Functional Roles in S-Adenosyl-L-Methionine Binding and Catalysis for Active Site Residues of the Thioestrepton Resistance Methyltransferase

Cullen L. Myers¹*, Emily G. Kuiper², Pei C. Grant¹,†, Jennifer Hernandez², Graeme L. Conn², and John F. Honek¹,‡

¹ Department of Chemistry, University of Waterloo, Waterloo, Ontario, N2L 3G1, Canada
² Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, U.S.A

Abstract

Resistance to the antibiotic thioestrepton, in producing Streptomyces, is conferred by the S-adenosyl-L-methionine (SAM)-dependent SPOUT methyltransferase Tsr. For this and related enzymes, the roles of active site amino acids have been inadequately described. Herein, we have probed SAM interactions in the Tsr active site by investigating the catalytic activity and the thermodynamics of SAM binding by site-directed Tsr mutants. Two arginine residues were demonstrated to be critical for binding, one of which appears to participate in the catalytic reaction. Additionally, evidence consistent with the involvement of an asparagine in the structural organization of the SAM binding site is presented.

Keywords
thioestrepton; SPOUT methyltransferase; S-adenosyl-L-methionine; isothermal titration calorimetry; enzyme kinetics

1. Introduction

The methylation of rRNA is vital to the structure and function of ribosomes across all domains of life [1]. Many of these modifications are carried out by methyltransferases that
recruit S-adenosyl-L-methionine (SAM) for highly specific methylations of RNA base targets. Such enzymes are in various instances responsible for rRNA methylations that bring about bacterial resistance to certain ribosome-targeting antibiotics, noteworthy examples of which can be found in clinically relevant classes such as aminoglycosides and macrolides [2]. This resistance mechanism also accounts for the majority of natural bacterial immunity to thiostrepton, the prototype of the ribosome-targeting thiopeptide antibiotics that have attracted renewed attention as a potential source for antimicrobial lead compounds [3-6].

Thiostrepton exhibits potent bactericidal effects against Gram-positive bacteria by binding the bacterial ribosome at the GTPase center on 50S subunit through interactions with ribosomal protein L11 and 23S rRNA, imposing conformational restrictions on L11 that perturb elongation factor activities and ultimately cause the arrest of protein synthesis at the translocation step of the elongation cycle [7-9]. Organisms that produce thiostrepton (S. cyaneus, S. laurentii) [10] express a SAM-dependent methyltransferase, Tsr, that catalyzes a 2'-O-ribose methylation of an adenine nucleotide (A1067; E. coli numbering) at the thiostrepton binding site [11], preventing its association and rendering the organisms resistant to its effects. Analogously, protection from the related thiopeptide nosiheptide, in the producing organism S. actuosus, is afforded by a methyltransferase (Nhr) that shares 74% sequence similarity with Tsr [12].

Crystal structures for Tsr and Nhr assign these enzymes to the SPOUT family of methyltransferases [11,12] that have thus far been found to exclusively target RNA bases [13], with the exception of a single protein SPOUT methyltransferase [14]. Apart from antibiotic resistance [11,12,15-18], known biological functions for enzymes from this class include tRNA modification [19] and roles in ribosome biogenesis [20,21]. Putative SPOUT methyltransferase genes have also been identified in the biosynthetic gene clusters of other thiopeptide-producing bacteria, which may indicate that SPOUT-enzyme RNA methylation is perhaps a more general form of thiopeptide resistance [22,23]. Structural studies show that SPOUT methyltransferases are functional homodimers, typified by an α/β Rossmann-like fold with a deep trefoil knot at the C-terminal end that binds SAM, and an active site near the dimeric interface that is constructed from residues contributed by both subunits [11,12,20,24-27]. Molecular models of RNA substrates bound to Tsr [11] or to other SPOUT methyltransferases [12,24,27] suggest that methyl transfer is accomplished by a single catalytic site, although these homodimeric enzymes appear capable of binding two SAM molecules.

Methylation of rRNA by Tsr, and by SPOUT enzymes in general, proceeds according to the scheme depicted in Fig. 1A. Implicit from this mechanism is the activation of the methyl group acceptor that precedes and facilitates nucleophilic attack. Detailed descriptions regarding this step of the catalytic mechanism, however, are limited to few examples notwithstanding considerable structural knowledge on this enzyme class. For instance, an Arg residue in the active site of the tRNA methyltransferase TrmH is thought to carry out deprotonation on the targeted guanine base that allows for 2'-O-ribose methylation [28], while N-methylation of the guanine targeted by Trm5 was shown to follow deprotonation at N1 by an active site Glu [29]. In a number of additional cases, Arg or Glu residues have
again been suggested for this role [18,19,25,27], but these proposals have broadly lacked substantiation through biochemical investigations.

Tsr is among the SPOUT methyltransferases for which the roles of active site amino acids in the catalytic mechanism and in interactions with SAM are unresolved. Moreover, Tsr catalysis is not well understood within the context of two simultaneously occupied cosubstrate (SAM)-binding sites. In this work, we have examined the interactions of SAM in the Tsr active site by investigating the catalytic and SAM-binding properties of structure-guided active site mutants. These studies have lead to the assignment of functional roles in SAM binding to specific residues, and highlighted the likely steps involved in activation of the methyl group acceptor in the Tsr catalytic mechanism.

2. Material and methods

2.1. Protein expression & purification

The pET28a plasmid construct bearing the Tsr gene fused to a sequence encoding an N-terminal hexahistidine tag followed by a thrombin protease cleavage site was previously created [11]. Single amino acid mutations (N129A/D, R135A/K, R165A/K, E220A/Q, K221A, S246A and N248A/D) were introduced into the *S. cyaneus* Tsr gene by using this plasmid as the template in PCR overlap extension reactions with vector-specific T7 primers and Tsr-gene-specific primers (Supplementary material, Table S1). Mutations were confirmed by DNA sequencing (Mobix Lab, McMaster University, Hamilton, Ontario, Canada). *E. coli* BL21 (DE3) pLysS transformed with plasmid bearing the wt or mutated Tsr gene was cultured at 37°C in Luria Bertani media supplemented with kanamycin (30 μg/mL) and chloramphenicol (34 μg/mL), and protein expression was induced by addition of IPTG (1 mM) after bacterial growth reached an OD_{600} of 0.6 – 0.8. Expression was continued for 4 hours and the cells harvested by centrifugation, then resuspended in buffer comprised of 50 mM Tris (pH 8.0), 500 mM KCl, 20 mM imidazole and 10% glycerol (v/v). Cells were lysed using an EmulsiFlex-C5 homogenizer (Avestin, Ottawa, Ontario, Canada) and the cellular debris removed by centrifugation. For Ni^{2+}-affinity purification of his-tagged proteins, the clarified lysate was applied to a 1 mL HisTrap HP column (GE Healthcare, Mississauga, Ontario, Canada) pre-equilibrated with resuspension buffer. The column was eluted with resuspension buffer to remove non-binding proteins, after which his-tagged proteins were obtained by elution with a buffer comprising 50 mM Tris (pH 8.0), 500 mM KCl, 500 mM imidazole and 10% glycerol (v/v), and then dialyzed overnight at 4°C against a buffer containing 50 mM Tris pH 7.5, 150 mM KCl, 10% glycerol (v/v). The his-tag was removed by subsequent incubation with thrombin protease (GE Healthcare) for 16 hours at 4°C. Cleaved and uncleaved protein were separated with the same chromatographic conditions as for obtaining his-tagged protein. Protein purity was assessed by SDS-PAGE and the expected molecular mass for mutant Tsr proteins was confirmed by positive ion ESI-MS. If necessary, further purification was performed by anion exchange chromatography using a MonoQ column (GE Healthcare); Tsr eluted between 250 and 300 mM of KCl during a linear KCl gradient (50 – 500 mM over 100 minutes) in a buffer containing 50 mM Tris (pH 8) and 10% glycerol (v/v). Purified, his-tag-cleaved protein was dialyzed as described above, then stored at −80°C for future use.
2.2. Size exclusion chromatography

Analytical size exclusion chromatography was performed in a buffer comprised of 50 mM Tris (pH 8.0), 150 mM KCl and 10% glycerol (v/v), using a Superdex-75 size exclusion column (GE Healthcare). A mixture of protein standards containing blue dextran, bovine serum albumin, carbonic anhydrase and cytochrome c was used for the estimation of relative molecular weight.

2.3. Methylation assays

Methylation of 16S/23S rRNA isolated from *E. coli* MRE600 (Roche Life Sciences, Laval, Québec, Canada) by purified, recombinant wt Tsr or its mutants was assessed using an enzyme-coupled fluorescent assay (Cayman Chemical Company, Ann Arbor, Michigan, USA) following established methods [21,30-32]. The principle of the assay is as follows: the adenine moiety is enzymatically cleaved from SAH generated as a by-product of methylation [33,34], and then enzymatically converted to urate with the stoichiometric production of H$_2$O$_2$ that is quantified by conversion of added 10-acetyl-3,7,-dihydroxyphenoxazine (ADHP) into the fluorescent compound 7-hydroxy-3H-phenoxazin-3-one (resorufin). Assays were performed according to the manufacturer’s specifications, except that the reaction buffer was supplemented with MgCl$_2$ (5 mM) and NH$_4$Cl (25 mM) and the pH adjusted to 7.5 to satisfy the optimum requirements for Tsr activity. These minor alterations did not affect the performance of the commercial assay, which was robust, displaying linearity with respect to time and enzyme concentration (Supplementary material, Fig. S1). Fluorescence measurements were carried out in black, 96-well microplates (Corning Life Science, Corning, NY, USA) using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, California, USA) that was calibrated to an internal standard. Excitation and emission wavelengths were 530 nm and 584 nm, respectively, with an emission filter at 570 nm. Assays monitoring catalytic activity under non-limiting substrate conditions contained 0.35 μM of 16S/23S rRNA, 1 mM SAM and 0.1 μM of enzyme. For the determination of enzyme kinetic parameters, SAM concentrations were varied from 0.005 mM to 0.5 mM and initial rate data fit by non-linear regression to equation 1 (GraphPad Prism 6.0); $v$ is the initial rate at a given SAM concentration [S], $K_M$ and $k_{cat}$ are the Michaelis constant and enzyme turnover, respectively and [E]$_t$ is total enzyme concentration.

$$v = \frac{k_{cat} [E]_t [S]}{K_M + [S]}$$

2.4. Isothermal titration calorimetry

Assessments of SAM binding for wt and mutant Tsr proteins mutants were performed with an ITC200 calorimeter (GE Healthcare, Mississauga, ON, Canada). Enzyme used for these experiments was dialyzed extensively against a buffer comprised of 50 mM Tris (pH 7.5), 75 mM KCl and 10% glycerol (v/v), and the dialysate was used to prepare fresh working solutions of protein and SAM. Titrations were performed at 25 °C and consisted of a single initial injection of 0.5 μL, followed by 29 injections of 1.25 μL of SAM (1.98 - 2.50 mM).
into a sample cell containing wt or mutant Tsr (0.0822 - 0.098 mM). Heats from dilution and mixing were obtained from injections of SAM into ITC buffer lacking protein. Thermodynamic data were analyzed using Origin 7.0 (GE Healthcare), and fit by non-linear regression to models for single or sequential binding sites.

Experiments measuring SAH binding were performed essentially as those described for SAM binding, but using an Auto-iTC200 microcalorimeter (GE Healthcare) and with the following adjustments: after dialysis against ITC buffer, Tsr was concentrated to 0.05 mM and working stocks of SAH (1 mM) were prepared using the final dialysate. Titrations were performed at 25 °C and consisted of a single initial injection of 0.5 μL followed by 15 injections of 2.4 μL.

2.5. Circular dichroism spectroscopy

Spectra were measured with a Jasco J715 spectropolarimeter (Jasco Analytical Instruments, Easton, Maryland, USA) in the low UV region (190 – 250 nm), with a bandwidth of 1 nm and a scanning speed of 100 nm/min. Spectra from 15 consecutive scans, performed at least twice, were averaged. Samples were prepared at 2.7 μM, in a buffer comprised of 50 mM Tris (pH 7.5), 150 mM KCl and 10% glycerol (v/v).

3. Results

3.1. Characterization of SAM binding and turnover by Tsr

Cocrystal structures of SPOUT methyltransferases with SAM, SAH or the methyltransferase inhibitor sinefungin show a cosubstrate/inhibitor molecule bound at the CTD of each subunit in the enzyme dimer [11,12,24,25,27]. We investigated this crystallographically observed stoichiometry by ITC, determining a 2:1 binding stoichiometry for SAM or SAH with the Tsr dimer on analysis of thermodynamic binding data with a model for a single set of identical sites; i.e. one binding site per enzyme subunit (Fig. 1B). The affinity of the methylation by-product SAH was noted to be 4 times higher than that of SAM (Fig. 1B). In the Tsr-SAM crystal structure, SAM adopts different conformations in structurally non-equivalent binding sites (Fig. 1C), which could manifest as differential binding parameters for the two sites. Binding data were therefore also evaluated with a model for sequential independent sites, as has been used elsewhere for characterizations of SAM binding by other dimeric methyltransferases [21,26]. Interestingly, this fit generated different SAM affinities for the two binding sites (K_D1 and K_D2 of 56.8 μM and 209.6 μM), while the other SAM binding parameters for the two sites were found to be comparable (ΔH1, −4.07 ± 0.11 (kcal/mol); ΔH2: −3.89 ± 0.17 (kcal/mol); ΔS1, 5.54 (cal.mol⁻¹.deg⁻¹); ΔS2, 3.50 (cal.mol⁻¹.deg⁻¹)). This fit, however, did not offer a statistically significant improvement over that from the single site model and furthermore, showed no distinction in SAH binding between the two sites. Together, these findings were taken to signify equivalent SAM binding at the two sites on the Tsr dimer despite the previously observed apparent differences in SAM binding conformation.

Equivalent binding of SAM at two binding sites in the Tsr homodimer conceivably allows for two catalytic sites in the enzyme, yet Tsr is thought to use a single catalytic center. To
examine this further, initial rate data for SAM turnover by Tsr was analyzed with various kinetic models, using *E. coli* 16S/23S rRNA as the methyl acceptor. Indeed, Tsr also efficiently methylates RNA oligonucleotides composed of the minimum target RNA sequence [11,35], but a ribosomal intermediate has been suggested to be the cellular target [36]. Hence, 16S/23S rRNA was used here to study Tsr activity in the context of a methyl acceptor that perhaps more closely resembles the biological substrate. In line with catalysis by a single active site, we observed hyperbolic Michaelis-Menten kinetics for SAM (Fig. 1D), with a $K_M$ comparable to that previously reported for Tsr-catalyzed methylation of total *E. coli* rRNA [36] or a 93 nt RNA substrate [35]. To investigate the influence of simultaneously occupied SAM binding sites on kinetics, additional analyses using non-Michaelis-Menten models were performed. Initial rate data did not fit models that describe cooperative, allosteric or effector kinetics [37], but a fit was obtained from a model that accounts for the presence of two independent substrate binding sites, one of which functions catalytically [37,38] (not shown). Still, as with assessments of SAM/SAH binding, this fit did not offer a statistically significant improvement over the single substrate (Michaelis-Menten) model. These findings therefore support the hypothesis of a single catalytic site and importantly, further suggest that SAM binding at the non-catalytic site does not affect overall enzyme turnover.

3.2. Effect of active site mutations on Tsr activity

The NTD of Tsr and related SPOUT enzymes has been implicated as being principally responsible for RNA binding [11,12,39]. To identify some of the critical interactions of SAM with amino acids in the Tsr active site, we introduced single amino acid mutations at a number of CTD amino acid residues in the vicinity of bound SAM as observed in the crystal structure (Fig. 2A, Supplementary Material Fig. S2). The catalytic activity of these Tsr variants was then monitored in the presence of a non-limiting concentration of SAM (~10-fold $K_M$), and an excess of 16S/23S RNA acceptor relative to Tsr. (Fig. 2B). Mutations to N129, R135 and R165 were most deleterious to activity; both N129 variants exhibited approximately 20% relative activity, and the Ala variants at R135 and R165 showed 30% and 35% relative activity, respectively. These effects from the Arg mutations matched those for equivalent mutations on the methylation of 29 and 60 nt RNA substrates by Nhr [12]. For further examinations into the nature of the contribution made by these Arg residues, especially the presence of electrostatic interactions, Lys mutants were also generated. For R135, activity remained similar (35%) to that of the corresponding Ala mutant, but activity for the Lys mutation of R165 was almost 2-fold greater (65%) than the Ala. Mutations to K221 and E220 resulted in an approximate 50% reduction to enzymatic activity, while modest effects were noted from mutations to S246 and N248.

3.3. Thermodynamics of SAM binding and kinetics of SAM turnover by select Tsr mutants

The survey of catalytic activity by Tsr variants showed the greatest effects arising from mutations to N129, R135 and R165. Filter-binding enzymatic assays where a 29 nt RNA served as the methyl acceptor also confirmed severely impaired activity by the mutants with Ala substituted at these positions (Supplementary material, Fig. S3). The relative activity of these mutants was lessened further with the 29nt RNA acceptor as compared to 16S/23S rRNA. This discrepancy was attributed to the significantly shorter sequence length of the
former, especially considering that methylation activity by Tsr (and Nhr) is influenced by the RNA acceptor length and consequently, tertiary structure [11,12]. We focused subsequent investigations on more in-depth examinations into the roles of these amino acids, first with evaluations of SAM binding. Consistent with effects on enzyme activity, SAM binding affinity was significantly reduced (approximately 2-6 fold increases in KD) for both N129 mutants and by Ala mutation of R135 or R165 (Table 1). The largest entropic penalties were observed from mutation of R135 and N129 to Ala and Asp, respectively, suggesting the binding sites in these mutants were the least accommodating to SAM. Tsr mutants with Lys substitutions for R135 or R165, however, exhibited SAM affinities similar to wt Tsr, with entropic losses offset by favorable changes to enthalpy (Table 1). Notably, SAH binding affinity was markedly less affected by individual mutants in this group of amino acids as compared to SAM (Table 2), possibly due to relaxed binding constraints in the absence of the methyl group and the absence of positive charge on the sulfur atom. Also, for Ala mutations of N129 and R135, the slight increase in SAH affinity accompanied by favorable entropic shifts, may have partly contributed to diminished activity by these mutants since SAH is known to inhibit the methylation reaction. Collectively, these results confirm that N129, R135 and R165 engage in interactions with SAM that are integral to its productive binding. With respect to R135 and R165, electrostatic interactions appear compulsory as impaired SAM binding resulting from Ala mutations at these sites correlated with the loss of enzyme activity, but affinity was restored by Lys substitutions presumably because of the presence of a side chain that mimics the charge properties of the native Arg. Unlike for R165, the Lys mutant of R135 did not recover catalytic activity, suggesting that the former is primarily involved in SAM binding, while the latter contributes an additional role to catalysis.

It seemed probable that this additional function of R135 was related to activation of the methyl group acceptor. We attempted further verification of this through the determination of enzyme kinetic parameters for Ala mutants of N129 and R135, in which the respective side chain functionalities would be abolished. However, activity by these variants could not be detected under the conditions of the kinetic assay (not shown), emphasizing their indispensable function. On the other hand, reductions to $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ observed for the Ala mutant of R165 offered additional support for a primary role in SAM binding for this residue (Figure 3A). Considering that R135 appears more suitably positioned for methyl acceptor activation than N129, the observations above are consistent with the catalytic mechanism depicted in Fig. 3B.

### 3.4. N129 is involved in the structural organization of Tsr

The orientation of the N129 side chain observed in the Tsr X-ray structure appears prohibitive to interactions with SAM, yet this amino acid was found essential for SAM binding and Tsr activity. As well, both N129 mutants exhibited a similar loss of SAM binding affinity in spite of their differing side chain properties. Such conflicting observations may be explained by changes to protein structure in response to the alteration of N129, as this amino acid is located in a region central to Tsr dimerization and construction of the SAM binding pocket [11] (Figs. 4A & 4B). We therefore looked for an effect on Tsr dimerization by the mutation of N129 to Ala, a substitution that would remove
the polar side-chain interactions at this position. On the basis of size exclusion chromatography, enzyme dimerization appeared unaffected (Fig. 4C) and so the effect of this mutation on protein secondary structure was examined by CD spectroscopy. This analysis showed a distinct divergence of the spectrum for the mutant from that of wt Tsr (Fig. 4D), indicative of differences in α-helical content.

4. Discussion

Tsr and related enzymes have been presumed to use a single catalytic site for methyl transfer. Here, the Tsr dimer was confirmed to bind two SAM molecules in vitro, but shown to exhibit Michaelis-Menten kinetics for SAM turnover, which suggests that a single catalytic SAM binding site operates independently of a non-catalytic binding site when both are occupied with SAM. This coincides with the recent findings by Yin and co-workers that showed catalytic activity for an Nhr heterodimer containing a functionally inactivated subunit [40]. However, it could not be determined whether this functional difference in Tsr is structurally predefined. The apo-structure for Tsr has not been reported, but apo- and SAM-bound structures of Nhr are largely similar [12], implying that the catalytic site is fixed and inherently defined. As well, although SAM binding by the Nhr heterodimer was not evaluated [40], our present findings predict that SAM would distinguish between two potential binding sites on the Nhr heterodimer and accordingly, catalysis would proceed from a structurally pre-defined catalytic site. Such structure-defined functional asymmetry in a homodimeric enzyme is exemplified by the E. coli glyoxylase I, where only one of the enzyme subunits is able to bind metal and function catalytically [41]. Nonetheless, mutually exclusive catalysis at either SAM binding site in Tsr cannot be excluded based on our findings. Indeed, that the cosubstrate binding sites on the Tsr dimer exhibit equivalent binding of SAM or SAH, with greater affinity for the latter, could imply that SAH release is a rate-limiting step in catalysis at either binding site. Moreover, considering that RNA binding to the Tsr dimer ostensibly follows that of SAM, it is possible that the catalytic site becomes designated from the association of RNA with the Tsr-SAM complex, which could allow for switching of the catalytic site when RNA substrates are encountered subsequent to dissociation of a methylated RNA product. Supporting evidence for this is seen by the demonstration of Tsr-induced structural changes within the RNA substrate that facilitate recognition [39]. Additional study is required to delineate the interplay between SAM and RNA binding on the catalytic activity of Tsr, particularly in the context of biologically relevant RNA acceptor substrates.

In agreement with previous studies, R135 and R165 were found essential for enzymatic activity and we have shown here that this could be linked to their importance in SAM binding. It is noteworthy to consider that the observed effects on enzyme activity resulting from mutations of these amino acids could in part be due to perturbed interactions with the RNA substrate. However, this seems in contradiction with structural evidence and molecular modeling that largely excludes these amino acids from such interactions [11,39]. In any event, our findings clearly demonstrate roles in SAM binding by these amino acids. Moreover, from the comparative effects from mutations with opposing side chain properties on SAM binding and enzyme activity, R135 emerged as the most likely candidate responsible for activation of the methyl group acceptor. Structural studies of SPOUT...
methyltransferases have often pointed towards Arg in this role, resembling its function as the catalytic base in the mechanisms of unrelated enzymes such as inosine 5-monophosphate dehydrogenase [42] and L-aspartate oxidase [43]. A factor common to these examples is the interaction of the catalytic Arg with a proximal carboxylate group that can be supplied by the enzyme substrate, and this is believed to exert steric and conformational effects on the guanidinium ion that result in a net deprotonated state with a lowered pKa that allows for proton abstraction [44]. Although pKa measurements of R135 were not attempted here, SAM binding was found here to be highly dependent upon R135, and it is thus surmised that the interaction between R135 and SAM promotes a proton-accepting guanidinium conformation that can lead to activation of the 2’-OH acceptor and facilitate transmethylation.

Intriguingly, N129 was found essential for SAM binding despite appearing unable to directly engage in meaningful interactions with SAM. The introduction of negative charge from the replacement of this amino acid with Asp might hinder SAM association, yet this explanation would not account for the loss of SAM binding observed for the corresponding mutation to Ala, which presents an uncharged, non-polar and smaller side-chain. N129 is highly conserved among SPOUT methyltransferases, residing on an equally conserved helix (helix 6) that is integral to the formation of the dimer interface and for the construction of the characteristic CTD knot [11] known to be essential for proper structural conformation and activity of enzymes in which it occurs [45]. Further, R135 is also located on this aforementioned helix. Hence, owing to its central location in a region of Tsr that supplies much of the structural elements required for SAM binding, N129 appears to play a pivotal role in the organization of Tsr active site architecture. It is also plausible that structural features reliant on N129 are required for proper association of the RNA substrate with the enzyme dimer. The alteration of N129 may therefore have ramifications on proper binding and/or positioning of the RNA substrate, further alluding to the likelihood of a functional interrelation between the interactions of Tsr with SAM and RNA.

In summary, this work has provided new details on SAM binding and turnover by Tsr. We propose that an active site Arg performs a key step in the catalytic mechanism, enabled by its interaction with SAM. Further, our findings implicate an active site-adjacent Asn as a structural lynchpin in the formation of the SAM binding site. Given the high degree to which these amino acids residues are conserved among SPOUT enzymes, the insights provided here are likely to be applicable to other enzymes of this class, and relevant to the continued understanding of the biological methylations they accomplish.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgements**

We are grateful to Drs. T. Dieckmann and G. Guillemette (University of Waterloo) for access to ITC and CD instrumentation, respectively. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada (JFH), the Ontario Graduate Scholarship program (CLM), the USDA National Institute of Food and Agriculture (Agriculture and Food Research Initiative Competitive Grant No. 2013-67011-21133) (EGK), the
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>alanine</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal ion affinity chromatography</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
</tr>
<tr>
<td>K_D</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>Lys</td>
<td>lysine</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosyl-L-homocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl-L-methionine</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
</tbody>
</table>

References


Highlights

- Tsr exhibits equivalent SAM binding at two structurally non-equivalent sites
- An arginine residue is implicated in the Tsr catalytic mechanism
- A central asparagine is integral to Tsr structure, SAM binding and catalysis
- Implications for a dynamic link in substrate/cosubstrate binding by SPOUT enzymes
Figure 1. SAM binding and turnover by Tsr

(A) General scheme for SAM-dependent transmethylation. “Nu” represents a generic nucleophilic acceptor on the substrate (e.g. activated hydroxyl, aromatic nitrogen) that is methylated by the electron deficient methyl group from SAM. The reaction proceeds via an energetically favored S_N2-type mechanism, with an inversion of stereochemistry at the methyl group, resulting in co-production of the methylated substrate and SAH. (B) Representative ITC measurements showing the background-subtracted heats/injection (top panels) and binding isotherms (bottom panels) for titrations of SAM and SAH with Tsr. Average integrated heats/injection from two independent experiments were fit by non-linear regression to a model for a single set of sites. Where reported, errors are the S.E. associated with the fit. (C) Overlay of SAM molecules in the conformations found when bound to each Tsr monomer as shown in the Tsr-SAM crystal structure [11]. The skeleton of SAM in each conformation is colored magenta and green, respectively. (D) Michaelis-Menten plot for the methylation of 0.35 μM 16S/23S rRNA by 0.1 μM Tsr. The fit was obtained by non-linear regression of the average initial rates from three independent experiments. Error bars are the S.D. and the S.E. associated with the fit is reported for K_M and k_cat.
Figure 2. Enzymatic activity of Tsr mutants

(A) Active site residues selected for mutation surround the bound SAM (spheres). (B) Catalytic activity of Tsr active site mutants with 16S/23S rRNA. Activity is expressed as the average initial rate of methylation from three independent experiments, normalized to that of native Tsr. Error bars are the S. E.
Figure 3. R165 functions in SAM binding

(A) Michaelis-Menten plot for the methylation of 16S/23S rRNA by the R165A mutant. Average initial rates from triplicate experiments were analyzed as with native Tsr. Error bars are the S.D. and S.E. associated with the fit is reported for $K_M$ and $k_{cat}$. (B) Proposed mechanism for SAM-dependent methylation of rRNA by Tsr. SAM is stabilized in the active site/binding pocket through H-bonding with R165. The interaction between R135 and the carboxylate from SAM promotes activation of the ribose 2‘-OH, leading to consequent attack by the SAM methyl group.
Figure 4. Structural significance of N129

(A) Surface representation of Tsr illustrating the contribution of helices 6 and 11 to the dimerization interface. Helices 6 and 11, and the loop corresponding to amino acids 234-247 are shown as a cartoon and SAM is shown as spheres. The arrowhead denotes the viewpoint shown in close-up in panel B. (B) Zoomed in view of the Tsr active site with the hydrophobic amino acid interface between helix 6 and helix 11 highlighted (orange). (C) Size exclusion chromatography of wt Tsr and the N129A mutant. The peak identities (left to right) for the molecular weight standards are as follows: blue dextran (2000 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa). Chromatograms show an identical elution volume for Tsr and the N129A variant, with an estimated relative molecular weight of 60 kDa (dimeric molecular weight of Tsr: 58364 Da). (D) Representative circular dichroism spectra for Tsr (dashed line) and N129A (solid line). The changes in ellipticity for the Ala variant of N129 indicate the absence of secondary structural features present in native Tsr.
Table 1

Parameters for SAM binding\(^a\) by Tsr and N129, R135 or R165 mutants

<table>
<thead>
<tr>
<th>Variant</th>
<th>Stoichiometry</th>
<th>±H (kcal/mol)</th>
<th>ΔS (cal.mol(^{-1}).deg(^{-1}))</th>
<th>(K_D) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>2.03 ± 0.02</td>
<td>−3.94 ± 0.71</td>
<td>4.7</td>
<td>107.1</td>
</tr>
<tr>
<td>N129A</td>
<td>2.19 ± 0.13</td>
<td>−8.95 ± 0.73</td>
<td>−15.3</td>
<td>493.0</td>
</tr>
<tr>
<td>N129D</td>
<td>1.87 ± 0.28</td>
<td>−21.31 ± 3.98</td>
<td>−57.2</td>
<td>411.5</td>
</tr>
<tr>
<td>R135A</td>
<td>2.22 ± 0.18</td>
<td>−43.50 ± 4.52</td>
<td>−134.0</td>
<td>613.5</td>
</tr>
<tr>
<td>R135K</td>
<td>1.89 ± 0.03</td>
<td>−9.36 ± 0.25</td>
<td>−14.4</td>
<td>142.3</td>
</tr>
<tr>
<td>R165A</td>
<td>1.71 ± 0.08</td>
<td>−8.16 ± 0.51</td>
<td>−10.8</td>
<td>188.7</td>
</tr>
<tr>
<td>R165K</td>
<td>2.13 ± 0.03</td>
<td>−6.52 ± 0.15</td>
<td>−4.0</td>
<td>97.1</td>
</tr>
</tbody>
</table>

\(^a\)Average integrated heats of injection from two independent titrations of SAM into solutions of Tsr mutants, after subtraction of heats from dilution and mixing, were fit by non-linear regression to a model for a single set of binding sites. The dissociation constant \(K_D\) is the reciprocal of the association constant \(K_A\) that is generated by Origin software. S.E. associated with the fit to the single sites model is reported for stoichiometry and AH. Binding parameters for wt Tsr are included for comparison.
Table 2
Parameters for SAH binding $^b$ by Tsr and mutants with Ala substituted for N129, R135 or R165

<table>
<thead>
<tr>
<th>Variant</th>
<th>Stoichiometry</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S$ (cal/mol$^{-1}$·deg$^{-1}$)</th>
<th>$K_D$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>2.09 ± 0.02</td>
<td>−19.2 ± 0.30</td>
<td>−43.1</td>
<td>24.4</td>
</tr>
<tr>
<td>N129A</td>
<td>2.45 ± 0.03</td>
<td>−13.0 ± 0.27</td>
<td>−21.6</td>
<td>16.4</td>
</tr>
<tr>
<td>R135A</td>
<td>2.70 ± 0.02</td>
<td>−15.2 ± 0.18</td>
<td>−28.3</td>
<td>11.0</td>
</tr>
<tr>
<td>R165A</td>
<td>2.03 ± 0.02</td>
<td>−18.5 ± 0.28</td>
<td>−40.7</td>
<td>20.5</td>
</tr>
</tbody>
</table>

$^b$ Titrations and analyses of thermodynamic data were performed as with SAM. S.E. associated with the fit is reported for stoichiometry and $\Delta H$. Binding parameters for wt Tsr are shown for comparison.