to the hyper-modified wybutosine via four successive reactions (Noma et al. 2006). In archaea, m1G37 is important for synthesis of cysteinyl-tRNA^Cys required as a substrate for decoding cysteine codons (Hauenstein et al. 2008; Zhang et al. 2008).

While m1G37 is conserved in tRNAs, the enzymes that catalyze its synthesis are unrelated in evolution. Eukaryotes and archaea employ the Trm5 enzyme (Bjork et al. 2001; Christian et al. 2004), whereas bacteria use the TrmD enzyme (Bystrom and Bjork 1982a,b). While both enzymes use AdoMet as the methyl donor to convert G37-tRNA to m1G37-tRNA, they are fundamentally distinct in structure (Ahn et al. 2003; Christian et al. 2004; Goto-Ito et al. 2008, 2009), in kinetics (Christian et al. 2010b), and in substrate recognition (Christian and Hou 2007; Lahoud et al. 2011; Sakaguchi et al. 2012). The lack of similarity between TrmD and Trm5 has led to the suggestion that specific targeting of TrmD, without affecting the Homo sapiens Trm5 (HsTrm5), would be a highly attractive strategy in developing the next generation of antibiotics (White and Kell 2004).

The potential of TrmD as a leading anti-microbial target emphasizes the need to better understand HsTrm5 for two reasons. First, when an inhibitor is identified for TrmD, it must be critically evaluated against HsTrm5 to eliminate cross-inhibition in order to ensure species-specific targeting. However, key aspects of the evaluation remain completely void for HsTrm5, including kinetic parameters of methyl
However, whether the overall structure with AdoMet and tRNA (Goto-Ito et al. 2008, 2009) and with sinefungin (an inactive AdoMet analog) and a ternary structure with AdoMet and tRNA (Goto-Ito et al. 2008, 2009) revealed only moderate homology between MjTrm5 and MtTrm5, showing 29% identity and 53% similarity, in contrast to 80% identity and 95% similarity among TrmDs. Also, there exist species-specific differences between archaeal and eukaryotic enzymes (Christian et al. 2010a), whose meaning is unknown. Furthermore, purification yields of HsTrm5 in bacteria have been noted as low (Brule et al. 2004), in contrast to high yields of MtTrm5 (Christian et al. 2004), indicating at least in part the possibility of protease sensitivity of the human enzyme relative to the archaeal enzyme.

Here we address the correlation between HsTrm5 and MtTrm5 in two parts, with the first using a kinetic approach to evaluate the conservation of catalytic properties of the two enzymes and the second using the tRNA-bound structure of the archaeal enzyme as a model to evaluate the overall similarity with the human enzyme. We show that, despite their modest sequence homology, the two enzymes are fundamentally similar in both the catalytic mechanism and overall structure, suggesting that the available structures of MtTrm5 can provide a useful model to study HsTrm5. This study provides direct information for HsTrm5 and a framework necessary and most relevant to human health in the anti-microbial targeting of TrmD.

RESULTS AND DISCUSSION

Protease sensitivity of HsTrm5 in Escherichia coli

HsTrm5 was expressed in Escherichia coli with a C-terminal His tag, based on the Kazusa sequence (Nagase et al. 2000). The Kazusa sequence differs from the annotated genomic sequence by lacking the N-terminal peptide V2LWILWRP9 of the genomic sequence, and by containing a K394 residue instead of glutamate (numbering based on the Kazusa sequence) (Supplemental Fig. S1) at a nonconserved position. The lack of the N-terminal peptide was to facilitate cloning, while the E394K substitution was in the Kazusa sequence and not an artifact. While the expression level of the recombinant HsTrm5 was high upon induction (MW: ∼60 kDa), only a small fraction was soluble, and the purity after elution from a metal resin was poor (15%–20%) (Supplemental Fig. S2A), consistent with a previous report (Brule et al. 2004). Further purification of the affinity-purified enzyme on monoS, while reaching 90% homogeneity, resulted in significant losses of protein quantity. Because the affinity-purified enzyme was as active as the monoS-purified enzyme on the transcript of human tRNA$^{\text{Gyc}}$ as a substrate (Fig. 1B; Supplemental Fig. S2B), we used the affinity-purified enzyme throughout this work.

Among the impurities in the affinity-purified enzyme, a 27-kDa protein was most persistent, which was also observed upon expression of MtTrm5 in E. coli but to a lesser extent (Christian and Hou 2007). An LC/MS/MS analysis of the smaller protein showed that it was a degradation product, representing the C-terminal half of the protein. Detailed MS/MS analysis showed that the cleavage sites occurred at the N terminus to M252, V254, M261, and T263 (Supplemental Fig. S3).
The persistent appearance of the 27-kDa protein suggested that the soluble fraction of HsTrm5 when expressed in E. coli was sensitive to proteolysis, which was one of the reasons for the poor yield of the enzyme relative to MjTrm5.

**Kinetic analysis of HsTrm5**

The affinity-purified HsTrm5 was examined for its steady-state activity on the transcript of human tRNA\(_{\text{Cys}}\), similar to the previous analysis of MjTrm5 with a transcript of the archael \(\text{tRNA}^\text{Cys}\) (Christian et al. 2004, 2010b; Sakaguchi et al. 2012). The transcript of human \(\text{tRNA}^\text{Cys}\) had a capacity of methylation to ~70% levels in extended time. The methylation reaction monitored methyl transfer from \(^3\text{H}-\text{methyl}\)-AdoMet to G37-tRNA, producing \(^3\text{H}-\text{m}^1\text{G37-tRNA}\) and releasing S-adenosyl homocysteine (AdoHcys). Steady-state activity as a function of AdoMet concentration with saturating tRNA revealed \(K_m\) (AdoMet) of 0.42 ± 0.08 \(\mu\text{M}\) and \(k_{\text{cat}}\) of 0.023 ± 0.003 sec\(^{-1}\), closely similar to values of MjTrm5 \([K_m\ (\text{AdoMet}) \text{ of } 1.0 \pm 0.1 \mu\text{M} \text{ and } k_{\text{cat}} \text{ of } 0.017 \pm 0.002 \text{ sec}^{-1}\]\) (Table 1; Christian et al. 2006). Separately, steady-state activity as a function of tRNA in the presence of saturating AdoMet revealed \(K_m\) (tRNA) of 0.47 ± 0.04 \(\mu\text{M}\) and \(k_{\text{cat}}/K_m\) (tRNA) of 0.05 ± 0.01 sec\(^{-1}\), also closely similar to values for MjTrm5 \([K_m\ (\text{tRNA}) \text{ of } 0.70 \pm 0.05 \mu\text{M} \text{ and } k_{\text{cat}}/K_m\ (\text{tRNA}) \text{ of } 0.024 \pm 0.003 \text{ sec}^{-1}\]\) (Table 1; Christian et al. 2006).

Pre-steady-state assays were also performed, with the enzyme in molar excess of AdoMet and with tRNA saturating to permit only one methyl transfer on the enzyme. The rate constant of each time course represented a composite term for all reaction steps from enzyme-substrates binding and up to and including the formation of \(\text{m}^1\text{G37-tRNA}\) (Johnson 1998). All time courses were well fit to a single exponential equation (Supplemental Fig. S4A), indicating rapid equilibrium binding. Control experiments confirmed that the mixing order did not affect the rate of methyl transfer. Fitting the data of \(k_{\text{obs}}\) as a function of enzyme concentration to a hyperbola (Supplemental Fig. S4B) revealed the dissociation constant \(K_d\) (AdoMet) (1.1 ± 0.1 \(\mu\text{M}\)), which is closely similar to the value of MjTrm5 (Christian et al. 2010b). Similarly, fitting the data of \(k_{\text{obs}}\) as a function of enzyme concentration in excess of tRNA and with saturating AdoMet revealed \(K_d\) (tRNA) of 1.8 ± 0.1 \(\mu\text{M}\) (Supplemental Fig. S4C,D), also closely similar to the \(K_d\) (tRNA) of 1.4 ± 0.1 \(\mu\text{M}\) of MjTrm5 (Table 2; Christian et al. 2010b). The maximum rate constant \(k_{\text{chem}}\) (0.09 ± 0.01 sec\(^{-1}\)), which included both the pre-chemistry isomerization of the enzyme and the chemistry of methyl transfer, was again similar to that of MjTrm5 (Table 2).

The \(K_d\) values of pre-steady-state assays were interpreted as the equilibrium dissociation constant, due to the rapid equilibrium binding conditions (Johnson 1998). This interpretation has been confirmed for MjTrm5, using the intrinsic enzyme tryptophan fluorescence as a probe for equilibrium binding of tRNA in the presence of saturating sinefungin (Sakaguchi et al. 2012). The addition of tRNA to the enzyme resulted in quenching of the intrinsic fluorescence and the measurement of the quench as a function of tRNA revealed a \(K_d\) (tRNA) of 1.3 ± 0.6 \(\mu\text{M}\), closely similar to the kinetic \(K_d\) (tRNA) determined for MjTrm5 (1.4 ± 0.1 \(\mu\text{M}\)) and for HsTrm5 (1.8 ± 0.1 \(\mu\text{M}\)). Based on the kinetic \(K_d\) of each enzyme as the equilibrium binding constant, the catalytic efficiency of methyl \(k_{\text{chem}}/K_d\) (tRNA) was closely similar for the two enzymes (0.05 ± 0.01 \(\mu\text{M}^{-1}\ \text{sec}^{-1}\) for HsTrm5 and 0.09 ± 0.01 \(\mu\text{M}^{-1}\ \text{sec}^{-1}\) for MjTrm5).

**The rate-determining step**

MjTrm5 previously showed a rapid burst of product synthesis in a pre-steady-state assay, followed by a slower and linear synthesis over time (Christian et al. 2010b). The burst suggested that product synthesis in the first turnover was fast, but that product release from the enzyme was slow, thus limiting the rate of subsequent turnovers. In contrast, TrmD showed a linear synthesis of product over time, suggesting that the slow step occurred before or during methyl transfer (Christian et al. 2010b). Similar to MjTrm5, HsTrm5 also showed a rapid burst of product synthesis followed by a steady-state rate (Fig. 2). Fitting the burst data yields \(k_{\text{chem}}\) of 0.13 ± 0.02 sec\(^{-1}\) and \(k_{\text{cat}}\) of 0.020 ± 0.002 sec\(^{-1}\), similar to values determined by separate steady-state and pre-steady-state analysis (Table 1). Extrapolation of the burst plot to the ordinate yielded an estimate for the active enzyme fraction at 15%–20%.

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**TABLE 1. Steady-state parameters of HsTrm5 relative to MjTrm5**

|        | \(K_m\) (AdoMet) (\(\mu\text{M}\)) | \(K_m\) (tRNA) (\(\mu\text{M}\)) | \(k_{\text{cat}}\) (sec\(^{-1}\)) | \(k_{\text{cat}}/K_m\) (tRNA) (\(\mu\text{M}^{-1}\ \text{sec}^{-1}\)) |
|--------|---------------------------------|-------------------------------|---------------------------------|---------------------------------|
| HsTrm5 | 0.42 ± 0.05                     | 0.47 ± 0.04                   | 0.023 ± 0.003                   | 0.05 ± 0.01                     |
| MjTrm5 | 1.00 ± 0.06                     | 0.70 ± 0.03                   | 0.017 ± 0.002                   | 0.02 ± 0.01                     |

Values for HsTrm5 were obtained from the average of at least three independent experiments. Values for MjTrm5 were taken from the published work (Christian and Hou 2007).

**TABLE 2. Pre-steady-state parameters of HsTrm5 relative to MjTrm5**

|        | \(K_d\) (AdoMet) (\(\mu\text{M}\)) | \(K_d\) (tRNA) (\(\mu\text{M}\)) | \(k_{\text{chem}}\) (sec\(^{-1}\)) | \(k_{\text{chem}}/K_d\) (tRNA) (\(\mu\text{M}^{-1}\ \text{sec}^{-1}\)) |
|--------|---------------------------------|-------------------------------|---------------------------------|---------------------------------|
| HsTrm5 | 1.1 ± 0.1                       | 1.8 ± 0.1                    | 0.09 ± 0.01                     | 0.05 ± 0.01                     |
| MjTrm5 | 0.8 ± 0.1                       | 1.4 ± 0.1                    | 0.12 ± 0.01                     | 0.09 ± 0.01                     |

Values for HsTrm5 were obtained from the average of three independent experiments. Values for MjTrm5 were taken from the published work (Christian et al. 2010b).
which corresponds closely to the purity of the affinity-isolated enzyme. This active-site fraction was used to correct the enzyme concentration as was done for *Mj*Trm5 (Christian et al. 2010b).

### The pH-activity profile

The high pK_a (\( \approx 9.5 \)) of the N^1_ imino proton of G37 suggested that enzyme-catalyzed deprotonation would be necessary for methyl transfer. Indeed the pH-activity profile of *Mj*Trm5 showed that catalysis was dependent on one proton transfer (Christian et al. 2010a), most likely the transfer of the N^1_ proton of G37 to a general base on the enzyme. A pH-activity profile of *Hs*Trm5 also revealed a steep increase of \( k_{\text{obs}} \) as the proton concentration was lowered, up to an asymptote at pH 8.0 (Fig. 3A). We measured \( k_{\text{obs}} \) at each pH, rather than \( k_{\text{diss}} \) and \( k_{\text{chem}} \), due to the inability to produce large quantities of *Hs*Trm5. The logarithmic plot of \( k_{\text{obs}} \) (log[\( k_{\text{obs}} \)]) versus pH revealed a slope of 0.5 (Fig. 3B), most consistent with one proton transfer upon deprotonation of the N^1_ of G37. The value of 0.5 suggests the possibility that the \( k_{\text{obs}} \) of the human enzyme might be a function of both \( k_{\text{chem}} \) and \( K_d \) (tRNA) and that only one of the two (probably \( k_{\text{chem}} \)) exhibited a dependence on pH. This is a notable difference from *Mj*Trm5, which displayed a value close to 1.0 (Christian et al. 2010a).

For the human enzyme, fitting the data of \( k_{\text{obs}} \) as a function of pH to a one-proton transfer equation revealed a pK_a of 7.1 ± 0.1 (Fig. 3A), higher by 0.6 units than the pK_a of *Mj*Trm5 (6.5 ± 0.2) (Christian et al. 2010a).

### Kinetic isotope effect

While the pH-activity analysis demonstrated the importance of one-proton transfer for catalysis, the structure of *Mj*Trm5 did not identify an appropriately positioned general base for proton abstraction of the N^1_ of G37. Instead, the general base for *Mj*Trm5 was proposed to be E185, which is strictly conserved as glutamate or aspartate but is located >5 Å away from the N^1_ of G37 (Christian et al. 2010a). The location of E185 far away from the N^1_ of G37 suggested that proton abstraction had occurred before the active-site structure was captured in the crystal, most likely during the induced-fit rearrangement of the enzyme with G37 of tRNA. In this model, the \( k_{\text{obs}} \) represented the induced-fit process rather than the chemical step of proton abstraction and thus would not be sensitive to the isotope effect at the N^1_ of G37. This hypothesis was confirmed for both *Mj*Trm5 and *Hs*Trm5 by evaluating the kinetic isotope effect of the methyl transfer reaction, where the N^1_ proton of G37 had been readily exchanged to deuterium in the presence of deuterium water (McConnell et al. 1983; Snoussi and Leroy 2001). Pre-steady-state kinetic assays showed that \( k_{\text{obs}} \) for *Mj*Trm5 (0.11 sec\(^{-1}\)) and \( k_{\text{obs}} \) for *Hs*Trm5 (0.012 sec\(^{-1}\)) remained unaffected in deuterium water relative to hydrogen water (Fig. 4A,B), indicating that the chemical step of proton abstraction was not the rate-determining step for these enzymes. The lack of an effect for both enzymes supports the notion that they share a common kinetic mechanism involving proton abstraction during a slower and rate-determining process of induced fit. This induced-fit and proton abstraction would require the action of the general base and a conserved arginine side chain to facilitate the rearrangement of electrons (Supplemental Fig. S5).

### Evaluation of the active-site structure

The conservation of kinetic features between *Hs*Trm5 and *Mj*Trm5 suggests the conservation of the active-site structure. We tested this hypothesis by evaluating mutations in *Hs*Trm5 (Table 3), based on the ternary structure of *Mj*Trm5 (Goto-Ito...
tRNA synthesis catalyzed by *Mj* Trm5 is virtually identical, as demonstrated by the close proximity of the N1 of G37.

Aspartate likely in the movement of the general base to the 24-fold decrease by the D172A mutation of the archaeal enzyme, supporting the notion that both enzymes engage this aspartate residue (D172 in *Mj* Trm5) in the flexible loop preceding the proposed general base.

Historically, the placement of the methyl group directly opposite from the N1-proton of G37 by R145 (*)Mj* Trm5, showing the enzyme stabilization of the O6-carbonyl of G37 by R145 (*Hs* Trm5) and the corresponding K264 of *Mj* Trm5 has a wider diversity (e.g., lysine, serine, glutamate, valine, and isoleucine). However, we found that the H162K mutation.

Evaluation of the overall structure

The conservation of catalytic features between *Hs* Trm5 and *Mj* Trm5, however, does not mean the conservation of their overall structure. To probe the similarity of their overall structure, we addressed the challenge of the protease sensitivity...
of the human enzyme relative to the archaeal enzyme. If the two enzymes share a similar structure, then a structure-guided site-specific substitution in the human enzyme to model the archaeal enzyme should confer an archaeal-like stability to the human enzyme. Because the protease-sensitive sites of the human enzymes M252, V254, M261, and T263 (Supplemental Fig. S3) are nonconserved and outside of the active site, this made the modeling more challenging. Modeling of these residues to the tRNA-bound structure of MjTrm5 showed that, while M252 and V254 are localized in a loop that stabilizes G37, M261 and T263 are in a helix more distant from G37 (Fig. 5A). We found that, indeed, the single M261L substitution that recapitulated the archaeal residue minimized the 27-kDa protease product upon enzyme expression in E. coli, indicating improved stability. In contrast, the T263L substitution had no effect, whereas the double substitutions (M261L and T261I) had the same effect as the single M261L substitution (Supplemental Fig. S6). Importantly, the single M261L substitution retained the full enzyme activity during modeling of the archaeal structure. For example, yeast Trm5 is localized in the nucleus, where it catalyzes m1G37 synthesis after tRNAPhe is modified with Cm32 and Gm34 (Nm = 2′-O-methylation in nucleotide N) in the cytoplasm and sent back to the nucleus (Murthi et al. 2010). The Trm5 product m1G37-tRNA is then re-exported to the cytoplasm for conversion of m1G37 to yW37 (Ohira and Suzuki 2011). Such a complex tRNA trafficking mechanism does not exist in archaea, suggesting the possibility that eukaryotic Trm5 may act on Cm32- and Gm34-modified tRNA and with high-resolution structures of HsTrm5 in complex with tRNA and an analog of AdoMet. Because of the importance of HsTrm5, efforts to obtain its structures should be given priority in order to produce insight into how eukaryotic Trm5 is diverged from its archaeal counterpart and how it is separated from the bacterial TrmD.

### MATERIALS AND METHODS

#### Cloning and expression of HsTrm5

The cDNA sequence for HsTrm5 was amplified from the KIAA 1393 plasmid of Kazusa DNA Research Institute (Kisarazu, Chiba, Japan).
This cDNA encodes F^3 to T^500 (Supplemental Fig. S1) and was cloned into the Ndel and Xhol restriction sites of pET22b. The protein sequence as expressed starts with M with encoded by the Ndel site, followed by F^5GRF^6 to T^500, and ends with a His-tag. The coding sequence differs from the genomic sequence (http://www.uniprot.org/uniprot/Q32P41) by having K394 instead of E394. The recombinant enzyme was expressed in E. coli BL21(DE3)-RIL and purified by affinity binding to the His-Link resin (Promega [H8820A] in a sonication buffer [20 mM HEPES at pH 7.5, 250 mM NaCl, 10 mM [β-mercaptoethanol, and 0.2 mM PMSF]). Mutations of the enzyme were created using the Quik-Change protocol and confirmed by DNA sequencing analysis. Enzyme mutants were assayed in the same way as the wild-type enzyme. Transcripts of HsRNA<sub>CV</sub> were synthesized by T7 RNA polymerase based on a DNA template created from two overlapping oligonucleotides (Zhang et al. 2008) and were purified by a denaturing 12% PAGE/7 M urea gel.

**Mass spectrometry analysis**

The analysis, performed as a service at the proteomic center of Ohio State University, is described in the legend to Supplemental Figure S3.

**Kinetic assays**

Steady-state kinetic assays of HsTrm5 were performed at 37°C in the same buffer as for MfTrm5 (Christian et al. 2004). Assays for tRNA parameters were performed with 25 μM [<sup>3</sup>H-methyl]-AdoMet (80 Ci/mmol) (PerkinElmer NET 155H), 0.25–6 μM of HsRNA<sub>CV</sub> transcript, and 50 nM of the enzyme, while those for AdoMet parameters were measured with tRNA saturated at 6 μM and 0.25–5 μM of AdoMet. Aliquots were removed at time points and precipitated in 5% TCA (trichloroacetic acid), and counts were measured and corrected for backgrounds and a quenching efficiency of ~55%. Parameters were determined from fitting data as a function of concentration to the Michaelis–Menten equation. Each value was determined from the average of at least three independent analyses, showing standard errors, which were obtained by dividing the standard deviation of each value by the root square of the sample size.

Pre-steady-state kinetics was monitored on an RQF3 KinTek instrument (Christian et al. 2010b). One syringe contained enzyme (0.5–10 μM) with tRNA (0.5 μM) and the other syringe contained [<sup>3</sup>H-methyl]-AdoMet (25 μM). Data were fit to the single exponential equation:

\[ y = y_0 + A \times (1 - e^{-k_{app} \times t}) \]

where \( y_0 \) is the y intercept, \( A \) is the scaling constant, \( k_{app} \) is the apparent or observed rate constant, and \( t \) is the time in seconds to determine \( k_{app} \). The analysis of \( k_{app} \) versus enzyme concentration was fit to the hyperbolic equation:

\[ y = k_{chem} \times E_0 / (E_0 + K_d) \]

where \( k_{chem} \) is the rate constant for the chemistry step, and \( E_0 \) is the enzyme concentration. Measurement of \( K_d \) (AdoMet) was as described (Christian et al. 2010b); one syringe contained enzyme (0.5–10 μM) with [<sup>3</sup>H-methyl]-AdoMet (0.3 μM), and the other syringe contained tRNA (6 μM). Data were fit to the single exponential equation as above.

Burst analysis was monitored as described (Christian et al. 2010b), upon rapid mixing of HsTrm5 (1 μM) in one syringe with tRNA (10 μM) and [<sup>3</sup>H]-AdoMet (25 μM) in the second syringe. The data were fit to the burst equation:

\[ y = y_0 + A \times \left(1 - e^{-k_1 \times t}\right) + k_2 \times E_0 \times t \]

where \( y_0 \) is the y intercept, \( A \) is the amplitude of the initial exponential phase, \( k_1 \) is the apparent rate constant of the initial exponential regression, \( k_2 \) is the apparent rate constant of the steady-state phase, and \( t \) is the time in seconds.

**The pH-activity profile**

The experiments were performed as described (Christian et al. 2010a). Buffers used for different pH values were as follows: sodium cacodylate (pH 5.9, 6.1); MES (pH 6.1, 6.3, 6.4, 6.6); MOPS (pH 6.6, 6.8, 7.0, 7.1); glycyglycine (pH 7.1, 7.3, 7.6, 8.1); and glycine (pH 9.1). If the pH was lower than the normal value, drops of 5 M KOH were added to the 5X solution such that the 1X solution became properly adjusted. Reactions at pH lower than 7.3 were monitored by hand sampling, while those at higher pH values were done on the RQF3 instrument. No differences in rate were observed for reactions run at pH values where two different buffers were used. HsTrm5 enzyme was used at 3 μM, 37°C, in the reaction buffer containing a final concentration of 0.1 M buffer, 0.1 M KCl, 6 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 4 mM DTT, and 0.024 mg/mL BSA. Upon rapid mixing with 0.5 μM tRNA and 25 μM AdoMet in the same buffer, the time course of m<sup>15</sup>G37 synthesis was monitored. Substrate saturation at pH 6.0, 8.1, and 9.8 was demonstrated by showing that the \( k_{obs} \) remained unchanged upon reducing the enzyme and tRNA concentrations by twofold. The consistency in the measured \( k_{obs} \) across all three pH values established that both tRNA and AdoMet were stable at all relevant pH values. The data were fit to the equation:

\[ k_{obs} = \frac{k_{A^-}\times K_{AH}\times pH}{1 + 10^{pK_a - pH}} \]

where \( k_{obs} \) is the observed reaction rate at a specific pH, \( K_{AH} \) is the activity of the protonated form of G37 (\( K_{AH} = 0 \)), \( k_{A^-} \) is the activity of the deionized form of G37, and \( K_a \) is the equilibrium constant for the dissociation of the proton.

**Kinetic isotope effect**

The solvent-exchangeable N<sup>1</sup> imino proton of G37 in tRNA was replaced with deuterium by incubating the tRNA (previously dried down) in D<sub>2</sub>O (Cambridge Isotope Laboratories) for a few minutes in one syringe of RQF3. The enzyme and AdoMet were incubated in the water-based buffer in the other syringe. Upon rapid mixing, the time course of methyl transfer was monitored by taking aliquots and measuring acid-precipitable counts. The kinetics of MfTrm5 was measured at the near-saturating 6 μM of the enzyme, while that of HsTrm5 was measured at 2 μM, due to the difficulty in obtaining large quantities of the latter. The average value of \( k_{obs} \) from two independent measurements was reported.

**Molecular modeling**

Figures were drawn with PyMol (PDB 2ZZN), corresponding to the ternary complex of MfTrm5·tRNA<sub>CV</sub>·AdoMet complex.
SUPPLEMENTAL MATERIAL
Supplemental material is available for this article.

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