Differentiating analogous tRNA methyltransferases by fragments of the methyl donor

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ABSTRACT

Bacterial TrmD and eukaryotic-archaeal Trm5 form a pair of analogous tRNA methyltransferase that catalyze methyl transfer from *S*-adenosyl methionine (AdoMet) to N¹ of G37, using catalytic motifs that share no sequence or structural homology. Here we show that natural and synthetic analogs of AdoMet are unable to distinguish TrmD from Trm5. Instead, fragments of AdoMet, adenosine and methionine, are selectively inhibitory of TrmD rather than Trm5. Detailed structural information of the two enzymes in complex with adenosine reveals how Trm5 escapes targeting by adopting an altered structure, whereas TrmD is trapped by targeting due to its rigid structure that stably accommodates the fragment. Free energy analysis exposes energetic disparities between the two enzymes in how they approach the binding of AdoMet versus fragments and provides insights into the design of inhibitors selective for TrmD.

Keywords: *S*-adenosyl methionine (AdoMet); adenosine; methionine; m¹G37-tRNA

INTRODUCTION

Analogous enzymes are thought to be independent inventions of nature (Galperin et al. 1998), arising from different phylogenetic lineages with unrelated structures to catalyze the same reaction. These enzymes are unlike the commonly found homologous enzymes, which share similar sequence and structural folds. The existence of analogous enzymes, present in different biological domains, suggests the attractive possibility of species-specific targeting. For example, the identification of a bacterial enzyme that is structurally distinct from its human counterpart would provide a promising target for the development of new antibacterial drugs. Such a strategy would circumvent the difficulty in targeting subtle differences between bacterial and human enzymes of homologous origins. However, despite the appeal of this idea, obstacles remain high with targeting analogous enzymes. The key reason is that while these enzymes possess different catalytic structures from each other, they recognize the same substrates and cofactors using tight networks of enzyme-substrate interactions that have been well de-

veloped and adapted over time with comparable substrate binding affinities. This presents severe challenges to the conventional approach of structure-based design of substrate analogs with sufficient selectivity between analogous enzymes. To date, no successful substrate analogs have been reported that target specific members of a pair of analogous enzymes.

Here we address this challenge by evaluating the determinants of the methyl donor AdoMet for selective inhibition of the analogous tRNA methyltransferases TrmD and Trm5, which catalyze the synthesis of the m¹G37 base 3' adjacent to the anticodon. TrmD is broadly conserved in all bacterial species, whereas Trm5 is found in eukaryotes and archaea but is absent from bacteria (Bjork et al. 1989, 2001; O'Dwyer et al. 2004). While both enzymes recognize AdoMet as the methyl donor and G37-tRNA as the acceptor, they share no structural homology. TrmD functions as a homodimer and binds AdoMet using a rare trefoil-knot fold (Ahn et al. 2003; Elkins et al. 2003), whereas Trm5 functions as a monomer and binds AdoMet using the popular protein fold known as the Rossmann fold (Goto-Ito et al. 2008, 2009). Importantly, crystal structures of these enzymes show that the AdoMet structure when bound to TrmD exists in an L-shaped bent conformation (Ahn et al. 2003), where the methionine moiety bends over the adenosine moiety, whereas the AdoMet structure when

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bound to Trm5 exists in an extended open conformation (Fig. 1; Goto-Ito et al. 2009). The two enzymes also differ in the recognition of G37 in tRNA (Christian and Hou 2007): TrmD requires only an extended anticodon stem-loop structure for activity, whereas Trm5 requires the intact tRNA structure. Furthermore, the rate-limiting step in the TrmD-catalyzed reaction occurs during the synthesis of m¹G37, whereas that for Trm5 occurs after synthesis (Christian et al. 2010b). The fundamental structural and kinetic differences between the two enzymes and their split along the evolutionary separation of bacteria from archaea and eukaryotes make this pair a paradigm to explore conceptual strategies for targeting analogous enzymes.

tRNA modification enzymes are attractive for targeting, because of their role in the adjustment of cell fitness and viability. Extensive cellular resources are dedicated to synthesize these enzymes, which are responsible for the over 100 modified nucleotides that have been identified in tRNA. While many nucleotide modifications in tRNA can be achieved by a specific enzyme, others are achieved by multiple enzymes in successive reactions (e.g., wybutosine; Noma et al. 2006). In general, inactivation of one modification enzyme for a tRNA often has no clear growth defect, although inactivation of additional enzymes can cause synthetic lethality (e.g., Persson et al. 1997; Urbonavicius et al. 2002). The synergistic effects on growth between tRNA modification enzymes, together with the observation that hypo-modifications accelerate tRNA degradation (Alexandrov et al. 2006; Chernyakov et al. 2008), provide an underpinning for the notion that tRNA modification enzymes function collectively as a device to regulate cellular processes and as a sensor of the cellular metabolic status in both bacteria and eukaryotes (Persson 1993).

While there are other analogous pairs of tRNA modification enzymes, none are as well characterized as TrmD and Trm5. For example, the synthesis of Cm32 and Um32 (Cm and Um = ribose 2'-O-methylation of C and U) in the anticodon loop is catalyzed by TrmJ in bacteria with a trefoil-knot structure but is catalyzed by Trm7 in eukaryotes with a Rossmann-fold structure (Purta et al. 2006). The synthesis of Cm56 in the T loop (Clouet-d'Orval et al.

2005; Renalier et al. 2005) is catalyzed by a single Trm56 enzyme in most organisms but by an RNA-protein (RNP) complex in some archaea, where the proteins in the complex perform this reaction in conjunction with an RNA guide made up of C/D boxes. The synthesis of Ψ35 (Ψ, pseudouridine) in the anticodon of tRNA^{Tyr} also involves analogous enzymes, with Pus7 acting as the single enzyme in eukaryotes and with an H/ACA-guided RNP in some archaea (Muller et al. 2009). However, detailed structural and kinetic information is lacking for these alternative pairs. Importantly, the reaction catalyzed by TrmD and Trm5 is rare in that it alone is required for optimal growth in bacteria (Bjork et al. 1989; Hagervall et al. 1993; O'Dwyer et al. 2004; Baba et al. 2006) and in eukaryotes (Bjork et al. 2001). This phenomenon is due to the essential role of m¹G37 in the prevention of translational errors on the ribosome (Hagervall et al. 1993). Although m¹A58 in the T loop of tRNA has also been shown to be essential for the viability of yeast (Anderson et al. 2000), due to its role in maintaining steady-state levels of eukaryotic initiator tRNA (Anderson et al. 1998), it is not uniformly present in human cells (Saikia et al. 2010) and is rare in bacteria, indicating that it is not a broad-based antimicrobial target. Also, while m¹A58 is synthesized by the TrmI enzyme in bacteria (Roovers et al. 2004; Varshney et al. 2004) but by a Trm6–Trm61 complex in eukaryotes (Anderson et al. 1998), the bacterial TrmI shares homology with the eukaryotic Trm61, indicating an orthologous relationship between the two enzymes.

High-resolution crystal structures of TrmD and Trm5 reveal that both enzymes have an extensive network of hydrogen-bonding (H-bonding) and hydrophobic interactions to recognize AdoMet. While different in spatial configuration, each network is elaborate and comprehensive in the stabilization of the N¹, N⁶, and N⁷ of the adenine ring, the 2'- and 3'-OH of the ribose, and the carboxyl and amino groups of methionine in the methyl donor. These stabilizing forces are well visualized in a binary structure of TrmD with AdoMet and are preserved in a product complex with S-adenosyl homocysteine (AdoHcy) (Ahn et al. 2003; Elkins et al. 2003). They are also clearly identified in a binary structure of Trm5 with sinefungin and in a ternary structure with AdoMet and tRNA (Goto-Ito et al. 2008, 2009). The extensive stabilizing networks established for AdoMet are consistent with the comparably high affinity of both enzymes for the methyl donor. Indeed, fluorescence binding analysis of a model pair consisting of *Escherichia coli* TrmD and archaeal *Methanococcus jannaschii* Trm5 reveals their K_d for AdoMet in the range of 1–3 μM (Christian et al. 2006, 2010b), which explains their similar catalytic efficiencies in steady state and in single turnover assays (Christian et al. 2004, 2010b).

AdoMet is the major biological methyl donor in diverse methyl transfer reactions, due to the attachment of an electrophilic methionine methyl group to a positively charged sulfur atom to provide a thermodynamically favorable

FIGURE 1. Two structures of AdoMet. (A) AdoMet when bound to TrmD (Ahn et al. 2003). (B) AdoMet when bound to Trm5 (Goto-Ito et al. 2009). Drawn based on the coordinates in the structures (PDBID: 1UAK and 2ZZN) by PyMol.

driving force for methyl transfer (Cantoni 1975). The two fragments of AdoMet, adenosine and methionine, are small and soluble and both are amenable to structural modifications through synthetic chemistry, thus emphasizing their potential for structure-guided drug development programs. Here we explore analogs of AdoMet in search of the determinants that are able to discriminate TrmD from Trm5, using kinetic analysis as a tool to monitor the synthesis of m¹G37. A clear advantage of kinetic analysis over fluorescence or other types of binding assays is the ability to examine the contribution of binding free energy and reactivity of all of the substrates, cofactors, and discrete states of the enzyme along the reaction coordinate. Also, by focusing on the m¹G37 reaction, kinetic analysis can identify AdoMet analogs that specifically inhibit the reaction, rather than those that inhibit other AdoMet-dependent reactions. For example, analogs that target TrmD can be separated by cross-reactivity analysis from those that target TrmH (Nureki et al. 2004) and TrmL (Benítez-Páez et al. 2010), which adopt the same trefoil-knot structure as in TrmD but synthesize Gm18 and Um34 on tRNA, respectively.

Using *E. coli* TrmD and *M. jannaschii* Trm5 as a pair of model enzymes, we find that neither naturally existing nor synthetic analogs of AdoMet are able to distinguish between the two enzymes; rather, we find that fragments of the methyl donor and their derivatives selectively inhibit TrmD more than Trm5. Crystal structural analysis of each enzyme in complex with the adenosine fragment reveals new insight into the molecular details of how the relatively inflexible structure of TrmD subjects the bacterial enzyme to targeting and inactivation, whereas the more fluid structure of Trm5 enables the eukaryotic-archaeal enzyme to escape targeting. Free energy analysis of binding provides evidence for an energetic penalty in the TrmD binding to AdoMet versus fragment that is not observed in the Trm5 binding. The disparities in binding energies between the two enzymes provide insight into their distinct AdoMet binding modes and suggest novel approaches toward the development of selective antibiotics targeting the bacterial enzyme. Together, this work demonstrates that the fragment-based drug discovery approach (Congreve et al. 2008; Hestekamp and Whittaker 2008), initially developed to identify small molecules that target individual proteins or RNAs, can be successfully exploited to differentiate the disparate substrate binding determinants of analogous enzymes.

RESULTS

Kinetic analysis of AdoMet analogs

To distinguish TrmD from Trm5, we sought to identify structural analogs of AdoMet that would more effectively compete for the binding site of the methyl donor in TrmD

than in Trm5, thus exhibiting selectivity against TrmD. In a competitive inhibition model, the maximum velocity ($V_{\max}$) of the reaction and the catalytic turnover k_{cat} ($= V_{\max}/[E]$, where $[E]$ = enzyme concentration) are unchanged, whereas the apparent affinity of the substrate to the binding site is decreased, leading to an increase in the apparent K_m of the substrate. We determined the inhibition constant K_i of an analog by measuring the apparent K_m (AdoMet) as a function of analog concentration and fitting the data to the competitive inhibition equation $\text{apparent } K_m = K_m \times (1 + \frac{[I]}{K_i})$, where K_m is the Michaelis constant of AdoMet and $[I]$ is the concentration of the analog inhibitor. Because K_m (AdoMet) differed substantially between TrmD and Trm5 (Fig. 2), we normalized K_i (analog) by K_m (AdoMet). Mathematically, K_i/K_m is equivalent to $IC_{50}/[S + K_m]$ (Hamilton-Miller 1966) and it is an approximation of $IC_{50}/[S]$ at saturating substrate concentration relative to K_m (AdoMet). Analog differentiation of TrmD from Trm5 was evaluated by the ratio of $[K_i/K_m (\text{Trm5})]/[K_i/K_m (\text{TrmD})]$, which was interpreted as the fold sensitivity of TrmD to the analog relative to Trm5 (Fig. 2).

E. coli TrmD was assayed at 37°C with an in vitro transcript of *E. coli* tRNA^{Leu}, while *M. jannaschii* Trm5 was assayed at 55°C with a transcript of *M. jannaschii* tRNA^{Cys}, using previously established conditions (Elkins et al. 2003; Christian et al. 2004). These transcripts were chosen for their optimal sequence contexts for TrmD and Trm5, respectively, and were each refolded by annealing and verified as tRNA substrates by demonstrating a capacity for methylation to ~70% in extended time courses (Christian et al. 2010b). Measurements of inhibition were exhaustively controlled: Enzymes were assayed at concentrations corrected by active-site titration and at levels that allowed linear synthesis of m¹G37 over time, while saturation of [³H]-AdoMet and tRNA was established at concentrations 10-fold higher than the previously determined K_d of each substrate (Christian et al. 2010b).

No differentiation by natural and synthetic AdoMet analogs

The naturally existing AdoMet analogs sinefungin and AdoHcy were evaluated as inhibitors of methyl transfer (Fig. 2). Sinefungin is an antifungal and antiparasitic antibiotic produced by *Streptomyces incarnates* that shares with AdoMet the adenosine fragment but differs by possessing an ornithine instead of a methionine fragment, whereas AdoHcy is the product of methyl transfer that lacks the methyl group of AdoMet. Kinetic analysis showed that, while both analogs were competitive inhibitors of AdoMet, they exerted similar effects on TrmD and Trm5 without discrimination. For example, analysis of sinefungin inhibition of m¹G37 synthesis as a function of AdoMet concentration revealed the same pattern for both enzymes: While increasing concentration of the inhibitor had no effect

AdoMet		$K_m(\mu\text{M})$				
		TrmD	Trm5			
		5.0 ± 0.8	0.42 ± 0.08			
Analog	Structure	$K_i(\mu\text{M})$		K_i/K_m		$K_i/K_m(\text{Trm5})$ $K_i/K_m(\text{TrmD})$
		TrmD	Trm5	TrmD	Trm5	
Sinefungin		0.62 ± 0.06	$(33 \pm 2) \times 10^{-3}$	0.12 ± 0.07	0.08 ± 0.02	0.7
AdoHcy		4.2 ± 0.4	0.49 ± 0.03	0.8 ± 0.5	1.2 ± 0.4	1.5
AdoPropen		25.9 ± 3.3	1.43 ± 0.07	5 ± 4	3.4 ± 0.9	0.6
AdoButyn		7.9 ± 0.7	3.1 ± 0.1	1.6 ± 0.8	7 ± 2	4.5
Adenosine		34.7 ± 0.9	64.2 ± 0.4	7 ± 1	153 ± 5	22
AETA		10.0 ± 0.6	14 ± 2	2.0 ± 0.7	34 ± 22	17
MeS-Adenosine		30 ± 1	1.2 ± 0.1	5.9 ± 1.3	2.8 ± 1.2	0.5
6-ChloroPurine		405 ± 35	461 ± 13	81 ± 43	$(1.1 \pm 0.2) \times 10^3$	14
Inosine		488 ± 14	$(1.34 \pm 0.02) \times 10^3$	97 ± 17	$(3.2 \pm 0.2) \times 10^3$	33
Methionine		$(18.2 \pm 0.7) \times 10^3$	$(6.7 \pm 0.6) \times 10^3$	$(3.6 \pm 0.9) \times 10^3$	$(16 \pm 7) \times 10^3$	4
MeS-Cysteine		$(33 \pm 3) \times 10^3$	$(44 \pm 5) \times 10^3$	$(6.5 \pm 1.2) \times 10^3$	$(104 \pm 67) \times 10^3$	16

FIGURE 2. Kinetic parameters for *E. coli* TrmD and *M. jannaschii* Trm5. Michaelis constants K_m (AdoMet) and inhibition constants (K_i) for all analogs were measured in steady state and are reported as mean values of at least two independent determinations ($\pm$ standard deviation values). K_i/K_m is calculated from respective values for each enzyme and the ratio $[K_i/K_m (\text{Trm5})]/[K_i/K_m (\text{TrmD})]$ is reported. Also see Supplemental Figure S1.

on the $V_{\max}$ or k_{cat} of the reaction, it progressively elevated the apparent K_m values of AdoMet (Fig. 3A,B), indicating competition of sinefungin for the AdoMet-binding site on both enzymes. Fitting the apparent K_m (AdoMet) as a function of sinefungin concentration to a linear equation derived from the competitive inhibition model returned the K_m (AdoMet) for TrmD ($5.0 \pm 0.8 \mu\text{M}$) and for Trm5 ($0.42 \pm 0.08 \mu\text{M}$) in the absence of the analog (the y -intercepts in Fig. 3C,D), both of which are closely similar to the values reported previously ($8 \mu\text{M}$ for *E. coli* TrmD and $1.0 \pm 0.1 \mu\text{M}$ for *M. jannaschii* Trm5) (Elkins et al. 2003; Christian et al. 2006). Extrapolation of the linear data to the x -intercepts returned the K_i (sinefungin) for TrmD ($0.62 \pm 0.06 \mu\text{M}$) and for Trm5 ($33 \pm 2 \text{ nM}$) (Fig. 3C,D). The K_i/K_m value calculated for TrmD (0.12 ± 0.07) and for Trm5 (0.08 ± 0.02) was closely similar, with the value for Trm5 slightly lower than the value for TrmD by 0.7-fold. Similar inhibition of the two enzymes was also observed for AdoHcy; analysis of K_i (AdoHcy) and K_m (AdoMet)

showed that the K_i/K_m value of TrmD (0.8 ± 0.5) is smaller than that of Trm5 (1.17 ± 0.37) by 1.5-fold.

Synthetic analogs with an allyl (AdoPropen) or propargyl (AdoButyn) group in place of the reactive methyl group of AdoMet were made by regio-specific chemical S-alkylation of AdoHcy (Dalhoff et al. 2006b). Both analogs have been shown as substrates for several DNA methyltransferases (Dalhoff et al. 2006a), including the N⁶A methyltransferase M.TaqI, the C⁵C methyltransferase M.HhaI, and the N⁴C methyltransferase M.BcnIB, for transfer of the side chains to diverse DNA substrates. While these analogs were not accommodated by *E. coli* TrmD or *M. jannaschii* Trm5 for transfer of their side chains to tRNA (data not shown), they were competitive inhibitors of AdoMet, permitting the determination of whether steric bulk in the vicinity of the sulfonium ion might engender inhibition specificity. Both analogs inhibited each enzyme more poorly than sinefungin or AdoHcy with an increased K_i , indicating that neither active site is particularly tolerant of steric bulk in the vicinity of the sulfonium ion. However, as with sinefungin and AdoHcy, the K_i/K_m values for TrmD and Trm5 were closely similar, although a noticeable difference was observed with AdoButyn with a Trm5/TrmD ratio of 4.5-fold.

Presumably, the rigid propargyl substituent in AdoButyn is more effective for inhibiting TrmD than Trm5.

Differentiation by fragments of AdoMet

To elucidate the determinants in AdoMet that interact with TrmD and Trm5, we tested adenosine and methionine fragments and their derivatives for competitive inhibition of methyl transfer. As expected, all of these fragments showed substantially higher K_i values for TrmD and Trm5 relative to natural or synthetic analogs of the parental methyl donor (Fig. 2), consistent with their smaller and less complex molecular structures. Importantly, all of the fragments tested, including adenosine, methionine, and most of the nucleoside and amino acid derivatives, showed selectivity against TrmD by exhibiting substantially lower K_i/K_m values to TrmD relative to Trm5. For example, adenosine inhibited TrmD with a K_i/K_m of 7 ± 1 and inhibited Trm5 with a markedly larger K_i/K_m of 153 ± 5 , a

FIGURE 3. Sinefungin inhibition of m¹G37-tRNA synthesis. (A) Analysis of initial rate of methyl transfer by TrmD (36 nM) as a function of AdoMet concentration (0–25 μM) in the presence of 20 μM *E. coli* tRNA^{Leu} and increasing concentration of sinefungin (0–4 μM). At each concentration of sinefungin, the value k_{cat} (in sec^{-1}) was calculated from $V_{\text{max}}/[E]$, where V_{max} was derived from fitting the data of V_0 as a function of AdoMet concentration to the Michaelis-Menten equation, while the value of apparent K_m (in μM) was derived from the same fit. Values of k_{cat} and apparent K_m at each sinefungin concentration are listed. The k_{cat} values remained constant with increasing concentration of sinefungin, while the apparent K_m values increased; this indicates that sinefungin was a competitive inhibitor of AdoMet. (B) Analysis of initial rate of methyl transfer by Trm5 (25 nM) as a function of AdoMet concentration (0–5 μM) in the presence of 6 μM *M. jannaschii* tRNA^{Cys} and increasing concentration of sinefungin (0–60 nM). (C) The concentration-dependent apparent K_m (AdoMet) for TrmD was fit to a linear equation to derive K_m (AdoMet) from the y -intercept and K_i (sinefungin) from the x -intercept. (D) The concentration-dependent apparent K_m (AdoMet) for Trm5 was fit to a linear equation to derive K_m (AdoMet) from the y -intercept and K_i (sinefungin) from the x -intercept.

difference of 22-fold. Substitution of the 5'-OH of adenosine with 5'-[2-aminoethyl thio] as in 5'-[2-aminoethylthio]-5'-deoxy-adenosine (AETA) (Goeddecke et al. 2001) selectively inhibited TrmD by 17-fold. Substitution of 6-NH₂ of adenosine with 6-chloro as in 6-chloropurine selectively inhibited TrmD by 14-fold. Substitution of 6-NH₂ with 6-carbonyl as in inosine selectivity inhibited TrmD by 33-fold. In these nucleoside derivatives, the selectivity against TrmD is due to preferential increases in the K_i to Trm5 relative to TrmD. For example, the K_i (inosine) for Trm5 is higher than the K_m (AdoMet) by 3200-fold, as compared to the 98-fold difference between the K_i (inosine) and K_m (AdoMet) for TrmD. Such preferential increases in the K_i for Trm5 were also observed for the amino acid derivatives, methionine and methylthio-cysteine (MeS-cysteine). The only exception among the fragment analogs without selectivity against TrmD was MeS-adenosine, due to a smaller increase in K_i for Trm5 relative to TrmD, presumably because the methylthio-substitution in the adenosine fragment confers an important determinant of interaction for Trm5.

The kinetic analysis is relevant for inhibition in vivo. The intracellular concentration of AdoMet in bacteria ($\sim 400 \mu\text{M}$)

(Javor 1993) is saturating relative to K_m (AdoMet) of *E. coli* TrmD ($5.0 \pm 0.8 \mu\text{M}$, Fig. 2). Similarly, the intracellular concentration of AdoMet in archaea and eukaryotes is likely saturating relative to K_m (AdoMet) of *M. jannaschii* Trm5 ($0.42 \pm 0.08 \mu\text{M}$, Fig. 2), based on the known intracellular concentration of AdoMet in humans ($\sim 60 \mu\text{M}$; de Ferra and Baglioni 1983). In both cases, the parameter K_i/K_m can be simplified as $IC_{50}/[S]$ to allow calculation of the IC_{50} value from $(K_i/K_m) \times [S]$ (the intracellular concentration of AdoMet). For example, in the case of adenosine, the IC_{50} (adenosine) calculated for TrmD ($7 \times 400 \mu\text{M}$) is smaller than IC_{50} (adenosine) calculated for Trm5 ($153 \times 60 \mu\text{M}$) by more than threefold in the respective cellular conditions, suggesting that the fragment has the capacity to selectively inhibit TrmD in vivo. This analysis reveals that, due to the ~ 6 -fold difference in the intracellular AdoMet concentration in bacteria and in humans, analogs that are selective against TrmD must exhibit at least a sixfold difference in K_i/K_m of TrmD relative to Trm5 to offset the difference in the intracellular concentrations of AdoMet in vivo. Given that a 10-fold difference in binding affinity is desirable for a selective inhibitor, this requires a 60-fold difference in K_i/K_m

when considering the cellular concentrations of AdoMet in humans and bacteria.

Kinetic K_d of adenosine to TrmD and Trm5

To independently evaluate the selective inhibition by fragments of AdoMet, we focused on the adenosine fragment and determined its affinity to *E. coli* TrmD and *M. jannaschii* Trm5 by measuring the kinetic K_d as a different probe than the steady-state K_i/K_m . The measurements of kinetic K_d were performed by rapid mixing of an enzyme-inhibitor complex with saturating AdoMet and tRNA to allow the formation of enzyme-substrate and enzyme-inhibitor complexes in rapid equilibria, so that the extent of methyl transfer in one turnover would provide a direct readout of the amount of the uninhibited enzyme capable of synthesis of m¹G37 (Fig. 4A,B). The time periods permitted for one turnover of *E. coli* TrmD and *M. jannaschii* Trm5 were respectively 11 and 8 sec, based on the single-turnover rate constant of each enzyme (Christian et al. 2010b). As the inhibitor concentration increased, the amount of the active enzyme available for methyl transfer progressively decreased. The data of m¹G37 synthesis as a function of adenosine concentration were well fit to a quadratic equation, revealing the kinetic K_d for TrmD ($372 \pm 37 \mu\text{M}$) and for Trm5 ($910 \pm 58 \mu\text{M}$) (Fig. 4A,B). As a control, the kinetic K_d (AdoMet) for *E. coli* TrmD was determined ($5.3 \pm 0.4 \mu\text{M}$) by a similar assay but without the inhibitor (Supplemental Fig. S2), while the kinetic K_d (AdoMet) for *M. jannaschii* Trm5 was previously determined ($0.44 \pm 0.09 \mu\text{M}$) (Christian et al. 2010b). For each enzyme, normalization of K_d (adenosine) by K_d (AdoMet) was used to evaluate the competence of the inhibitor to compete for the AdoMet-binding site during the rapid binding equilibria of one methyl transfer. Analysis of this normalized ratio revealed a preferential binding of adenosine to TrmD than to Trm5 by 29-fold (Fig. 4C), similar to the 22-fold preference in the steady-state analysis of $[K_i(\text{adenosine})]/[K_m(\text{AdoMet})]$ (Fig. 2).

X-ray structural analysis of adenosine-bound TrmD and Trm5 complexes

To gain structural insight into the selective binding affinity of adenosine to TrmD rather than to Trm5, crystal structures of the two enzymes were solved in complex with the

FIGURE 4. Determination of kinetic K_d (adenosine). (A) Determination of kinetic K_d (adenosine) for *E. coli* TrmD. Kinetic analysis was performed in single turnover conditions by rapid mixing of TrmD ($0.4 \mu\text{M}$) and adenosine (0.08 – 8 mM) in one syringe with tRNA ($10 \mu\text{M}$) and AdoMet ($60 \mu\text{M}$) in the second syringe (shown in the scheme). The reaction was quenched after one turnover (11 sec) by acid and the amount of product synthesis was determined. Fitting the data of concentration-dependent product synthesis to a quadratic binding equation revealed the K_d (adenosine) for TrmD. (B) Determination of kinetic K_d (adenosine) for *M. jannaschii* Trm5. Kinetic analysis was performed in single turnover conditions by rapid mixing of Trm5 ($0.4 \mu\text{M}$) and adenosine (0.2 – 17 mM) in one syringe with tRNA ($16 \mu\text{M}$) and AdoMet ($60 \mu\text{M}$) in the second syringe (shown in the scheme). The reaction was quenched after one turnover (8 sec) and the amount of product synthesis was determined. Fitting the data of the concentration-dependent product synthesis to a quadratic binding equation revealed the K_d (adenosine) for Trm5. Error bars correspond to standard deviation from three independent measurements. The inset in each graph shows the order of mixing of reagents and the reaction time. (C) A summary of K_d (AdoMet) and K_d (adenosine) for *E. coli* TrmD and *M. jannaschii* Trm5. The K_d (AdoMet) for Trm5 was previously determined (Christian et al. 2010b). The ratio of K_d (adenosine)/ K_d (AdoMet) was calculated for both enzymes to reflect the competitive binding of adenosine for the AdoMet binding site. Values in parentheses correspond to the normalized ratios of Trm5 relative to TrmD. Also see Supplemental Figure S2.

fragment (detailed information summarized in Supplemental Table S1). The TrmD structure was solved at $2.25 \text{ \AA}$ resolution with the *Haemophilus influenzae* enzyme, which shares 83% sequence identity and 91% similarity with *E. coli* TrmD. As seen in the previous structures of TrmD (Ahn et al. 2003; Elkins et al. 2003), the two active sites in the dimeric *H. influenzae* enzyme were symmetrically assembled in the dimer interface, with each consisting of residues from the AdoMet-binding motifs in the N-terminal domain of one monomer and residues from the tRNA-binding motifs in the C-terminal domain of the second monomer. A comparison with the previous AdoMet-bound structure of TrmD (Ahn et al. 2003) revealed that the two adenosine molecules bound in the new structure were in virtually overlapping positions as the adenosine moiety of the previous structure (Fig. 5A). The overlapping occupancy of adenosine in the adenosine-pocket of the AdoMet-binding site was also preserved in the AdoHcy-bound structures of TrmD (Ahn et al. 2003; Elkins et al. 2003). The network of H-bonds that stabilizes AdoMet was maintained in its entirety to stabilize adenosine in the new structure, including those linking adenosine N¹, N⁶,

FIGURE 5. Structural analysis of adenosine-bound TrmD and Trm5 complexes. (A) Superposition of the C α atoms of adenosine in the adenosine-bound (magenta) complex onto the structure of the AdoMet-bound complex of *H. influenzae* TrmD (orange, PDB ID: 1UAK). Adenosine and AdoMet are colored in blue and cyan, respectively. Enzyme residues that form the interacting network with AdoMet are depicted, showing H-bonds (dashed lines) with the methyl donor. These residues are in overlapping positions in the two structures. (B) Superposition of the C α atoms of adenosine in the adenosine-bound (magenta) complex onto the sinefungin-bound complex of *M. jannaschii* Trm5 (orange, PDB ID: 2YX1). Adenosine and sinefungin are colored in blue and cyan, respectively. Enzyme residues that form the interacting network with sinefungin are depicted, showing H-bonds (dashed lines) with the AdoMet analog. The superposition reveals a shift of adenosine toward the methionine pocket by 0.6 Å with the sugar flexed away from the active site by 1.35 Å. Residues with the most significant shifts are those that contact G37-tRNA in the D2 domain, including Y177 and R136. (C) A ribbon diagram showing the superposition of the overall adenosine-bound and AdoMet-bound structures of *H. influenzae* TrmD. (D) A ribbon diagram showing the superposition of the overall adenosine-bound and sinefungin-bound structures of *M. jannaschii* Trm5.

and N⁷ with, respectively, the amide group of I133, the carbonyl groups of G134 and Y136, and the amide of L138 in the main chain. As in the previous structures, the ribose oxygen of adenosine in the new structure was stabilized by the main-chain amide of G140 and G141, while the 2'- and 3'-OH groups were stabilized respectively by the main-chain of L87 and side-chain of Y86. However, minor changes were observed in the new structure relative to the previous structures, which were primarily associated with the conformation of the residues involved in the stabilization of the methionine moiety of AdoMet, including S170, D177, and H180, all of which made adjustments to occupy the space left open by the absence of the amino acid. Overall, a pairwise superposition of the C α -atoms of the AdoMet- and adenosine-bound complexes revealed identical structures (Fig. 5C) with a root-mean-square deviation (RMSD) of 0.18 Å for 217 of the 236 atoms aligned. The conformation of the adenosine-bound N-terminal domain was essentially unchanged in the two structures (RMSD of 0.13 Å for the 147 atoms aligned), while the movement of the C-terminal domain was slightly more pronounced (RMSD

of 0.28 Å for the 66 atoms aligned). The latter movement may be due to the disordering of residues 161–171 in both structures. The superposition also showed that adenosine binding to TrmD created few changes to the extensive dimer interface that forms the active site. Thus, the structure of TrmD appears rigid enough that it can use the same active site and overall conformation to accommodate adenosine in the adenosine pocket of the AdoMet- or AdoHcy-binding site.

The adenosine-bound Trm5 structure was solved at a 3.05 Å resolution with the *M. jannaschii* enzyme. Trm5 consists of three domains: an N-terminal D1 domain that contacts the tertiary core (elbow) region of the tRNA L shape in a ternary complex of the enzyme with tRNA and AdoMet (Goto-Ito et al. 2009); a D2 domain that recognizes G37 and the anticodon loop; and a D3 domain that stabilizes AdoMet binding and catalyzes methyl transfer. Due to the lack of a Trm5 structure bound to AdoMet, the new structure was compared to the sinefungin-bound structure of the enzyme (Goto-Ito et al. 2008). This comparison revealed that, in contrast to TrmD, significant misalignment of adenosine with the sinefungin site of Trm5 was observed, primarily due to a 0.6 Å translation and 0.5 Å downward shift of adenosine toward the methionine pocket

(Fig. 5B). The movement of adenosine disturbed the H-bond and hydrophobic network established for the adenosine fragment in the sinefungin-bound structure by weakening or abolishing the enzyme interactions with the adenine ring through D251, V252, and I224 and the interactions with the ribose through F203, D223, and L266. More substantial disruptions were observed in the conformation of the residues in the D2–D3 domain responsible for recognition of G37-tRNA, including N265, P267, and Y177 that played a role in stabilizing the purine base, R136, R181, and R186 that stabilized the conformation of the anticodon loop, and E185 that was proposed to deprotonate N¹ of G37 before methyl transfer (Christian et al. 2010a). The other catalytic residue R145 that presumably stabilized O⁶ of G37 during deprotonation (Christian et al. 2010a) was in a disordered region. All of these residues are important for the reaction mechanism of Trm5, and their displacements from the positions in the AdoMet- or sinefungin-bound complex due to adenosine binding indicated a coordinated dismantling of the active site. Displacement in the D1 domain was also observed (Fig. 5D), most notably for residues that

recognized the backbone structure of the tRNA tertiary core (e.g., K10, R16, and K27). However, because D1 is flexibly linked to the D2–D3 domain, its displacement in the new structure from the sinefungin-bound structure might be due to crystal packing effects. An overall pairwise superposition was thus performed only with the C α atoms of the D2–D3 domains in the present structure relative to the sinefungin-bound structure, which revealed a 0.46 Å RMSD (based on alignment of 236 out of 253 residues), significantly higher than the 0.18 Å value obtained with TrmD. The sinefungin position in the sinefungin-bound structure differed slightly from the AdoMet position in the ternary complex of Trm5–AdoMet–tRNA. An overall pairwise superposition performed with the C α atoms of the D2–D3 domains in the present structure relative to the ternary complex structure revealed an even higher RMSD value of 0.65 Å.

DISCUSSION

While analogous enzymes exist in unrelated structures, they recognize the same substrates using distinct but well-developed interacting networks to catalyze the same reaction. Identifying substrate analogs that can preferentially bind to one enzyme but not the other has proven to be a significant challenge. The challenge is clearly illustrated for the TrmD and Trm5 pair, which as we show here cannot be distinguished by several previously well-characterized natural and synthetic AdoMet analogs. Instead, fragments of AdoMet, particularly adenosine and its purine derivatives, show discerning and specific inhibition of TrmD. To our best knowledge, this work is the first and successful application of the fragment-based structure-activity analysis to differentiate the substrate binding determinants of analogous enzymes.

Free energy analysis

To gain insight into the AdoMet binding determinants of TrmD and Trm5, the free energy of binding the methyl donor by these enzymes relative to the analogs studied here was calculated and summarized (Table 1), based on the experimentally determined K_m and K_i values (Fig. 2). Although TrmD and Trm5 differ in the rate-limiting step in the control of their catalytic turnover, they display the same catalytic turnover rate (Christian et al. 2010b), suggesting that the use of K_m and K_i values for free energy calculation was reasonable for both enzymes. In addition, the K_m and K_d values of AdoMet are closely similar for both enzymes, and the K_i/K_m ratios of adenosine are closely similar to the K_d ratios (Figs. 2, 4). Free energy calculations indicate that TrmD interacts with AdoMet with lower affinity than does Trm5 (−7.5 vs. −9.6 kcal/mol, respectively), and that the difference in the apparent binding free energy between the two enzymes remains more or less the same in all of the natural and synthetic analogs as well as in the MeS-adenosine analog, which is the only fragment that

TABLE 1. Calculation of binding free energy

Chemical	ΔG^0 (kcal/mol)	
	TrmD	Trm5
AdoMet	−7.5	−9.6
Sinefungin	−8.8	−11.2
AdoHcy	−7.6	−9.5
AdoPropen	−6.5	−8.8
AdoButyn	−7.2	−8.3
Adenosine	−6.3	−6.3
AETA	−7.1	−7.3
MeS-Adenosine	−6.4	−8.9
6-ChloroPurine	−4.8	−5.0
Inosine	−4.7	−4.3
Methionine	−2.5	−3.3
MeS-Cysteine	−2.1	−2.0

Free energy of binding ΔG^0 (kcal/mol) is calculated from $\Delta G^0 = RT \ln (K_m \text{ or } K_i)$, where K_m and K_i are interpreted as dissociation constants, R is the gas constant $1986 \text{ cal K}^{-1} \text{ mol}^{-1}$, and T is the absolute temperature. Values of K_m and K_i are taken from Figure 2, based on the kinetic determination of *E. coli* TrmD at 37°C (310 K) and *M. jannaschii* Trm5 at 55°C (328 K). This analysis shows that adenosine binding accounts for 85% of the binding energy of AdoMet to TrmD ($6.3/7.5 = 84\%$), but only 65% of the binding energy of AdoMet to Trm5 ($6.3/9.6 = 66\%$).

lacks selectivity. Interestingly, free energy calculations indicate that TrmD interacts with similar affinity as does Trm5 with fragments or fragment derivatives that show selectivity. However, additivity analysis of TrmD reveals that the sum of the free energies of binding the adenosine and methionine fragments ($[-6.3] + [-2.5] = -8.8 \text{ kcal/mol}$) is smaller than the free energy of binding AdoMet by 1.3 kcal/mol, suggesting the existence of an energetic penalty in the binding of AdoMet relative to fragments. In contrast, additivity analysis of Trm5 reveals that the sum of the free energies of binding adenosine and methionine ($[-6.3] + [-3.3] = -9.6 \text{ kcal/mol}$) is the same as the free energy of binding AdoMet, suggesting that the fragment free energies of interaction for Trm5 are additive. The disparity in free energy additivity of TrmD and Trm5 is likely attributable to the unusual bent conformation of AdoMet when bound to TrmD in contrast to the extended conformation when bound to Trm5 (Fig. 1). The bent conformation of AdoMet is also observed in the structures of sinefungin and AdoHcy when bound to TrmD (Ahn et al. 2003; Elkins et al. 2003), while the extended conformation is preserved in the structure of sinefungin when bound to Trm5 (Goto-Ito et al. 2008). The consistent bent conformation in AdoMet and in natural analogs when bound to TrmD suggests that the energetic penalty in the binding of these compounds by TrmD is involved in the entropic costs associated with stabilizing the bent conformation, preventing the enzyme from full utilization of the binding energy of its component fragments. In contrast, the additivity of binding by Trm5 suggests that the enzyme can capture the full

spectrum of the binding free energies from the component fragments in a way that is facilitated by the extended conformation of the bound AdoMet.

The different binding determinants of AdoMet versus fragments to TrmD and to Trm5 provide a basis to understand the fragment selectivity. Even if a fragment binds with the same binding energy to both enzymes, the overall energetic contribution of the fragment relative to AdoMet binding is different for the two enzymes. For example, while adenosine binds with the same energy to both TrmD and Trm5 (-6.3 kcal/mol), it accounts for 84% of the total binding energy of AdoMet to TrmD (-7.5 kcal/mol), but only 66% of the total binding energy of AdoMet to Trm5 (-9.6 kcal/mol). The greater contribution of adenosine binding to the TrmD–AdoMet binding interaction than to the Trm5–AdoMet interaction suggests a more stable binding of the fragment to TrmD than to Trm5. Analysis of the thermodynamic K_d as determined by kinetic measurements under rapid binding equilibrium (Fig. 4) confirms that the adenosine affinity to TrmD is 2.4-fold higher than the affinity to Trm5 ($K_d = 372 \pm 37$ and 910 ± 58 μ M, respectively). This difference, after normalization by the inherent 12-fold difference of AdoMet affinity to the two enzymes ($K_d = 5.2 \pm 0.4$ and 0.44 ± 0.09 μ M, respectively), is enlarged to 29-fold, meaning that the fragment is 29-fold more stable in the AdoMet-binding site on TrmD than on Trm5 each time it outcompetes against AdoMet for the binding site. Due to the rapid binding equilibria under which the binding constants were measured, the K_d ($= k_{\text{off}}/k_{\text{on}}$) values can be used to calculate k_{off} s assuming that k_{on} is diffusion controlled. This calculation suggests that the adenosine fragment on TrmD is more stable than on Trm5 with a 29-fold longer duration time. Direct measurements of fragment binding kinetics to TrmD and Trm5 will more precisely assess the difference in binding stability. Nonetheless, part of the stability of the fragment on TrmD may be provided by the inherent structural rigidity of the trefoil-knot fold, as suggested by a biophysical analysis of a model knotted protein (King et al. 2010). Conversely, part of the instability of the fragment on Trm5 may derive from the inherent structural fluidity of the enzyme, which is indicated by a recent study of Trm5 during catalysis (Christian et al. 2010a) and is supported by the higher B factors associated with Trm5 relative to TrmD in structural analysis (Supplemental Table S1).

Combining free energy analysis with structural analysis, we propose a model to explain the selectivity of adenosine targeting to TrmD. In this model (Fig. 6), the adenosine fragment binds to the adenosine pocket of TrmD in an overlapping position, trapping the enzyme in an inactive state that is likely stabilized by the rigidity of the enzyme structure. In contrast, the fragment binds to the adenosine pocket of Trm5 in a misfit position, which is corroborated with global structural perturbation in the active site and

FIGURE 6. A model of adenosine targeting TrmD and Trm5. In the active state, TrmD and Trm5 each possess an AdoMet-binding site, consisting of an adenosine and a methionine pocket. The AdoMet site is located in a dimer interface of TrmD comprised of the N-terminal domain of monomer 1 (N₁) and the C-terminal domain of monomer 2 (C₂), whereas it is located between the D2 and D3 domains of the monomeric Trm5. In the adenosine-bound inactive state, TrmD maintains the same structure and binds adenosine in the already-established adenosine pocket, whereas Trm5 shifts its adenosine pocket and binds adenosine in a misfit conformation, leading to structural alterations of the enzyme. In TrmD, the stable binding of adenosine causes delayed release of the ligand, whereas in Trm5 the unstable binding of adenosine can lead to rapid release of the fragment, allowing refolding of the enzyme to its native structure. The chemical structure of AdoMet is depicted below with a blue box showing the adenosine fragment and a red box showing the methionine fragment. The AdoMet-binding site is shown in white, whereas the adenosine ligand is shown in dark blue.

throughout the D2–D3 domains. The inherent structural flexibility of Trm5 may promote rapid release of the fragment, facilitating the return to the native structure and explaining the escape from targeting.

Insight into selective targeting TrmD

Fragments of enzyme substrates, due to their smaller chemical space and easier accessibility to exploration, have been suggested as an attractive starting point to build fragment libraries for drug screening that may have the potential to supersede traditional high throughput screening of larger molecules. Fragment hits usually achieve only low mM to high μ M affinities with the possibility for further elaboration into more potent inhibitors. The elaboration can take place in one of four directions: evolution of initial fragments by building larger and more complex molecules that form additional interactions with the target protein; chemically linking two or more fragments that bind to separate but close enough sites on the target protein to generate a larger and higher-affinity molecule; using the target protein as a template to assemble fragments with complementary functional groups so as to react together to generate a larger molecule with a higher potency; and optimization of initial fragments based on properties other than just the binding potency. Recent work has seen successful examples in all of these approaches each with the potential to develop from initial fragments into high-affinity leads (Rees et al. 2004).

In the pursuit of selective targeting of TrmD, we show here that the enzyme has the ability to fully accommodate adenosine to the adenosine pocket in a conformation indistinguishable from that in AdoMet or AdoHcy, suggesting that the energetic penalty upon binding AdoMet is due to the inability to completely capture the free energy of binding the methionine portion of the molecule. Indeed, the methionine portion of AdoMet when bound to TrmD adopts an unusual conformation that places the sulfonium ion in close proximity to the bridging 4-oxo group of the ribose, resulting in the bent conformation. This raises the possibility that a fragment-linking approach that links the adenosine fragment with derivatives of the methionine fragment in structural or topological constraints that stabilize the bent conformation of AdoMet may offer an attractive route to develop selective targeting. This possibility provides a strong impetus for further studies aimed at solving additional TrmD structures in complex with methionine. The new structural information should provide a basis for a fragment-linking approach to generate analogs of AdoMet that exhibit enhanced affinity to TrmD, or reduced affinity to Trm5, or a combination of the two.

MATERIALS AND METHODS

Synthesis of m¹G37-tRNA was monitored by the incorporation of the [³H-methyl] group of AdoMet into tRNA and acid precipitation on filter pads as described (Christian et al. 2010a). Trm5 was assayed with the transcript of *M. jannaschii* tRNA^{Cys}, while TrmD was assayed with transcript of *E. coli* tRNA^{Leu}. *K_m* (AdoMet) was determined from steady-state analysis of initial rate (*V*₀) of methyl transfer as a function of AdoMet concentration and fitting the data to the Michaelis-Menten equation. *K_i* (analog) was determined from analysis of the apparent *K_m* (AdoMet) as a function of the analog concentration and fitting the data to the competitive inhibition equation. Determination of *K_d* (adenosine) was performed in single turnover conditions upon rapid mixing of a solution of saturating tRNA and AdoMet with a solution of enzyme and adenosine on a Kintek RQF-3 apparatus. The reaction was quenched exactly after one single turnover cycle and the amount of m¹G37-tRNA was plotted against adenosine concentration. The data were fit to a quadratic equation to derive *K_d* (adenosine). Determination of *K_d* (AdoMet) for TrmD was performed similarly in single turnover conditions but in the absence of adenosine. Details of structural analysis of the adenosine-bound TrmD and Trm5 structures are described in Supplemental Table S1.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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