A Novel Motif for S-Adenosyl-L-methionine Binding by the Ribosomal RNA Methyltransferase TlyA from *Mycobacterium tuberculosis*  

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Marta A. Witek, Emily G. Kuiper, Elizabeth Minten, Emily K. Crispell, and Graeme L. Conn

From the Department of Biochemistry, the Biochemistry, Cell and Developmental Biology Program, and the Microbiology and Molecular Genetics Program, Graduate Division of Biological and Biomedical Sciences, Emory University School of Medicine, Atlanta, Georgia 30322

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Capreomycin is a potent ribosome-targeting antibiotic that is an essential component of current antituberculosis treatments, particularly in the case of multidrug-resistant *Mycobacterium tuberculosis* (Mtb). Optimal capreomycin binding and Mtb ribosome inhibition requires ribosomal RNA methylation in both ribosome subunits by TlyA (Rv1694), an enzyme with dual 2′-O-methyltransferase and putative hemolytic activities. Despite the important role of TlyA in capreomycin sensitivity and identification of inactivating mutations in the corresponding *Mtb* gene *tlyA*, which cause resistance to capreomycin, our current structural and mechanistic understanding of TlyA action remains limited. Here, we present structural and functional analyses of Mtb TlyA interaction with its obligatory co-substrate for methyltransferase activity, S-adenosyl-L-methionine (SAM). Despite adopting a complete class I methyltransferase fold containing conserved SAM-binding and catalytic motifs, the isolated TlyA carboxyl-terminal domain exhibits no detectable affinity for SAM. Further analyses identify a tetrapeptide motif (RXWV) in the TlyA interdomain linker as indispensable for co-substrate binding. Our results also suggest that structural plasticity of the RXWV motif could contribute to TlyA domain interactions, as well as specific recognition of its two structurally distinct ribosomal RNA targets. Our findings thus reveal a novel motif requirement for SAM binding by TlyA and set the stage for future mechanistic studies of TlyA substrate recognition and modification that underpin Mtb sensitivity to capreomycin.

*Mycobacterium tuberculosis* (Mtb), the etiological agent of tuberculosis (TB), infects approximately one-third of the world population and resulted in an estimated 9.6 million new cases of active TB disease and 1.5 million deaths in 2014 (1). Of further significant concern is the rising number of TB cases involving Mtb strains that are either multidrug-resistant, defined as being resistant to the first line antibiotics isoniazid and rifampicin, or extensively drug-resistant, defined as being additionally resistant to any fluorquinolone and at least one of the three injectable second line drugs: amikacin, kanamycin, or capreomycin (2, 3).

The cyclic aminoglycoside-like peptide antibiotic capreomycin targets the mycobacterial ribosome at the interface of the small and large subunits (4) and requires ribosomal RNA (rRNA) methylation for optimal binding and thus inhibition of ribosome function. Resistance to capreomycin in *Mtb* can arise via mutation of *tlyA*, the gene encoding the protein TlyA (Rv1694), a proposed virulence factor for *Mtb* with dual hemolytic and rRNA methyltransferase activities (5–8). Resistance to ribosome-targeting drugs is generally associated with the addition of methyl groups rather than their loss (9–11). Thus, TlyA belongs to a unique group of methyltransferases for which the loss of function confers bacterial antibiotic resistance. Additionally, because many bacterial genera lack *tlyA*, the potent antibiotic activity of capreomycin is specific against *Mtb* (6, 12). However, treatment of TB has become problematic not only because of the side effects of aminoglycosides but also because of the increased incidence of virulent, capreomycin-resistant *Mtb* strains generated by inactivation of *tlyA* (6, 13). Despite the critical role of TlyA in capreomycin sensitivity and identification of inactivating mutations that cause resistance, our current understanding of TlyA structure and mechanism of action remains limited.

The S-adenosyl-L-methionine (SAM)-dependent methyltransferase activity of TlyA results in ribose 2′-OH methylation of two cytidine residues: 16S rRNA C1409, which is located within the 30S (small) ribosomal subunit “decoding center,” and 23S rRNA C1920, which is present in a highly conserved region of the 50S (large) ribosomal subunit near the subunit interface (4, 6, 14). Despite the importance of TlyA methyltransferase activity in capreomycin action and resistance, many molecular details of the TlyA mechanism of action remain

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The atomic coordinates and structure factors (codes SEOV, SKYG, and SK52) have been deposited in the Protein Data Bank (http://wwpdb.org/).

1. Both authors contributed equally to this work.

2. To whom correspondence should be addressed; Dept. of Biochemistry, 4135 O. Wayne Rollins Research Center, 1510 Clifton Rd. NE, Atlanta, GA 30322. E-mail: gconn@emory.edu.

3. The abbreviations used are: Mtb, *Mycobacterium tuberculosis*; NTD, amino-terminal domain; CTD, carboxyl-terminal domain; His-TlyA, hexahistidine-tagged *Mtb* TlyA protein; ITC, isothermal titration calorimetry; rRNA, ribosomal RNA; SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-homocysteine; TB, tuberculosis; Ulp, ubiquitin-like protease; PDB, Protein Data Bank.
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FIGURE 1. Recombinant MtboHis-TlyA (Rv1694) is well folded and active in vitro against both 30S and 50S ribosomal subunit substrates. A. CD spectrum of His-TlyA and results of secondary structural deconvolution (inset). MRW, mean residue molar ellipticity. B, RT analysis of in vitro 2′-O-methylation by TlyA (solid arrowheads) of residues C1409 (16S rRNA, left) and C1920 (23S rRNA, right). The positions of nearby modifications normally present in E. coli are also indicated (open arrowheads).

largely unknown, including interaction with co-substrate SAM and how TlyA recognizes and methylates its two structurally distinct substrates (23S and 16S rRNA). Therefore, detailed molecular studies of TlyA are urgently required to better understand this resistance determinant and its contribution to capreomycin susceptibility.

Here, we demonstrate experimentally that TlyA folds into two stable structural domains connected by a protease-sensitive linker, with rRNA binding and SAM binding/methyltransferase activities expected to reside in the amino- and carboxyterminal domains (NTD and CTD), respectively. A high resolution X-ray crystal structure of the TlyA CTD reveals a Class I methyltransferase fold containing all expected conserved SAM binding and catalytic motifs. Surprisingly, however, this isolated protein domain has no detectable affinity for SAM. Further structural and functional studies reveal a novel tetrapeptide motif (RXWV) in the region linking the two structural domains as indispensable for co-substrate binding. Finally, our results also suggest that structural plasticity within this interdomain linker could play a role in TlyA recognition of its two structurally distinct rRNA targets.

Results

Construct Design, Protein Expression, and Purification of TlyA for Structural Studies—A plasmid encoding amino-terminally hexahistidine-tagged MtboTlyA (His-TlyA) was generated for heterologous expression in Escherichia coli, and the resulting protein was purified to near homogeneity using Ni²⁺ affinity and gel filtration chromatographies. The CD spectrum of His-TlyA is consistent with that of a well folded protein with a mixed α/β structure (Fig. 1A). We additionally showed our purified recombinant His-TlyA to be active in methylation of both target nucleotides, C1920 (23S rRNA) and C1409 (16S rRNA), by primer extension analysis of in vitro methylated E. coli 50S and 30S subunits (Fig. 1B).

We next attempted to crystallize full-length His-TlyA to solve its high resolution X-ray crystal structure, but efforts to obtain suitable crystals were unsuccessful. Therefore, His-TlyA was treated with various proteases with the goal of identifying stable fragment(s) of TlyA better suited to structural studies. The endopeptidase GluC, a serine proteinase that selectively cleaves peptide bonds carboxyl-terminal to glutamic acid residues (15), produced two stable fragments of ~10 and ~20 kDa (Fig. 2A). Based on the observed digestion pattern and inspection of the TlyA sequence and homology model (16), we identified glutamic acid 59 (Glu⁵⁹) as the most likely site of GluC cleavage. The TlyA homology model predicts Glu⁵⁹ to be surface-exposed in an unstructured region that links the predicted NTD and CTD (Fig. 2B), and GluC cleavage after this residue would result in products of 7.9 or 6.2 kDa (NTD; with or without the hexhistidine tag) and 21.8 kDa (CTD), correlating well with the observed products. Cleavage products of similar sizes were also previously observed for TlyA treated with proteinase K, which was hypothesized to target a site within the interdomain linker (5). To test whether Glu⁵⁹ was indeed the GluC cleavage site, a TlyA-E59A variant was generated, and the purified protein similarly was subjected to GluC cleavage. Although GluC cleavage was not abolished, the pattern of fragments produced from the variant protein was altered, suggesting that Glu⁵⁹ is the major, but not the only, cleavage site recognized by the protease (Fig. 2C). We next asked whether these two GluC-derived TlyA fragments remain stably folded and associated by applying partially cleaved protein to a gel filtration column. Two major peaks were observed (Fig. 2D), with the earlier eluting protein corresponding to the remaining uncleaved full-length His-TlyA protein. Although eluting at a volume corresponding to a significantly lower molecular mass entity, the later peak was found to contain both stable fragments (Fig. 2, D and E), suggesting that the TlyA NTD and CTD remain stably folded and associated following GluC cleavage.

We conclude from these results that treatment of His-TlyA with GluC produces two stable protein fragments that likely correspond to the predicted TlyA NTD and CTD and that these domains have sufficient affinity that they remain associated following cleavage under the solution conditions used. Based on these observations, we generated a new expression construct for crystallographic studies of the TlyA methyltransferase domain (CTD) beginning at residue Ser⁶⁴, which immediately follows the predicted interdomain linker and corresponds to the first amino acid of α-helix 1 (α1) in the TlyA homology model (Fig. 2F). Crystals of TlyA CTD suitable for structural determination formed within 5 days and diffracted to 1.7 Å resolution.

The TlyA CTD Adopts a Class I Methyltransferase Fold—The structure of TlyA CTD was solved using a TlyA homology model, and unambiguous electron density allowed modeling of amino acids Ser⁶⁴–Pro⁹⁶, producing a final refined model with \( R_{work} / R_{free} \) of 0.188/0.218 (also see Table 1). The TlyA CTD structure contains a RrmJ/FtsJ Rossmann-like methyltransferase fold comprising seven β-strands (with topology...
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The TlyA CTD structure additionally confirms the location of the proposed TlyA catalytic tetrad Lys<sup>69</sup>/Asp<sup>154</sup>/Lys<sup>182</sup>/Glu<sup>238</sup> (16, 18).

The TlyA CTD domain overlays well with other Class I methyltransferases including the archetypical member of the TlyA family, the 2'-O-methyltransferase RrmJ, and the DNA C5-methyltransferase Hhal (19, 20). Structural superimposition of TlyA-CTD with other class I methyltransferases was used to model the likely location of SAM in the TlyA binding pocket. Although most features of protein secondary structure overlay well between TlyA and RrmJ (PDB code 1EIZ), severe clashes were observed when the RrmJ bound SAM was placed within the TlyA CTD via protein superimposition because of differences in the more variable loops that link the core β-strands. In contrast, modeling using the SAM-bound structure of either the Hhal DNA methyltransferase (PDB code 2HMY) or RlmM 2'-O-methyltransferase (PDB code 4B17) (21) places SAM into the TlyA binding pocket with no significant clashes (shown for Hhal in Fig. 3B). The modeled SAM is positioned on top of the loop connecting TlyA core strand β1 and α2, which contains the SAM-binding motif I<sup>90</sup>GASTG<sup>94</sup>. The central Ser residue of an atypical SAM-binding motif of TlyA is oriented toward the modeled SAM and positioned to directly hydrogen bond with the ribose 3'-OH. Additionally, by adopting a different rotomeric state, the hydroxyl of residue Thr<sup>93</sup> within this motif would be positioned for interaction with the carboxyl end of the modeled SAM. Finally, the SAM adenine moiety is modeled within a largely hydrophobic pocket on the surface of TlyA comprising the side chains of residues Val<sup>113</sup>, Ala<sup>136</sup>, and Ile<sup>158</sup>; the backbone of Gly<sup>114</sup> and Asn<sup>135</sup> (Fig. 3C). Thus, the TlyA CTD structure possesses the expected features necessary for interaction with the obligatory methyltransferase co-substrate SAM.

We additionally note that the proposed TlyA catalytic residue Asp<sup>154</sup> is positioned adjacent to the transferable methyl group of the modeled SAM (Fig. 3, B and C). Interestingly, two aromatic amino acids, Tyr<sup>115</sup> and Phe<sup>157</sup>, also line the SAM binding pocket but are oriented into the solvent. These residues could play an important functional role in recognizing the rRNA substrate and coordinating the target nucleotide for catalysis as seen with NpmA-30S complex (22).

The Isolated TlyA CTD Protein Does Not Bind SAM—To begin examining TlyA-co-substrate interactions, we used isothermal titration calorimetry (ITC) to compare the binding of SAM and the methylation reaction by-product S-adenosylhomocysteine (SAH) to the full-length enzyme. His-TlyA bound both SAM and SAH with similar affinities in the low micromolar range (Fig. 4A and Table 2), comparable with other rRNA methyltransferases (23, 24). Surprisingly, however, despite retaining a complete class I methyltransferase SAM-binding fold with the expected conserved motifs, the isolated TlyA CTD protein did not exhibit detectable binding of SAM (Fig. 4B and Table 2). In contrast, GluC-cleaved full-length His-TlyA (His-TlyA<sub>GluC</sub>), i.e. the co-purified NTD and CTD fragments (Fig. 2D), bound SAM with similar affinity to the intact protein (Fig. 4C and Table 2). Together, these data indicate that the methyltransferase fold of our TlyA CTD construct is not sufficient for SAM binding, and thus, one or more elements of the amino-
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TABLE 1
X-ray data collection and refinement statistics for TlyA CTD structures

| PDB code    | CTD          | RAWVCTD (form 1 loop) | RAWVCTD (form 2 helix) |
|-------------|--------------|-----------------------|------------------------|
|             | CTD          | RAWVCTD (form 1 loop) | RAWVCTD (form 2 helix) |
|             | PDB code     | Resolution (Å)        | Resolution (Å)         |
|             | 5EOV         | 47.93–1.70 (1.73–1.70) | 53.04–1.90 (1.97–1.90) |
|             | 5KYG         | 47.98–2.18 (2.24–2.18) |
| Space group | P4,2,2       | 1.000                 | 1.000                  |
| Cell dimensions | a, b, c (Å)   | 90, 90, 90            | 90, 90, 90            |
| Resolution (Å) | Rmerge (%)    | 0.109 (0.871)         | 0.121 (0.763)         |
|                | Rfree (%)     | 0.025 (0.234)         | 0.035 (0.762)         |
|                | Rmerge (%)    | 65.2 (3.3)            | 45.8 (3.6)            |
|                | Rfree (%)     | 100.0 (100.0)         | 99.9 (99.9)           |
|                | Redundancy    | 19.2 (13.7)           | 12.8 (9.1)            |
|                | Completeness (%) | 100.0 (100.0)         | 99.9 (99.9)           |
|                | Protein       | 1,587                 | 1,579                  |
|                | Water         | 38.7                  | 38.9                   |
|                | Ligand        | 44.9                  | 44.8                   |
|                | B-factors     | 1.09                  | 1.51                   |
|                | Ramachandran plot | 98.0                   | 98.0                   |
|                | Favorable (%) | 34.6                  | 37.8                   |
|                | Outliers (%)  | 30.2                  |                       |
|                | Root mean square deviations | Bond lengths (Å)   | Bond angles (°)       |
|                |              | 0.007                 | 0.011                  |
|                |              | 1.09                  | 1.51                   |
|                |              | 0.00                  | 0.00                   |
|                |              | 1.09                  | 1.51                   |

a Unless otherwise noted, the values in parentheses are for the highest resolution shell.

b Rmerge = \(\frac{\sum(hkl) \times (f_{o}(hkl) - f_{c}(hkl))}{\sum(hkl) \times f_{o}(hkl)}\), where \(f_{o}\) and \(f_{c}\) are the observed and calculated structure factors, respectively. Rfree applies to the 10% of reflections chosen at random to constitute the test set.

c CC(1) = \(\frac{\sum(hkl) - \sum(hkl) f_{c}(hkl)}{\sum(hkl) \times f_{o}(hkl)}\), where \(f_{o} f_{c}\) is the observed and calculated structure factors, respectively.

d Root mean square deviations:
- Bond lengths (Å)
- Bond angles (°)

FIGURE 3. The TlyA CTD adopts a class I methyltransferase fold. A, two orthogonal views of the TlyA CTD crystal structure with the conserved seven-stranded β-sheet core (left) and six surrounding α-helices (right) highlighted in purple, model of the TlyA CTD interaction with SAM (yellow; from PDB 2HMY) shown in the same orientation as A. Residues highlighted are from the SAM binding motif I90GASTG94 (pink backbone, purple side chains) and Asp154 of the proposed TlyA catalytic tetrad (green). C, amino acids proposed to make up the SAM binding pocket in TlyA, colored as in B.

terminal 1–63 residues of TlyA must also play a critical role in SAM co-substrate binding.

The RAWV Tetrapeptide Interdomain Linker Is Critical for TlyA CTD-SAM Interaction—To assess the potential contribution of the TlyA NTD to SAM binding, an amino-terminal domain expression construct was created corresponding to residues 1–63 ending with the RAWV tetrapeptide domain linker sequence (NTD\textsuperscript{RAWV}; Fig. 2F). Expression and purification of the NTD\textsuperscript{RAWV} required an amino-terminal SUMO fusion tag that was removed using the ubiquitin-like protease (Ulp) prior to use in experiments. Using this new construct, we first tested whether the NTD\textsuperscript{RAWV} and CTD proteins interact, recapitulating the retained association of the GluC-derived NTD and CTD fragments of His-TlyA (Fig. 2, D and E). The NTD\textsuperscript{RAWV} and CTD proteins were mixed with 1:1 stoichiometry and applied to a gel filtration column under identical conditions as used previously for the full-length and GluC-cleaved His-TlyA. In contrast to GluC-cleaved His-TlyA, each individual protein domain eluted as a separate peak with no evidence for their direct association (Fig. 5A). Additionally, no binding was detected by ITC when SAM was titrated into in the sample cell containing NTD\textsuperscript{RAWV}/CTD mixture (data not shown). Thus, separate expression of the NTD\textsuperscript{RAWV} and CTD proteins and in vitro reconstitution failed to recapitulate the observed domain association and SAM binding affinity of the GluC-cleaved full-length His-TlyA protein.

We reasoned that the inability of the separately expressed TlyA domains to interact and bind SAM (either the CTD alone or as an NTD\textsuperscript{RAWV}/CTD mixture) might arise from an inappropriate choice of domain boundary in our expression constructs. Although the NTD and CTD derived from full-length TlyA by GluC cleavage appear to remain strongly associated (Fig. 2, D and E), we determined that dialysis against high salt (1 M NaCl) containing buffer and subsequent application to the gel filtration column was sufficient to isolate a sample highly enriched for TlyA CTD\textsuperscript{GluC} (Fig. 5B). This CTD\textsuperscript{GluC} bound SAM with an affinity essentially identical to full-length His-TlyA despite depletion of the NTD fragment (Fig. 5C and Table
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FIGURE 4. The isolated TlyA methyltransferase domain (CTD) does not bind SAM. ITC analysis of TlyA-SAM interaction for titration by SAM into the cell containing His-TlyA (A), tag-free CTD (B), and His-TlyA after cleavage with GluC (His-TlyAGluC) (C).

TABLE 2
Substrate binding affinity of full-length TlyA and individual domains

| Protein         | Ligand | Binding affinity, $K_D$ (µM) |
|-----------------|--------|-----------------------------|
| His-TlyA        | SAM    | 23.4 ± 2.9                  |
| His-TlyA        | SAH    | 39.2 ± 7.3                  |
| His-TlyAGluC    | SAM    | 17.3                        |
| CTD             | SAM    | No binding                  |
| CTD$^\text{GluC}$ | SAM   | 17.9                        |
| RAWVCTD         | SAM    | 20.0 ± 1.1                  |
| NTD$^{\text{RAWV}}$ | SAM   | No binding                  |

* The values are the average $K_D$ values from three independent experiments ± S.D., except for GluC-cleaved proteins, which were performed once.

2), indicating that the majority of the NTD is dispensable for TlyA interaction with co-substrate. We therefore prepared a new TlyA CTD expression construct corresponding to the precise fragment produced by GluC at the predicted Glu$^{59}$ cleavage site, thus placing the RAWV tetrapeptide interdomain linker sequence on the amino terminus of the CTD (residues 60–268, $^{\text{RAWV}}$ CTD; Fig. 2F). Remarkably, addition of the RAWV sequence in $^{\text{RAWV}}$ CTD restored wild-type SAM binding affinity to the isolated domain protein (Fig. 5D and Table 2). Thus, the RAWV tetrapeptide sequence appears essential for SAM binding in TlyA.

Given the predicted location of the RAWV sequence in the region linking the two domains of TlyA, we next evaluated the possibility that this tetrapeptide motif might also contribute to TlyA domain interaction only when present on the CTD. An additional construct was therefore generated corresponding to the amino-terminal 1–59 amino acids of TlyA (NTD; Fig. 2), indicating that the majority of the NTD is dispensable for TlyA interaction with co-substrate. We therefore prepared a new TlyA CTD expression construct corresponding to the precise fragment produced by GluC at the predicted Glu$^{59}$ cleavage site, thus placing the RAWV tetrapeptide interdomain linker sequence on the amino terminus of the CTD (residues 60–268, $^{\text{RAWV}}$ CTD; Fig. 2F). Remarkably, addition of the RAWV sequence in $^{\text{RAWV}}$ CTD restored wild-type SAM binding affinity to the isolated domain protein (Fig. 5D and Table 2). Thus, the RAWV tetrapeptide sequence appears essential for SAM binding in TlyA.

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Trp$^{62}$ and Val$^{63}$ Are the Most Critical Residues for SAM Binding—BLAST search and multiple sequence alignment of the $^{\text{RAWV}}$ CTD sequence in UniProt revealed that the tetrapeptide sequence is strongly conserved in the top 250 TlyA homologs. In particular, position 62 is most highly conserved as a tryptophan, and position 63 is invariant as either a valine or alanine (Fig. 6A). Proteins identified as TlyA homologs include TlyA 2’-O-methyltransferases, RrmJ methyltransferases, cyto-toxins/hemolysins, cytochrome c oxidase subunit II, and TlyA family members. Similar analysis of all SAM-binding proteins within this set of homologs (i.e. either TlyA or RrmJ methyltransferases) revealed an almost identical pattern of sequence conservation as for all 250 proteins (Fig. 6B). In contrast, among Mycobacterial methyltransferases and hemolysins, Arg$^{60}$ was more conserved, and Trp$^{62}$/Val$^{63}$ were invariant. TlyA homologs from all other species were more variable with valine and proline conserved at position 60 and alanine conserved at position 63 (Fig. 6C). Finally, among six functionally characterized TlyA methyltransferases with overall identities of 38–100% to $^{\text{Mtb}}$ TlyA (25), Val$^{63}$ was found to be invariant, with either a tryptophan or tyrosine present at position 62 (Fig. 6E). Based on this conservation, we predicted Arg$^{60}$ and, in particular, Trp$^{62}$ and Val$^{63}$ may play important roles in TlyA function.

To begin experimentally testing the impact of the RAWV tetrapeptide sequence on the TlyA-SAM interaction, individual single amino acid substitutions were made of each at the four residues within full-length TlyA. Each variant was expressed and purified similarly to the wild-type protein, and CD spectroscopy was used to confirm that none of the substitutions resulted in gross changes to the protein fold (data not shown). The SAM binding affinity of each variant was then measured by ITC as before (Table 3). Arg$^{60}$ substitution with
either Ala or Glu modestly impacted SAM binding affinity (~3-fold decrease), whereas an A61V variant had wild-type affinity for the co-substrate. In contrast, substitution of Trp62 with Phe or Ala resulted in 4- and 10-fold reduction in SAM binding affinity, confirming a more significant role for Trp62 in SAM binding and suggesting that the aromatic nature of the side chain is important given the lesser impact of the Trp to Phe substitution. Finally, the most pronounced decrease in SAM binding affinity was observed for the V63A variant (20-fold), pointing to a critical role for this hydrophobic residue in SAM binding.

Structural Plasticity of the RAWV Motif—Because Trp62 and Val63 are not predicted by our TlyA CTD structure or the TlyA homology model to interact with SAM, we next wanted to determine their local structural environments to assess how they might stabilize amino acids that directly contact SAM. Two different crystallization conditions were identified for TlyA RAWVCTD that produced distinct crystal forms with the same space group and similar cell dimensions but that differed slightly in their packing within the crystal lattice. In both crystal forms of RAWVCTD, the presence of a symmetry-related molecule near the SAM binding site precluded obtaining a structure of SAM-bound RAWVCTD via soaking of preformed crystals with SAM, and efforts to complex crystals by direct co-crystallization were unsuccessful. Nevertheless, the two structures of RAWVCTD offer further insight into the potential molecular mechanism by which the RAWV motif influences SAM binding. Although the core methyltransferase fold in the two structures is essentially identical (aligning with 0.27 Å root mean square deviation for 160 residues), the structure and position of the RAWV motif varies significantly, adopting an unstructured loop in one crystal (form 1 loop) and an extension of one of the methyltransferase domain in the second (form 2 helix; Fig. 7, A and C).

### Table 3

| TlyA variant | Binding affinity, $K_d$ | Decrease compared with wild type (fold) |
|--------------|-------------------------|----------------------------------------|
| R60A         | 87.2 ± 8.5             | 3.6                                    |
| R60E         | 62.8 ± 23              | 2.7                                    |
| A61V         | 21.1 ± 4.3             | 0                                      |
| W62A         | 234 ± 56               | 10                                     |
| W62F         | 98.5 ± 27              | 4.3                                    |
| V63A         | 47.0 ± 19              | 20                                     |
| T93A         | 66.9 ± 2.63            | 2.9                                    |

$^a$ The values are the average $K_d$ values from three independent experiments ± S.D.
In the form 1 loop, electron density allowed modeling of Trp^{62} and Val^{63} peptide backbone and also the Trp^{62} side chain (Fig. 7B). In the form 2 helix, clear density was observed for the peptide backbone of the RAWV motif, as well as side chains of residues Ala^{61}–Val^{63} (Fig. 7D). In the form 2 helix structure, extension of α1 by the RAWV tetrapeptide sequence positions Trp^{62} to interact with the backbone of Lys^{254} and Gly^{255} of a symmetry-related molecule. This arrangement is similar to that of the CTD structure (which lacks Trp^{62}) in which an extended α1 would be accommodated, if the RAWV sequence were present, without clashing with a symmetry-related molecule. In contrast, the altered crystal packing of the form 1 loop restricts the ability of the RAWV sequence to extend α1. Strikingly, the RAWV sequence instead adopts the same structure as observed for the corresponding region (sequence LRYV) in hemolysin proteins from *Streptococcus thermophilus* (PDB code 3HP7) and *Lactococcus lactis* (PDB code 3OPN) with Trp^{62} overlaying with Tyr^{62} and Tyr^{36}, respectively, and stabilized by a hydrophobic interaction with Val^{99}/Val^{99} (TlyA/3HP7/3OPN; Fig. 7E). Although detailed interpretations are potentially complicated by the influence of crystal packing contacts on the position of the RAWVCTD amino terminus residues, the two structures nonetheless reveal that the *Mtb* TlyA RAWV motif is capable of adopting two strikingly different conformations. We speculate that these two conformations might reflect an important functional transition in TlyA; for example, if the relative orientation of the NTD and CTD is altered by interaction with ribosomal subunit substrate. An important final question, however, is whether one or both of the observed conformations of the RAWV motif can provide a suitable mechanistic explanation for its contribution to SAM affinity.

Comparison of the two RAWVCTD structures and the CTD structure reveals changes in the regions surrounding the SAM binding pocket in addition to those in the RAWV sequence structure itself (Fig. 7F). In the α-helical conformation, Trp^{62} is rotated by 180° from its position in the form 1 loop structure. On the opposite side of the SAM binding pocket, a shift in the peptide backbone is also observed for the loops containing...
Thr^{134} and Tyr^{115}, with movements of 3.1 and 3.9 Å for their Cα atoms, respectively, upon comparison of the form 1 loop with the form 2 helix structure. Because Tyr^{115} interacts with symmetry-related molecules in each structure, we cannot eliminate the possibility that this structural change is influenced in part by crystal packing. However, it is noteworthy that although the Tyr^{115} backbone moves away from the SAM molecule, its side chain is reoriented closer to the SAM pocket such that it could contribute to positioning of SAM or the target nucleotide in the TlyA active site.

The most striking differences between the two RAWV-CTD structures surround Val^{63}, the residue most critical for SAM affinity. In the extended αI of the form 2 helix structure, Val^{63} is shifted 4.4 Å toward Thr^{93} of the SAM binding motif I (Fig. 7G). Additional small differences are seen in the positions of both Thr^{93} and Ser^{92} Cα, shifted 1.4 and 1.9 Å, respectively, toward the expected position of the bound SAM. We also note that in both the form 1 loop and, to a lesser extent, the CTD structures, the Thr^{93} side chain is oriented with its hydroxyl group oriented away from the SAM pocket (Fig. 7H). In contrast, in the form 2 helix structure, the Thr^{93} side chain is reoriented with its hydroxyl group pointing into the SAM pocket and positioned to interact directly with the co-substrate carboxylate group (Fig. 7J).

To evaluate the direct contribution of Thr^{93} to SAM binding by TlyA, we measured the SAM binding affinity of a T93A variant. TlyA T93A bound SAM with ~3-fold lower affinity compared with the wild-type enzyme (Table 3), suggesting that Val^{63} does not exert its effect exclusively by reorienting Thr^{93} for SAM binding. Instead, Val^{63} must play a more general role in optimally organizing the SAM-binding pocket that underpins the unexpected contribution of the RAWV motif, and this residue in particular, to TlyA-SAM binding affinity.

Discussion

In the present study we have shown that the TlyA methyltransferase contains a class I Rossmann-like methyltransferase fold with a complete SAM-binding motif. However, the methyltransferase domain (amino acids 64–268) is not competent for SAM binding, and we identified an additional tetrapeptide motif RXWV as essential for SAM interaction with the methyltransferase domain. In particular, the final two amino acids, Trp^{62} and Val^{63}, have the most pronounced impacts on SAM affinity. Our structural studies illustrated a role for these amino acids in organizing the conserved GXGXG SAM-binding motif I when they are part of an extended α-helix of the TlyA methyltransferase domain.

Whether the RAWV motif is an important determinant of SAM binding when present in methyltransferases other than TlyA is an open question. To date, the structures of two other methyltransferases with similar sequences have been determined: Rm1 (RAW^{36}X) and human fibrillarin (RAW^{138}X). In fibrillarin, any contribution of the RAWX sequence to SAM binding would be indirect as the motif is not part of the first α-helix of the methyltransferase domain. However, in contrast to both fibrillarin and TlyA, the RAWX sequence of Rm1 is located in the α-helix, which precedes the first β-sheet of the methyltransferase domain, and the side chain of Ala^{137} defines one part of the SAM binding pocket. The potentially direct influence of the RAWX sequence on co-substrate binding by Rm1 is thus in contrast to the indirect influence we have identified in TlyA.

Our structures and functional data suggest that formation of an extended α1 helix by the RAWV motif, and in particular, the movement of Val^{63} is necessary to promote formation of an optimal SAM binding pocket in TlyA. However, the question remains why TlyA should require this additional motif outside the conserved SAM-binding fold. Characterized TlyA orthologs (Fig. 6E) have both high conservation of the Trp^{62}/Val^{63} dipeptide of the RXWV motif and an atypical SAM-binding motif, 9^6GA^7STG^94. Thus, one possibility is that TlyA may exploit the RAWV motif to compensate for the effects of a SAM binding motif I divergent from the canonical GXGXG sequence. In other homologs that also deviate from the GXGXG motif, such as Rm1 (59GAAPG^63) and RlmM (219GACPG223) (19, 21), the larger side chain is accommodated through its orientation away from the bound SAM molecule, allowing the backbone carboxyl group to be positioned within hydrogen bonding distance of the SAM 3′-OH. In contrast in TlyA, the bulkier Ser^{92} points toward the SAM binding pocket, potentially either creating or disrupting interactions important for SAM binding. Our structures suggest that the formation of an extended helical structure and repositioning of Val^{63} within α1 promotes movement of residues Ser^{92} and Thr^{93} toward the SAM 3′-OH and carboxylate, respectively. These changes induced by Val^{63} thus appear to optimally organize the SAM binding pocket and underpin the requirement of the RXWV motif in SAM binding by TlyA.

The RXWV motif connects the methyltransferase domain to the TlyA amino-terminal domain, which is predicted to adopt an S4 ribosomal protein binding fold (16) and likely plays a critical role in rRNA recognition (25). However, precisely how TlyA recognizes its two different nucleotide targets, 16S rRNA C1409 and 23S rRNA C1920, remains to be elucidated. Individual fully assembled 30S and 50S ribosomal subunits are the optimal TlyA substrates (25). Although both C1409 and C1920 reside within RNA helical structures with similar sequences, their overall structural context differs significantly. As such, TlyA must accurately recognize two structurally different substrates. C1409 is located in a region of h44, which packs near multiple 16S rRNA helices to form a complex RNA tertiary surface in the assembled 30S subunit, whereas C1920 is contained within a stem-loop (23S rRNA H69) that protrudes from the surface of the free 50S subunit (26). Could the structural plasticity we observe in the TlyA interdomain linker (RXWV tetrapeptide) also play a role in TlyA recognition of its two substrates? A mechanism of this type has been described for the tRNA G37 methyltransferase TrmD, in which an interdomain linker transitions from being structurally disordered to helical upon substrate binding (27). To begin exploring this idea, we manually appended the TlyA NTD homology model onto each of our two TlyA RAWV-CTD structures followed by geometry minimization to reveal the potential for the NTD to be oriented in two very different ways (Fig. 8). On the form 1 loop structure, the NTD is more loosely associated with the CTD and in a similar orientation to hemolysin structures and the full-length TlyA homology model, which is based on a hemolysin template (16). In contrast, modeled on the form 2 helix structure, the
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NTD is significantly repositioned and packs more closely against the TlyA CTD surface surrounding the SAM binding pocket, more consistent with our observation that the domain fragments from GluC-cleaved His-TlyA remain tightly associated. From this preliminary modeling, we speculate that TlyA may employ a mechanism in which specific recognition via the NTD of its two different substrates may be mediated via a common conformational change in the RXWV interdomain linker. In future studies to test this idea, it would also be interesting to determine whether GluC-cleaved or reconstituted NTD-RAWV-CTD TlyA proteins are able to efficiently methylate ribosomal subunits. Substitutions of some positively charged residues in the TlyA NTD are known to have differential impacts on the activity of TlyA against the 30S and 50S substrates (25). Thus, an appealing feature of a TlyA substrate recognition mechanism exploiting the structural plasticity of the RXWV tetrapeptide motif is that interactions made by different regions of the amino-terminal S4 domain, with either 30S or 50S, could lead to a common signal via the interdomain linker for correct substrate recognition and activation of methyltransferase activity.

In summary, the present study has revealed the unexpected but critical importance of the RXWV tetrapeptide motif in the TlyA interdomain linker for SAM binding. Our structures of the RAWV-CTD and modeling further suggest a potential role for the structural plasticity of the RXWV motif in regulating communication between the TlyA domains and in specific substrate recognition. Further structural studies of TlyA and its complexes with both 30S and 50S subunits are next needed to fully understand the molecular details of specific substrate recognition and the role of the RXWV motif in the activity of this important antibiotic resistance-associated enzyme.

Experimental Procedures

TlyA Construct Design and Site-directed Mutagenesis—An E. coli codon optimized gene encoding TlyA (Rv1694; UniProt P9W[63]) from Mtb (strain ATCC 25618/H37Rv) was obtained by chemical synthesis (GeneArt) and subcloned into a modified pET44 plasmid (28) for expression of protein with a thrombin-cleavable aminoterminal hexahistidine tag (His-TlyA). Screening for proteolytic fragments of His-TlyA suitable for structural studies with the Proti-Ace kit (Hampton Research) identified the endopeptidase GluC (Staphylococcus aureus Protease V8) as producing two stable domain fragments, and this observation was used as a guide to produce additional domain constructs (see “Results” for details). Plasmids encoding His-CTD (amino acids 64–268), His-RAWV-CTD (amino acids 60–268), and full-length His-TlyA with single amino acid substitutions were generated using a whole plasmid PCR protocol (29). Constructs for expression of amino-terminal domain proteins SUMO-NTD (amino acids 1–59) and SUMO-NTDRAWV (amino acids 1–63) were generated by PCR amplification of the corresponding coding region in pET44-His-TlyA using primers containing Bsal and XbaI sites for ligation of the amplicon into the pE-SUMOpro vector (LifeSensors).

TlyA Protein Expression and Purification—E. coli BL21 (DE3) cells transformed with protein-encoding plasmid were grown at 37 °C in terrific broth supplemented with ampicillin (100 μg/ml) to mid-log phase (A600 = ~0.4–0.6), induced with 0.5 mM isopropyl β-d-1-thiogalactopyranoside, and grown for an additional 3–4 h. Following harvesting by centrifugation, the cells were resuspended in 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole and lysed by sonication. The resulting soluble fraction was applied to a HisTrap HP column (GE Healthcare) equilibrated in the same buffer. The column was washed with 10 column volumes of 50 mM sodium phosphate (pH 8.0) buffer containing 300 mM NaCl and 20 mM imidazole and subsequently eluted with the same buffer, but containing 250 mM imidazole. Further purification was accomplished using a Superdex 75 16/60 gel filtration column (GE Healthcare) equilibrated in gel filtration buffer (20 mM Tris, pH 8.0, 10 mM magnesium acetate, 250 mM ammonium chloride, 6 mM β-mercaptoethanol, and 10% glycerol). All domain proteins and sequence variants were expressed and purified in the same way as full-length His-TlyA.

For removal of the amino-terminal hexahistidine tag, protein was mixed overnight with thrombin (Sigma-Aldrich; 5 units/1 mg of fusion protein) and passed over tandem HiTrap benzamidine FF and HisTrap HP columns (GE Healthcare). To remove the His-SUMO tag, His-SUMO-NTD and His-SUMO-NTDRAWV were incubated with Ulp and passed over a HisTrap HP column. After tag cleavage and initial purification step, cleaved TlyA or TlyA domains were concentrated and purified in a second gel filtration chromatography step (as described above).

CD Spectroscopy—CD spectroscopy was performed for full-length wild-type His-TlyA and variants with single amino acid substitutions in the RAWV tetrapeptide sequence on a Jasco J810 spectropolarimeter using solution conditions and instrument settings as described previously (30). Spectra (260–190 nm) were collected at 20 °C. Averaging and background correction were performed using the Spectra Manager software provided with the instrument and analysis of TlyA secondary structure was accomplished using the CDSSTR deconvolution algorithm via Dichroweb (31).

RT Analysis of 16S and 23S rRNA Methylation—Methylation of 30S and 50S was determined using RT assays with E. coli MRE600 30S and 50S subunits purified as described previously (25, 32). In brief, E. coli cells were lysed using a French press, and the 50S and 30S ribosomal subunits fractionated by sucrose gradient centrifugation and individually isolated by pelleting of
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the respective fractions. For the methylation assay, 200 pmol of purified His-TlyA was incubated for 1 h at 37 °C with 100 pmol of ribosome subunit (30S or 50S) in the presence of 1 mM SAM in 10 mM HEPES-KOH buffer (pH 7.5) containing 10 mM MgCl₂, 50 mM NH₄Cl, and 5 mM β-mercaptoethanol. The reaction was terminated by phenol/chloroform extraction followed by ethanol precipitation to recover 16S or 23S RNA from the 30S or 50S ribosomal subunits, respectively. The reaction products were analyzed by RT using 3²P-labeled DNA primers complementary to E. coli 16S rRNA nucleotides 1457–1473 (5’-CAAGGTGAACCC-3’) and 23S rRNA nucleotides 1964–1980 (5’-CATTAGCCATCTGTC-3’). The 2’-O-ribose methylation on C1409 and C1920 was observed with low dGTP concentration (0.5 mM) in the presence of 75 μM each of dATP, dTTP, and dCTP. Extension products were run on 10% PAGE-urea gels and visualized using Typhoon Trio phosphor-imaging system (GE Healthcare).

Protein Crystallization and Structure Determination—His-CTD (6 mg/ml) was crystallized at 20 °C in 0.2 M HEPES (pH 7.5) and 2.5 mM NaCl using the vapor diffusion method. His-RAWVCTD (8 mg/ml) was crystallized in the same way but using 8% tacsimate (pH 8.0) and 20% PEG 3350 (form 1 loop) or 0.3 M HEPES (pH 7.5) and 2.8 mM NaCl (form 2 helix). The crystals were cryo-protected with reservoir solution supplemented with 20% glycerol and flash frozen by plunging in liquid nitrogen. The data were collected at the Southeast Regional Collaborative Access Team ID-22 beamline at the Advanced Photon Source at the Argonne National Laboratory. The initial structure of His-CTD was solved by molecular replacement using a homology model of TlyA amino acids 59–265 based on the structure of His-CTD (Ala61 with Trp62 of form 1 loop, Glu59 with Arg of form 2 helix CTD). Each model of the TlyA (NTD homology model-CTD structure)-SAM complex was then regularized using geometry minimization in Phenix (35).

Author Contributions—G. L. C. conceived and coordinated the study. M. A. W. and E. G. K. designed, performed, and analyzed the results of all experiments. E. K. C. contributed to the performance and analysis of the experiments shown in Fig. 1. E. M. contributed to the performance and analysis of the experiments shown in Fig. 2. E. G. K., M. A. W., and G. L. C. wrote the manuscript, and all authors reviewed the results and approved the final submitted version.

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