RNA methylation by Radical SAM enzymes RlmN and Cfr proceeds via methylene transfer and hydride shift

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RlmN and Cfr are Radical SAM enzymes that modify a single adenosine nucleotide—A2503—in 23S ribosomal RNA. This nucleotide is positioned within the peptidyl transferase center of the ribosome, which is a target of numerous antibiotics. An unusual feature of these enzymes is their ability to carry out methylation of amidine carbons of the adenosine substrate. To gain insight into the mechanism of methylation catalyzed by RlmN and Cfr, deuterium labeling experiments were carried out. These experiments demonstrate that the newly introduced methyl group is assembled from an *S*-adenosyl-L-methionine (SAM)-derived methylene fragment and a hydrogen atom that had migrated from the substrate amidine carbon. Rather than activating the adenosine nucleotide of the substrate by hydrogen atom abstraction from an amidine carbon, the 5′-deoxyadenosyl radical abstracts hydrogen from the second equivalent of SAM to form the SAM-derived radical cation. This species, or its corresponding sulfur ylide, subsequently adds into the substrate, initiating hydride shift and *S*-adenosylhomocysteine elimination to complete the formation of the methyl group. These findings indicate that rather than acting as methyltransferases, RlmN and Cfr are methyl synthases. Together with the previously described 5′-deoxyadenosyl and 3-amino-3-carboxypropyl radicals, these findings demonstrate that all three carbon atoms attached to the sulfonium center in SAM can serve as precursors to carbon-derived radicals in enzymatic reactions.

enzymatic methylation | RNA modification

To evade the action of antibiotics, pathogenic bacteria have evolved specific defense mechanisms, including modification of antibiotic targets (1). The recent identification of *cfr*, the chloramphenicol-florfenicol resistance gene in clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), is a particularly severe example of bacterial resistance caused by target modification. The acquisition of this gene renders five important classes of antibiotics ineffective in treating infections (2, 3), including the entirely synthetic oxazolidinone antibiotic linezolid, an important therapeutic option and often the last line of defense in the treatment of infections caused by MRSA (4, 5). The drug resistance enzyme Cfr and its closely related homolog RlmN are enzymes that modify the 23S component of the ribosomal RNA (2, 3, 6, 7). The substrate of both enzymes is a single adenosine nucleotide—A2503—positioned within the catalytically crucial peptidyl transferase center of the ribosome, a common binding site of numerous antibiotics (8–10). RlmN and Cfr modify the substrate adenosine by adding methyl groups to C2 and C8 amidine carbons, respectively (Fig. 1). Commonly, RNA methylation is achieved by addition of substrate nucleophile (either a heteroatom or an enzyme-bound enolate) into the electrophylic methyl group of *S*-adenosyl-L-methionine (SAM) (11–13). By their reactivity and electronic demands, RlmN and Cfr substrate sites are distinct from other known methylation substrates in RNA, implying a unique mechanism of methylation.

RlmN and Cfr belong to the Radical SAM superfamily of proteins as predicted by the presence of the characteristic CX_3CX_2C motifs in their primary sequences (2). A common catalytic intermediate in the reactions catalyzed by Radical SAM enzymes is a

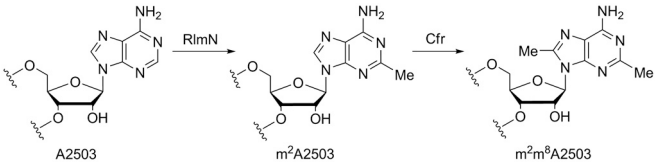

Fig. 1. Ribosomal RNA methylation by RlmN and Cfr.

5′-deoxyadenosyl radical (5′-dA·), generated by iron-sulfur cluster-mediated reductive cleavage of SAM (14, 15). This potent oxidant typically starts the enzymatic reaction by abstracting the hydrogen atom from the substrate, initiating a subsequent enzyme-specific transformation (16). Alternatively, 5′-dA· can also abstract a hydrogen atom from the enzyme which performs catalysis (17–19). In our previous work, we have demonstrated that both RlmN and Cfr have spectral features characteristic of Radical SAM enzymes (20). Furthermore, we showed that both enzymes are active only when assayed under strictly anaerobic conditions and in the presence of a reductant, observations consistent with membership in the Radical SAM superfamily. Additional evidence that these enzymes follow a Radical SAM pathway was obtained by detecting the formation of the characteristic by-products methionine and 5′-deoxyadenosine (5′-dA) that accompany the formation of methylated nucleotides implying the intermediacy of 5′-dA·. We also observed the transfer of radioactivity from [*methyl*-$^3H_3$]-SAM into the RNA product and identified *S*-adenosyl-L-homocysteine (SAH) as another reaction by-product. These observations led to the proposal that, in analogy to other Radical SAM enzymes, RlmN and Cfr could activate the adenosine substrate via hydrogen atom abstraction from the amidine carbons (20). The resulting radical would then undergo methylation by a second molecule of SAM. However, this route would require the homolytic cleavage of a C–H bond from aromatic carbon atoms in the substrate and the intermediacy of an energetically unfavorable σ-radical, questioning the feasibility of direct substrate activation. To further investigate the mechanism of methylation catalyzed by RlmN and Cfr, we set out to determine the source of the hydrogen atom abstracted by 5′-dA·.

Results and Discussion

To test the possibility that 5′-dA· activates the substrate by hydrogen atom abstraction from the RNA substrate, a substrate containing deuterium at the C2 carbon of adenosine was prepared and used in the reaction with the C2-modifying enzyme RlmN. If A2503 were indeed activated for methylation by hydrogen

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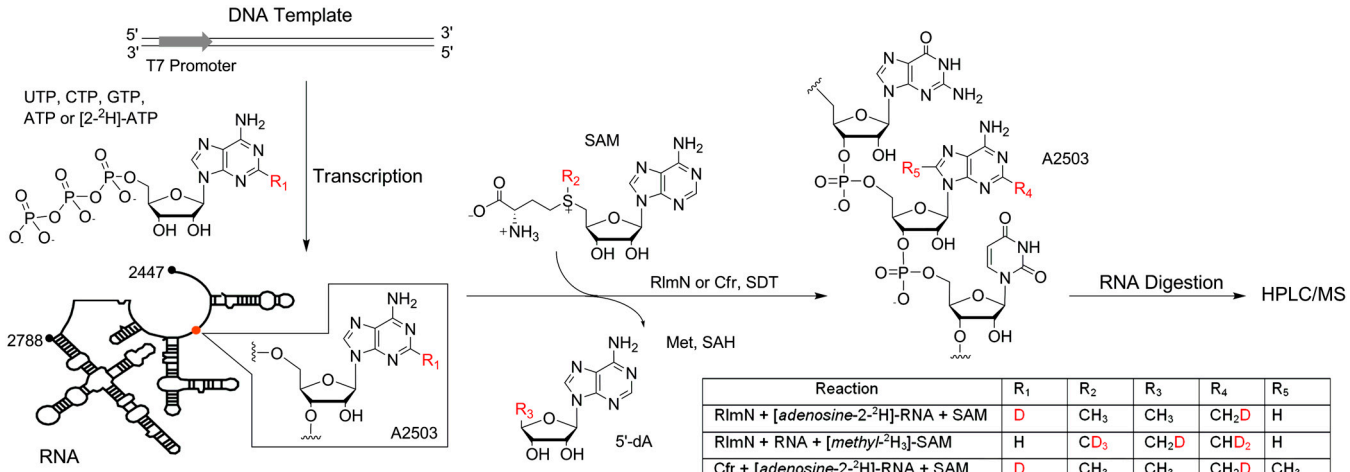
atom abstraction from its C2 carbon, reaction with deuterium-labeled substrate would form [5′-^{2}H]-dA. The deuterium-labeled substrate used in these experiments was an RNA fragment encompassing nucleotides 2447–2788 of 23S rRNA, prepared by in vitro transcription using [2-^{2}H]-ATP as a source of adenosine (Fig. 2), resulting in substitution of all adenosine nucleotides with [2-^{2}H]-A. This RNA fragment is known to undergo methylation by both RlmN and Cfr (Fig. S1). The deuterium-labeled RNA was incubated with RlmN in the presence of SAM, sodium dithionite (SDT), dithiothreitol (DTT), and magnesium chloride, and the reaction mixture subsequently subjected to enzymatic RNA digestion to yield single nucleosides (Fig. 2). The obtained 2-methyladenosine (m^{2}A) product and 5′-dA were isolated from the reaction by reversed-phase HPLC, and their masses determined by mass spectrometry. Analysis of mass spectra established that 5′-dA did not contain deuterium (Fig. 3*A*, $[M + H]^+ = 252$, identical to that of 5′-dA from the control reaction performed with unlabeled 2447–2788 rRNA fragment, Fig. S2*A*), indicating that the intermediate 5′-dA· does not directly activate the substrate. In contrast, analysis of the mass of the C2-methylated adenosine product indicated that the deuterium from the substrate was retained in the product ($[M + H]^+ = 283$, one mass unit higher than that of m^{2}A isolated from the control reaction with unlabeled 2447–2788 rRNA fragment, Fig. S2*A*). To determine the site of deuterium incorporation, this product was further analyzed by tandem mass spectrometry, establishing that the deuterium atom resides in the newly incorporated methyl group (Fig. S3).

To further probe this unusual labeling pattern, RlmN was incubated with unlabeled RNA substrate (oligonucleotides 2447–2788) but in the presence of SAM containing a trideuterated methyl group ([*methyl*-^{2}H$_3$]-SAM), and the reaction products analyzed as described above (Fig. 3*B*). The mass of C2 methylated adenosine ($[M + H]^+ = 284$ Da) showed incorporation of two deuteriums into the product, while the 5′-dA isolated from this reaction had a $[M + H]^+$ mass of 253 Da, consistent with incorporation of one deuterium. Together, these findings confirm that 5′-dA· does not abstract a hydrogen atom from the RNA substrate. Rather, isotope incorporation pattern suggests that abstraction occurs from the SAM and that the remaining two hydrogens from the methyl group of SAM are incorporated into the product. The observed minor amounts of unlabeled m^{2}A ($m/z = 282$) and 5′-dA ($m/z = 252$) could be attributed to the reaction with unlabeled SAM that copurified with RlmN. Although our improved purification protocol significantly decreases formation of these products, small amounts of unlabeled products persisted in the reactions even when performed with RlmN that had been preincubated with 20-fold excess of [*methyl*-^{2}H$_3$]-SAM. Additionally, unlabeled 5′-dA could also arise from the abortive cleavage of SAM that is uncoupled from product formation, as evidenced in other Radical SAM enzymes (21–23).

We next investigated whether the Cfr-catalyzed methylation shows an analogous deuterium incorporation pattern. Because Cfr methylates both the C8 and C2 carbons of A2503 in 23S rRNA isolated from the *rlmN* knockout strain of *Escherichia coli* (20), we first confirmed that the in vitro transcribed 2447–2788 fragment of 23S rRNA undergoes double methylation by Cfr (Fig. S2*B*). Because deuterium at the C8 position of adenosine readily exchanges with the solvent (24), we could not use an adenosine substrate doubly labeled at C2 and C8. Instead, these experiments were carried out with the in vitro transcribed 2447–2788 RNA bearing deuterium at the C2 position of the adenosines. The incubation of this RNA with Cfr and unlabeled SAM resulted in the formation of unlabeled 5′-dA and monodeuterated m^{2}m^{8}A, as indicated by $[M + H]^+$ peaks at 252 Da and 297 Da, respectively (Fig. 3*C*). When the Cfr was incubated with [*methyl*-^{2}H$_3$]-SAM and unlabeled 2447–2788 RNA fragment, the obtained 5′-dA was monodeuterated, while the product m^{2}m^{8}A contained four deuteriums ($[M + H]^+$ of 253 and 300 Da, respectively) (Fig. 3*D*). As in the RlmN-catalyzed reaction, these observations indicated that 5′-dA· does not remove a hydrogen atom from the RNA substrate but rather from the cosubstrate SAM. Additionally, only two hydrogen atoms from the cosubstrate SAM are incorporated into the methyl group in the product, with the third hydrogen (or deuterium) atom coming from the carbon that undergoes methylation.

Together, these labeling experiments indicate that RlmN and Cfr use an unprecedented mechanism of methylation, tailored to the electronic properties of the adenosine substrate (Fig. 4). This unique reactivity is enabled by the ability of the enzymes to use SAM in two distinct roles: as a source of 5′-deoxyadenosyl radical and as a source of a methylene fragment incorporated into the newly formed methyl group in the product. Following the formation of 5′-dA· via reductive cleavage of SAM1, rather than carrying out the energetically challenging activation of the RNA substrate, this radical removes a hydrogen atom from SAM2. Energetically, the hydrogen atom abstraction from SAM2 is likely to be more favorable than the homolytic cleavage of amidine C–H bonds in the substrate adenosine [calculated bond



Reaction	R_1	R_2	R_3	R_4	R_5
RlmN + [*adenosine*-2-^{2}H]-RNA + SAM	D	CH_3	CH_3	CH_2D	H
RlmN + RNA + [*methyl*-^{2}H$_3$]-SAM	H	CD_3	CH_2D	CHD_2	H
Cfr + [*adenosine*-2-^{2}H]-RNA + SAM	D	CH_3	CH_3	CH_2D	CH_3
Cfr + RNA + [*methyl*-^{2}H$_3$]-SAM	H	CD_3	CH_2D	CHD_2	CHD_2

Fig. 2. Outline of deuterium incorporation experiments in RlmN- and Cfr-catalyzed rRNA methylation. The red dot indicates the position of A2503 in the secondary structure of 2447–2788 RNA fragment. SDT = sodium dithionite; Met = methionine; SAH = *S*-adenosyl-L-homocysteine.

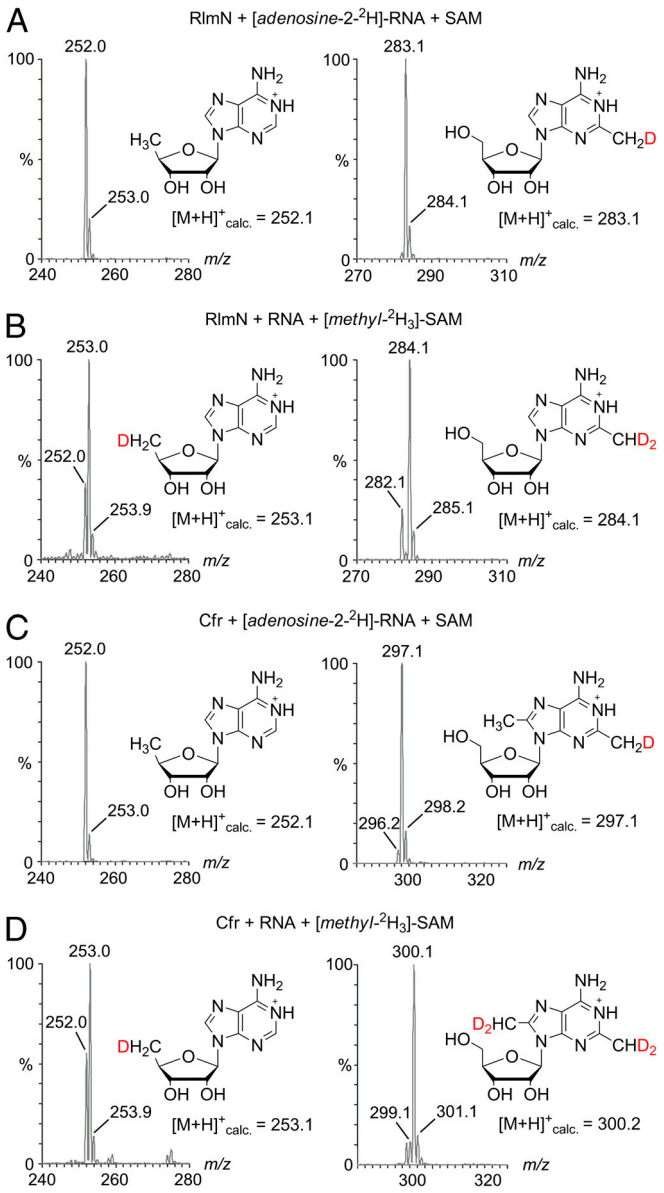


Fig. 3. Liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) analysis of deuterium incorporation in the RlmN and Cfr reaction products. (*A*) RlmN reaction using deuterium-labeled RNA and unlabeled SAM; (*B*) RlmN reaction using unlabeled RNA and deuterium-labeled SAM; (*C*) Cfr reaction using deuterium-labeled RNA and unlabeled SAM; (*D*) Cfr reaction using unlabeled RNA and deuterium-labeled SAM. The in vitro transcribed 2447–2788 RNA fragment was used as substrate in the reactions.

dissociation energies (BDEs) of 105 kcal · mol^{-1} for C2—H and 113 kcal · mol^{-1} for C8—H] (25, 26). To our knowledge, the precise value for BDE of C—H bonds in the methyl group of SAM has not been measured or calculated. In general, it is believed that α-heteroatom substituents stabilize methyl radicals through a three-electron interaction between the unpaired electron at the radical center and a lone pair on the heteroatom substituents (27). The magnitude of this mode of stabilization of a methyl radical by an α-sulfonium group, if any, is unclear. Calculations suggest that, at least in the case of $(CH_3)_2$—S^+—CH_3, the stability of the corresponding methyl radical is similar to that of the unsubstituted methyl radical (approximately 103–105 kcal · mol^{-1}) (28), a value still lower than the BDEs of amidine C—H bonds. Once formed, this SAM2-derived radical cation intermediate adds into the amidine carbon of the substrate. One electron reduction of the intermediate results in an adduct poised to undergo a hydride shift (Fig. 4). Hydride migration from the substrate carbon to the newly introduced methylene, enabled by the rearomatization of adenine and the leaving group ability of SAH, completes the formation of the methyl group. Precedent for radical addition to the amidine carbon of adenosine is found in the anaerobic photolysis of adenosylcobalamin which results in the addition of 5′-dA· to C8 of adenosine to form 8,5′-cyclic-5′-deoxyadenosine (29), as well as in the nonenzymatic methylcobalamin-mediated radical methylation of 2′-deoxyguanosine (30). In light of the unknown timing of the second electron transfer, the same outcome could be envisioned if the SAM2-derived radical cation is first reduced by one electron to a sulfur ylide, a proposed intermediate in cyclopropanation of polarized double bonds (31), prior to the addition into the amidine carbon of the RNA substrate.

In conclusion, Radical SAM enzymes RlmN and Cfr employ a unique mechanism of methylation, where the methyl group in the product originates from a SAM-derived methylene fragment and the hydrogen atom from the carbon that undergoes methylation. Aromatization of the product and expulsion of SAH provide the driving force for the observed hydride shift. This unprecedented reactivity is enabled by the ability of the enzymes to use SAM in two distinct roles: as a source of the canonical 5′-deoxyadenosyl radical and as a source of the methylene group. In light of these findings, RlmN and Cfr are more accurately described as methyl synthases rather than methyltransferases. In contrast to other known SAM-dependent methyltransferases, this mode of SAM-mediated methylation does not require an electron-rich substrate. The observed methylene transfer as a precursor to the methyl group assembly is reminiscent of thymidylate synthases (32–34). Despite this similarity, the mechanisms of incorporation of the methylene fragments are significantly different, as thymidylate synthases use N^5, N^{10}-methylene-tetrahydrofolate as a cosubstrate, and add the methylene group to nucleophilic carbon atoms, indicating that RlmN and Cfr use a unique mechanism to incorporate methyl groups. Together with observations that SAM is a precursor to 5′-dA· (14, 15) and the recently described 3-amino-3-carboxypropyl radical (35), our findings demonstrate that all three carbon atoms alpha to the sulfonium center in SAM can serve as precursors to carbon-derived radicals in enzymatic reactions.

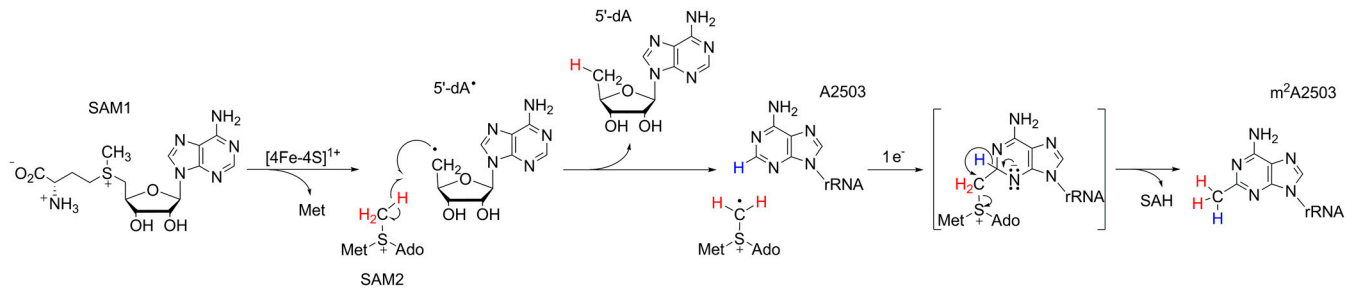


Fig. 4. Proposed mechanism of RNA methylation by the Radical SAM enzyme RlmN.

Materials and Methods

General. All anaerobic experiments were performed in the glovebox (MBraun) under an atmosphere consisting of 99.997% N_2 with less than 2 ppm O_2. All chemicals were analytical grade or the highest quality commercially available and were used without further purification unless otherwise noted. *S*-Adenosyl-L-[methyl-$^3H_3$]methionine (10 Ci/mmol) ([*methyl*-$^3H_3$]-SAM) was purchased from Perkin-Elmer. *S*-Adenosyl-L-[methyl-$^2H_3$]methionine (99.9% ^2H) ([*methyl*-$^2H_3$]-SAM) and adenosine 5′-triphosphate (2-^2H, 97%) ([2-^2H]-ATP) were purchased from C/D/N Isotopes and Cassia, respectively. Enzymes and biochemical reagents used in T7 RNA transcription were from New England Labs.

Preparation of RlmN and Cfr. RlmN and Cfr proteins were overproduced, purified, and reconstituted for their iron-sulfur clusters according to the previously reported procedure (20) with the following modification: after chemical reconstitution of the iron-sulfur cluster, the proteins were further purified by FPLC on a HiLoad 26/60 Superdex 200 Prep grade column (GE Healthcare Life Sciences) using 50 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl. The fractions containing protein were combined and concentrated to ~50 μM before being stored at −80 °C.

Preparation of Truncated rRNA Substrates. 23S rRNA fragment 2447–2788 was used as the substrate for RlmN- and Cfr- mediated methylation. The RNA substrate was generated by in vitro transcription using PCR product as a template. The primers used for PCR amplifications are F-2447: 5′-GAAATTAATACGACTCACTATAGGGATAACAGG-3′ and R-2788: 5′-GGAGAACTCATCTCGGGGCAAG-3′. The forward PCR primer included the T7 RNA polymerase promoter sequence TAATACGACTCACTATAGG. Segments of the 23S rRNA gene were amplified using the plasmid pKK3535 as a template. PCR products were purified using the Qiagen PCR purification kit and subsequently used for in vitro transcription.

For the in vitro transcription, 20 μg of DNA template were placed in 500 μL solution containing buffer (40 mM Tris-HCl, pH 7.9, 16 mM $MgCl_2$, 2 mM spermidine), 3 mM each rNTP, 20 mM DTT, 0.8 U/μL RNase inhibitor, and 3 U/μL T7 RNA polymerase. Reactions were incubated at 37 °C for 4 h, followed by addition of 40 units (U) of DNase RQ1 (Promega) and 30 min incubation at 37 °C. The transcribed RNA was purified by Qiagen RNeasy midi kit, and stored at −80 °C. The size and purity of RNA were verified by denaturing Urea-PAGE electrophoresis.

[2-^2H]adenosine-substituted 2447–2788 RNA fragment ([*adenosine*-2-^2H]-RNA) was prepared in the same manner except ATP was replaced by [2-^2H]-ATP in the T7 transcription reaction. The deuterium enrichment in [*adenosine*-2-^2H]-RNA was verified by mass spectral analysis of the nucleosides isolated from digested RNA.

Nonradioactive Methylation Reaction. RlmN/Cfr methylation reactions contained 10 mM $MgCl_2$, 2 mM DTT, 2 mM SDT, 3 μM (300 pmol) of RNA fragment 2447–2788, 30 μM SAM, and 4.5 μM RlmN (or 6 μM Cfr) in 1 mL of reaction buffer (Tris-HCl 50 mM, pH 8.0, NaCl 50 mM). All the reaction components were made anaerobic by repetition of vacuum and flushing with nitrogen prior to mixing in an MBraun glovebox. The reaction was initiated by addition of enzyme, and incubated at 37 °C for 60 min.

HPLC Separation of Reaction Products. The RlmN/Cfr methylation reaction mixture was subjected to stepwise RNA digestion by nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase (36). The digested samples were loaded onto a Luna analytical C18 column (10 μm, 4.6 mm× 250 mm) (Phenomenex), in a solvent system consisting of 40 mM ammonium acetate, pH 6.0 (A), and 40% aqueous acetonitrile (B). The nucleosides were eluted at a flow rate of 1 mL/min with a step gradient of 0% B (0–2 min), 0–25% B (2–27 min), and 25–60% B (27–37 min), and detected by their UV absorption at 256 nm. 2-methyladenosine (m^2A) from RlmN reaction, 2,8-dimethyladenosine (m^2m^8A) from Cfr reaction, and 5′-deoxyadenosine (5′-dA) from both reactions were collected at their known retention times (20). Adenosine was also collected and used as a standard in the subsequent Liquid chromatography/mass spectrometry (LC/MS) analysis. All collected products were lyophilized prior to MS analysis.

MS Analysis of Reaction Products. The lyophilized products were dissolved in water, and analyzed by a Waters Alliance HPLC, equipped with a Waters 2487 diode array detector and a Waters/Micromass ZQ single-quadruple mass spectrometer (Waters). The sample solutions were loaded onto an Xterra MS C18 column (3.5 μm, 2.1 × 50 mm) (Waters) and eluted at a flow rate of 0.2 mL/min with a 6-min gradient from 100% water to 20% aqueous acetonitrile (both containing 0.1% formic acid). Data processing was done with MassLynx software version 4.0. All the recorded mass spectra were calibrated externally using adenosine as a standard. The MS/MS analysis of the deuterated 2-methyladenosine isolated from the RlmN reaction that used [*adenosine*-2-^2H]-RNA and unlabeled SAM was performed at the University of California, San Francisco (UCSF) Mass Spectrometry Facility.

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