RlmN and Cfr are Radical SAM Enzymes Involved in Methylation of Ribosomal RNA

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Abstract: Posttranscriptional modifications of ribosomal RNA (rRNA) nucleotides are a common mechanism of modulating the ribosome's function and conferring bacterial resistance to ribosome-targeting antibiotics. One such modification is methylation of an adenosine nucleotide within the peptidyl transferase center of the ribosome mediated by the endogenous methyltransferase RlmN and its evolutionarily related resistance enzyme Cfr. These methyltransferases catalyze methyl transfer to aromatic carbon atoms of the adenosine within a complex 23S rRNA substrate to form the 2,8-dimethylated product. RlmN and Cfr are members of the Radical SAM superfamily and contain the characteristic cysteine-rich CX_3CX_2C motif. We demonstrate that both enzymes are capable of accommodating the requisite [4Fe-4S] cluster. *S*-Adenosylmethionine (SAM) is both the methyl donor and the source of a 5′-deoxyadenosyl radical, which activates the substrate for methylation. Detailed analyses of the rRNA requirements show that the enzymes can utilize protein-free 23S rRNA as a substrate, but not the fully assembled large ribosomal subunit, suggesting that the methylations take place during the assembly of the ribosome. The key recognition elements in the 23S rRNA are helices 90−92 and the adjacent single stranded RNA that encompasses A2503. To our knowledge, this study represents the first *in vitro* description of a methyl transfer catalyzed by a member of the Radical SAM superfamily, and it expands the catalytic repertoire of this diverse enzyme class. Furthermore, by providing information on both the timing of methylation and its substrate requirements, our findings have important implications for the functional consequences of Cfr-mediated modification of rRNA in the acquisition of antibiotic resistance.

Introduction

The bacterial ribosome, composed of ribosomal RNA (rRNA) and ribosomal proteins, is a target of many clinically useful antibiotics.[1,2] The rRNA is the key functional and structural component of the ribosome. Most of the protein synthesis inhibitors that interfere with the functions of the ribosome interact with rRNA and establish immediate contacts with nucleotides. One of the main mechanisms that renders bacterial pathogens resistant to ribosome-targeting drugs is the acquisition of rRNA methyltransferases which modify specific nucleotide residues located in antibiotic binding sites. The recently described Cfr methyltransferase belongs to this class of enzymes.[3,4] Evidence obtained in *in vivo* studies indicates that Cfr methylates the C8 atom of A2503 of 23S rRNA (*E. coli* numbering), located in the peptidyl transferase center of the

bacterial large ribosomal subunit.[5] Such modification renders cells resistant to several important classes of ribosomal antibiotics that act upon the peptidyl transferase center, including phenicols, pleuromutilins, streptogramins A, lincosamides, and the recently developed oxazolidinones.[3,4,6,7] The recent discovery of the *cfr* gene in a hospital isolate of *Staphylococcus aureus*, where it is present on mobile genetic elements,[8] raises the possibility of its rapid spread among human pathogens. The spread of *cfr*-based resistance may compromise several key antibiotics, including the oxazolidinone linezolid, which is often used as the last line of defense against multidrug-resistant bacterial infections.[9,10] The drug resistant methyltransferase Cfr is a close homologue of a methyltransferase RlmN.[11] RlmN is an endogenous cellular enzyme which modifies the same

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Scheme 1. Formation of a 5′-Deoxyadenosyl Radical via a Reductive Cleavage of S-Adenosyl-L-methionine (SAM)
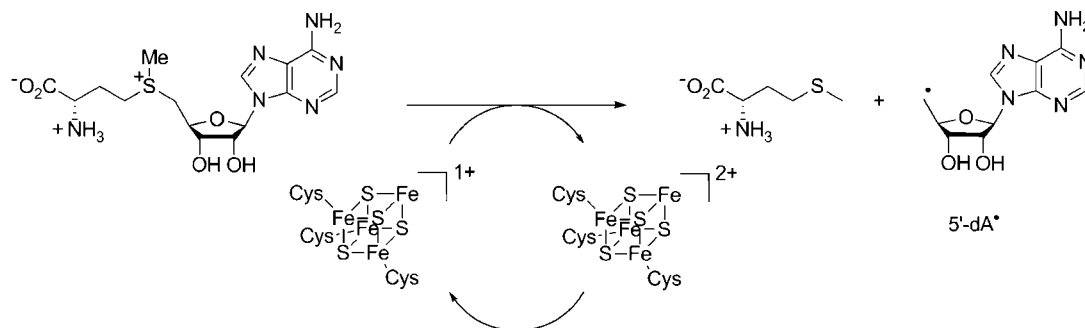
nucleotide (A2503), but at the C2 atom of the adenine base. Such 'native' posttranscriptional modifications of rRNA residues are presumed to play an important role in modulating and optimizing ribosomal function, although the precise roles of most modifications, including the RlmN-mediated C2 methylation of A2503, remain largely obscure.[11,12]

Nothing is known about the substrate requirement of these two enzymes. Yet knowledge of the substrate at which these enzymes act may have a critical impact on understanding the functional consequences of their actions. The rRNA-modifying enzymes can act at different steps of ribosome assembly. Some enzymes can modify fully assembled ribosomal subunits.[13,14] Others catalyze the rRNA modification only during ribosome assembly because the target site of their action becomes sequestered in the mature ribosome. Thus the extent of rRNA modification may be dramatically influenced by the mode of action of the enzyme and the rate of ribosome assembly. Modulating the degree of the rRNA modification could be used as an epigenetic mechanism of control of gene expression. On the other hand, the extent of antibiotic resistance conferred by rRNA modification should critically depend on the fraction of cellular ribosomes which has undergone modification by the resistance enzyme. Since the kinetics of ribosome assembly are affected by many antibiotics, understanding the nature of the substrate upon which the resistance rRNA methyltransferase acts may guide the antibiotic treatment regimens.

RlmN and Cfr represent an intriguing pair of evolutionarily related rRNA modifying enzymes with completely different functions: one (RlmN) is an endogenous enzyme used by the cell to refine functions of the ribosome in protein synthesis, and the other (Cfr) is an acquired methyltransferase that protects cells from the action of antibiotics. Besides the important biological functions of the two enzymes, the enzymatic mechanisms which are thought to be used by these methyltransferases squarely place RlmN and Cfr among the most interesting types of RNA-modifying enzymes. Most of the enzymes which methylate cellular RNA targets use S-adenosylmethionine (SAM) as a donor of electrophilic methyl groups to methylate nucleophilic oxygen atoms within the ribose moieties or nitrogen atoms within RNA nucleotide bases. RlmN and Cfr are unique as they perform enzymatically unprecedented methyl transfer to sp²-hybridized carbon atoms of an adenosine nucleotide.

RlmN and Cfr belong to the Radical SAM superfamily, which is characterized by the presence of the cysteine-rich CX_3CX_2C motif.[15−18] Enzymes belonging to the Radical SAM superfamily use this motif to ligate a unique four iron−four sulfur cluster (4Fe−4S), which, upon reduction to the +1 oxidation state, donates one electron to the bound SAM, leading to the formation of a 5′-deoxyadenosyl radical (5′-dA•) and methionine (Scheme 1). The resulting 5′-deoxyadenosyl radical is a potent oxidant which then initiates a radical transformation by performing hydrogen atom abstraction from its substrate. Subsequent catalytic steps, specific to the given Radical SAM enzyme, result in a variety of outcomes such as amine shifts (lysine aminomutases),[19−23] nucleotide reduction (ribonucleoside triphosphate reductase III),[24−26] sulfur insertion (biotin synthase, lipoyl synthase),[27−32] oxidative decarboxylation (coproporphyrinogen oxidase),[33−35] methylthiolation,[36−39] and complex rear-

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rangements,[40] among others. Overall, members of this superfamily participate in more than 40 distinct biochemical transformations,[16] and the full catalogue of functions carried out by these enzymes is yet to be determined.

Despite the importance of RlmN in modulating protein synthesis and Cfr in the generation of multiantibiotic resistant phenotypes, no biochemical studies on the *in vitro* activity of Radical SAM methyltransferases have been reported to date. Purification of these enzymes in their active forms and elucidation of their catalytic mechanism will provide critical insight into the functional principles of Radical SAM methyltransferases and may pave the way for combating Cfr-based antibiotic resistance. Understanding the substrate requirements of RlmN and Cfr may further lead to detailed structural analyses of methyltransferase-RNA recognition features.

Herein, we describe the *in vitro* reconstitution of the methyltransferase activity of RlmN and Cfr and propose a mechanism for the highly chemically challenging C−H to C−C bond conversion. To our knowledge, this study is the first *in vitro* description of a methyl transfer reaction to a carbon center catalyzed by a Radical SAM enzyme and adds a new function to the catalytic repertoire of these enzymes.

Materials and Methods

General. All anaerobic experiments were performed in the glovebox (MBraun, Stratham, NH) 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-^{3}H]methionine and [2,8-^{3}H]ATP were purchased from Perkin-Elmer (Waltham, MA). Culture medium ingredients were purchased from Difco (Detroit, MI). DNA minipreps were performed using the Wizard DNA purification kit from Promega (Madison, WI). All electrophoresis materials were purchased from Bio-Rad Lab (Hercules, CA). Enzymes used in plasmid construction and characterization were from Fermentas (Glen Burnie, MD).

Construction of the Expression Plasmids for RlmN and Cfr. The *rlmN* gene was PCR-amplified from genomic DNA of the *E. coli* strain K12 using primers RlmN Cloning 1 and RlmN Cloning 2 (Table S1, Supporting Information), which included the NdeI and XhoI restriction sites. The PCR product was purified, digested with NdeI and XhoI, and cloned into the corresponding sites of the vector pET-21a (Novagen, Madison, WI). The RlmN protein encoded in the resulting construct, pET21a-*rlmN*, is C-terminally His$_6$-tagged.

The *cfr* gene was PCR-amplified from the previously constructed plasmid pMS2,[7] using primers Cfr Cloning 1 and Cfr Cloning 2 (Table S1) which carried the Bpu1102I and NdeI sites. The PCR product was initially cloned into the pGEM-T vector (Promega). Once the *cfr*-positive clone was identified and the cloned gene sequenced, *cfr* was excised using NdeI and Bpu1102I restriction enzymes and cloned into the corresponding sites of the pET-15b vector (Novagen). A spontaneous mutation in the *cfr* stop codon

that was incidentally generated during the cloning procedure was corrected by site-directed mutagenesis using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The Cfr protein expressed from the resulting construct, pET15b-*cfr*, is N-terminally His$_6$-tagged.

Expression, Purification, and Reconstitution of RlmN and Cfr. A 5 mL culture of *E. coli* BL21 (DE3)/pET21a-*rlmN* or pET15b-*cfr* was grown overnight at 37 °C in Luria-Bertani (LB) medium containing ampicillin (100 μg/mL) and was used to inoculate 2 L of the same medium. When OD$_{600}$ reached 0.4−0.6, the incubation temperature was lowered to 18 °C and isopropyl-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.2 mM to induce gene expression. FeCl$_3$ was also added to a final concentration of 0.2 mM to provide a sufficient amount of iron for iron−sulfur cluster assembly. After incubation for an additional 24 h at 18 °C, cells were harvested by centrifugation (5000 × *g*, 10 min) at 4 °C and stored at −80 °C. The typical yield was 5 g of wet cells per liter of culture. Subsequent purification was carried out at 4 °C, and all buffers were degassed and saturated with nitrogen before use in an anaerobic glovebox.

Thawed cell pellets (∼10 g) were resuspended in 50 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 300 mM NaCl) and subjected to 10 × 30 s ultrasonic bursts (50% cycle, power level 5), with a 1 min cooling interval between each blast. Cell debris was removed by centrifugation (30 000 × *g*, 30 min), and the supernatant was mixed by slow agitation with 10 mL of Ni-NTA resin (Qiagen, Valencia, CA) for 1 h at 4 °C. The slurry was poured into a column and subsequently washed with lysis buffer supplemented with 20 mM and then 40 mM imidazole (50 mL each). The brownish protein was eluted with elution buffer (50 mM Tris-HCl, 10% glycerol, 300 mM NaCl, 250 mM imidazole, pH 8.0).

The brownish fractions that contained the desired proteins, as indicated by SDS-PAGE, were separately pooled (∼20 mL). The [4Fe−4S] cluster was further reconstituted by treating the fractions with 350 μM DTT, 140 μM cysteine, and 150 μM Na$_2$S. While the mixture was gently stirred, FeCl$_3$ was slowly added to a final concentration of 450 μM. The stirring was continued for another 2 h at room temperature, and the proteins were subsequently concentrated in an Amicon concentrator with YM-10 membrane (Millipore, Billerica, MA) to less than 1 mL prior to loading on a 30 mL P6 desalting column (Bio-Rad). The reconstituted proteins were eluted from the column using 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl and 10% glycerol. The collected proteins were further purified by FPLC on a 5 mL Hi-Trap Q HP column (GE Healthcare Life Sciences) using buffer A (50 mM Tris-HCl, pH 8.0, 50 mM NaCl) and buffer B (50 mM Tris-HCl, pH 8.0, 1 M NaCl). The FPLC buffers for Cfr purification also contained 10% glycerol. The column was run with 100% buffer A for 5 mL, followed by a linear gradient of 0 to 40% B in 20 mL. The flow rate was 1.5 mL/min, and the detector was set to 280 and 410 nm. The eluted proteins were diluted with FPLC buffer A and concentrated in an Amicon concentrator prior to storage at −80 °C. The concentrations of the as-purified proteins were determined by the method of Bradford using bovine serum albumin as the standard.[41] The relative molecular weight and purity of enzyme samples were determined using SDS-PAGE as described by Laemmli.[42] The average protein yield was ∼1.2 mg/L for RlmN and ∼0.3 mg/L for Cfr.

Preparation of RlmN and Cfr Mutants. Mutagenesis of conserved cysteine residues in the CxxxCxxC motif of RlmN and Cfr to alanines was carried out using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The oligonucleotides used for mutagenesis were purchased from Bioneer Inc. (Alameda, CA) (Table S2). The constructed mutant plasmids were amplified in *E. coli* TOP10 strain and purified with the Qiaprep Spin Miniprep Kit

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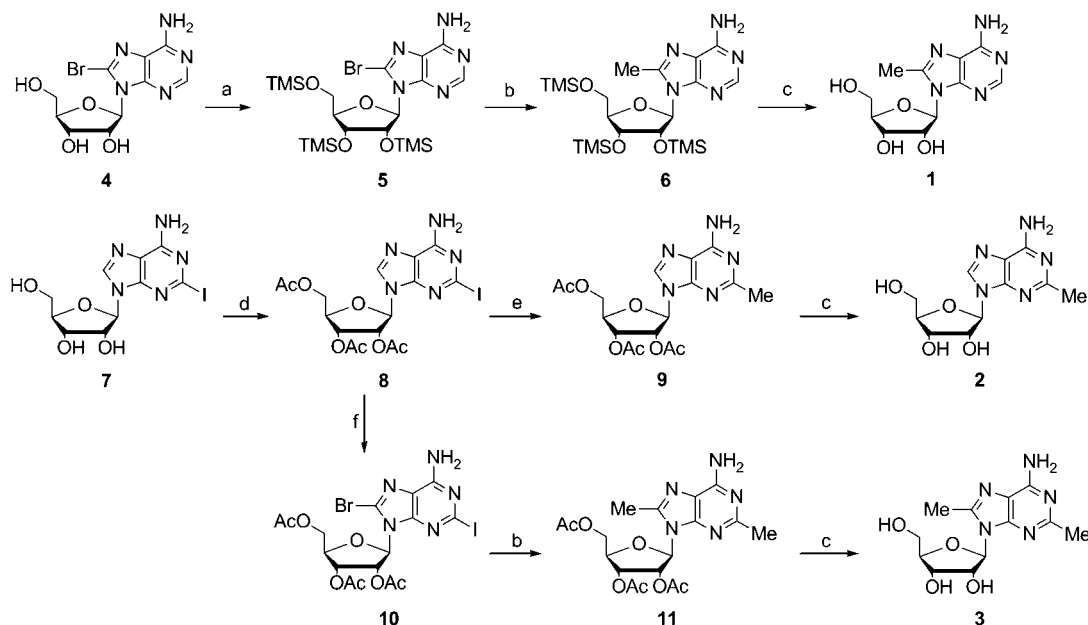
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Scheme 2. Chemical Synthesis of Methyladenosine Standards[a]

[a] Reagents and conditions: (a) HMDS, dioxane, reflux, 8 h; (b) NMP, Pd(Ph$_3$)$_4$, Me$_4$Sn, 120 °C, 20 h; (c) K$_2$CO$_3$, MeOH, 23 °C, 12 h; (d) Ac$_2$O, Et$_3$N, 4-DMAP, MeCN, 23 °C, 1 h; (e) NMP, Pd(Ph$_3$)$_4$, Me$_4$Sn, 110 °C, 12 h; (f) Br$_2$, 10% NaH$_2$PO$_4$, dioxane, 23 °C, 12 h.

(Qiagen) and subsequently verified by DNA sequencing (Elim Biopharmaceuticals, Hayward, CA). The mutant plasmids were then used to transform *E. coli* BL21(DE3) cells (Novagen) which were used for protein expression. The RlmN/Cfr mutants were expressed, purified, and subjected to iron–sulfur cluster reconstitution in the same manner as that for the wild type enzymes, except that the final FPLC purification step was omitted due to the small quantities of proteins obtained.

Iron Content Determination.[43] For iron content analysis, 1 mL of RlmN or Cfr sample was mixed with 500 μL of reagent A (1:1 of 4.5% KMnO$_4$/1.2 N HCl) and incubated at 60 °C for 2 h. The sample was cooled, and 100 μL of reagent B (8.8 g of ascorbic acid, 9.7 g of ammonium acetate, 80 mg of ferrozine, 80 mg of neocuproine, and H$_2$O to 25 mL total volume) were added followed by vigorous vortexing. The absorption at 562 nm was recorded after 1 h of incubation at room temperature. The iron content was finally determined by comparing the reading to a standard curve.

Preparation of Truncated rRNA Substrates. 23S rRNA fragments that encompassed A2503 were generated by *in vitro* transcription using PCR products as templates. All forward PCR primers included the T7 RNA polymerase promoter sequence TAATACGACTCACTATAGG, followed by several nucleotides corresponding to specific segments of 23S rRNA. The primers used for PCR amplifications are listed in Table S3. Segments of the 23S rRNA gene were amplified using the plasmid pKK3535 as a template. PCR products were cleaned using the SV Wizard Kit (Promega) and subsequently used for *in vitro* transcription. For the shortest truncation, 2496–2507, the template for *in vitro* transcription was prepared by annealing 400 pmol each of primers 2496 Short and 2507 Short in 40 μL of water. The mixture of the two primers was incubated at 100 °C for 1 min and then allowed to cool down to room temperature over 20 min.

For the *in vitro* transcription, 10 μg of DNA template were placed in 100 μL of a solution containing buffer (40 mM Tris-HCl, pH 8.0, 22 mM MgCl$_2$, 5 mM DTT, 1 mM spermidine), 2.5 mM each rNTPs, 20 mM DTT, 0.2 U/μL RiboLock RNase inhibitor (Fermentas), and T7 RNA polymerase. Reactions were incubated at 37 °C for 3 h, until the reactions turned cloudy. 2 U/μg (20 μL) of RQ1 DNase (Promega) were added, and incubation at 37 °C

continued for 30 min. 102 μL of water were added to each reaction, followed by 22.6 μL of Stop Solution (5 M ammonium acetate, 100 mM EDTA). Once the solutions turned clear, 1 volume of isopropanol (for truncations longer than 86 nucleotides) or 2.5 volumes of ethanol (for truncations shorter than 86 nucleotides) were added, and the reactions were incubated at −20 °C for 20 min or at −80 °C overnight. Samples were spun at 4 °C for 20 min at 21 000 × g. Pellets were resuspended in 200 μL of 0.3 M sodium acetate, pH 5.5 and extracted with phenol, phenol/chloroform, and chloroform. RNA was precipitated with 3 volumes of ethanol overnight at −80 °C. The samples were pelleted, washed with 70% ethanol, resuspended in water, and stored at −80 °C.

Chemical Synthesis of Methyladenosine Standards. The overall strategy for preparing the methyladenosines **1**–**3** is depicted in Scheme 2. The synthesis of 8-methyladenosine (**1**) and 2-methyladenosine (**2**) followed previously published protocols.[44,45] 2,8-Dimethyladenosine (**3**) was prepared starting with 2′,3′,5′-tri-*O*-acetyl-8-bromo-2-iodoadenosine (**10**), obtained by a known protocol.[45] **10** (100 mg, 0.17 mmol) was dissolved in *N*-methyl-2-pyrrolidone (NMP, 1 mL), and Pd(Ph$_3$)$_4$ (20 mg, 17 μmol) and Me$_4$Sn (100 μL, 0.7 mmol) were added, all under the protection of an argon atmosphere. The mixture was heated at 120 °C, and the formation of 2′,3′,5′-tri-*O*-acetyl-2,8-dimethyladenosine (**11**) was continuously monitored by TLC (CH$_2$Cl$_2$–MeOH, 98:2). After 12 h, the mixture was cooled and the product, **11**, was extracted with EtOAc and purified by silica gel column chromatography. The acetyl protecting groups were removed by treatment with K$_2$CO$_3$ in MeOH, and the final product, **3**, was obtained in 65% yield after purification on a silica gel column. 2,8-Dimethyladenosine (**3**): ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.21 (2H, s, NH$_2$), 6.39 (1H, m, OH), 5.77 (1H, d, J = 7.1 Hz, H-1′), 5.41 (1H, d, J = 7.1 Hz, OH), 5.27 (1H, d, J = 4.0 Hz, OH), 4.81 (1H, m, H-2′), 4.18 (1H, m, H-3′), 4.06 (1H, br. s, H-4′), 3.74 (1H, m, H-5′), 3.60 (1H, m, H-5′), 2.54 (3H, s, CH$_3$), 2.39 (3H, s, CH$_3$). ESI MS *m/z* 296 (M + H)$^+$.

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Activity Assays for RlmN and Cfr. A typical activity assay for RlmN/Cfr contained 10 mM MgCl$_2$, 2 mM sodium dithionite (SDT), 1.5 μCi *S*-adenosyl-L-[methyl-^{3}H]methionine ([^{3}H-methyl]-SAM, 10.0 Ci/mmol), 1 μM RlmN (or 2 μM Cfr), and 0.2 μM (10 pmol) of purified rRNA in 50 μL of Tris-HCl buffer (100 mM, pH 8.0). All the reaction components were made anaerobic by bubbling or purging with argon prior to mixing in an MBraun glovebox. The reaction was initiated by addition of [^{3}H-methyl]-SAM, and after 30 min of incubation at 37 °C, the reaction mixture was transferred onto a 23 mm Whatman DE81 filter paper disk (GE Healthcare Life Sciences). The paper discs were thoroughly washed with a 5% trichloroacetic acid (TCA) solution by gentle swirling (3 × 5 min), dried in air, and placed inside scintillation vials. Scintillation fluid was added, and counts were taken in a Beckman-Coulter LS6500 multipurpose scintillation counter (Fullerton, CA).

HPLC Separation and Identification of Methylated Adenosines. The methylated rRNA from RlmN/Cfr assay mixtures was purified using the Qiagen RNeasy Mini Kit. Subsequently, the purified rRNA was enzymatically digested to mononucleosides using nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase.[46] The digested samples were loaded onto a Luna analytical C18 column (10 μm, 4.6 mm × 250 mm) (Phenomenex, Torrance, CA), 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 the retention times of enzymatic products were compared to those of synthetic standards.[47] The mononucleosides and the synthetic methyladenosines were detected by their UV absorption at 256 nm, while the ^{3}H-labeled methylated adenosines were detected by a Packard radiomatic 515TR flow scintillation analyzer (Perkin-Elmer).

HPLC Detection of Methionine Formation. The RlmN/Cfr assay mixtures were treated with OPA reagent (5 mM *ortho*-phthalaldehyde, 2% β-mercaptoethanol, 0.3% sodium borate, pH 10.5).[48] The derivatized samples were analyzed on a Phenomenex Luna analytical C18 column (10 μm, 4.6 mm × 250 mm) at a flow rate of 1 mL/min using a linear gradient of 30% to 80% methanol in 50 mM NaH$_2$PO$_4$, pH 5.1, over 40 min. An OPA derivative of commercial methionine was detected by monitoring the absorption at 338 nm, while ^{3}H-labeled methionine produced in reaction mixtures was detected by a Packard radiomatic 515TR flow scintillation analyzer.

Enzymatic Synthesis of [2,8-^{3}H-Adenosyl]-SAM. The *E. coli* strain DM22(pK8) that overproduces SAM synthetase was a generous gift from Dr. G. Douglas Markham, Fox Chase Cancer Center (Philadelphia, PA). SAM synthetase was prepared in the form of crude lysate of DM22(pK8) cells using the protocol of Broderick.[49] The ability of as-prepared SAM synthetase to produce SAM was initially confirmed using unlabeled ATP and methionine. To prepare the tritium-labeled [2,8-^{3}H-adenosyl]-SAM, a 100 μL reaction containing 50 mM KCl, 20 mM MgCl$_2$, 1 mM EDTA, 10 μM methionine, 0.1 unit inorganic pyrophosphatase, and 15 μCi [2,8-^{3}H]ATP (27.8 Ci/mmol) in 100 mM Tris-HCl pH 8.0 was initiated by the addition of 5 μL of SAM synthetase crude lysate. Following incubation, the ^{3}H-labeled SAM was bound to ion-exchange resin Dowex-50WX8 in the Na$^+$ form (Bio-Rad Lab); washed sequentially with H$_2$O, 0.1 M NaCl, and 1 M HCl; and eluted with 6 M HCl.[50] After removal of the solvent, the identity and purity of synthetic SAM were verified by coelution with commercial SAM on a Phenomenex Luna analytical C18 column

(10 μm, 4.6 mm × 250 mm) using a linear gradient (1 mL/min) from 85% eluant A (0.1 M NaH$_2$PO$_4$, 2% acetonitrile, 8 mM 1-heptanesulfonic acid, pH 2.65) to 100% eluant B (0.1 M NaH$_2$PO$_4$, 26% acetonitrile, 8 mM 1-heptanesulfonic acid, pH 3.25) over a 30 min span. The ^{3}H-labeled synthetic SAM was detected by a Packard radiomatic 515TR flow scintillation analyzer, while the coeluted commercial SAM was detected by monitoring the absorption at 256 nm.

HPLC Detection of 5′-Deoxyadenosine Formation. The RlmN/Cfr assays were performed using newly synthesized [2,8-^{3}H-adenosyl]-SAM. The reaction mixtures were analyzed on a Phenomenex Luna analytical C18 column (10 μm, 4.6 mm × 250 mm) using the conditions that were previously developed for the detection of methylated adenosines. The ^{3}H-labeled 5′-deoxyadenosine (5′-dA) produced in the reaction was detected by a Packard radiomatic 515TR flow scintillation analyzer, while the coeluted commercial 5′-dA was detected by monitoring the absorption at 256 nm.

HPLC Detection of *S*-Adenosylhomocysteine Formation. The reaction mixtures from the RlmN/Cfr assays using [2,8-^{3}H-adenosyl]-SAM were derivatized with OPA reagent and analyzed on a Phenomenex Luna analytical C18 column (10 μm, 4.6 mm × 250 mm) using the conditions that were previously developed for the detection of methionine. The ^{3}H-labeled *S*-adenosylhomocysteine (SAH) produced in the reaction was detected by a Packard radiomatic 515TR flow scintillation analyzer, while the coeluted OPA derivative of commercial SAH was detected by monitoring the absorption at 338 nm.

Results

Expression, Purification, and Reconstitution of RlmN and Cfr. The *rlmN* gene was amplified from *E. coli* genomic DNA and cloned into a pET-21a expression vector, and the resultant recombinant plasmid (pET21a-*rlmN*) was introduced into the *E. coli* BL21(DE3) strain, allowing for the production of C-terminally His$_6$-tagged RlmN. To prepare N-terminally His$_6$-tagged Cfr protein, the *cfr* gene was amplified from the plasmid pMS2,[7] cloned into a pET-15b expression vector, and used to transform *E. coli* BL21(DE3) cells. Protein expression from both constructs was induced as described in Materials and Methods, and both proteins were purified under anaerobic conditions by nickel affinity chromatography, resulting in the isolation of brownish proteins. For each of the proteins, the as-isolated protein fractions were treated with DTT (350 μM), cysteine (140 μM), Na$_2$S (150 μM), and FeCl$_3$ (450 μM) to allow for reconstitution of the iron−sulfur cluster. The reconstituted proteins were further purified by ion-exchange chromatography under anaerobic conditions, resulting in homogeneous dark brown proteins. The purified and reconstituted proteins migrated at approximately 43 kDa (RlmN) and 37 kDa (Cfr) on an SDS-PAGE gel (Figure 1A), in good agreement with the calculated molecular weight of the polypeptides (43.1 and 39.9 kDa, respectively). The protein concentration was determined by the method of Bradford, and the iron content by spectrophotometry after treatment with ferrozine. The UV/vis spectra, with absorbance maxima at approximately 410 nm, tailing to longer wavelengths, are consistent with the presence of an iron−sulfur cluster (Figure 1B, C).[39,51,52] These spectral characteristics are in agreement with the iron content determined for both proteins after reconstitution (RlmN: 3.98 ± 0.16; Cfr: 6.79 ± 0.42 mols of iron per mole of protein), sufficient to support formation of a [4Fe−4S] cluster.

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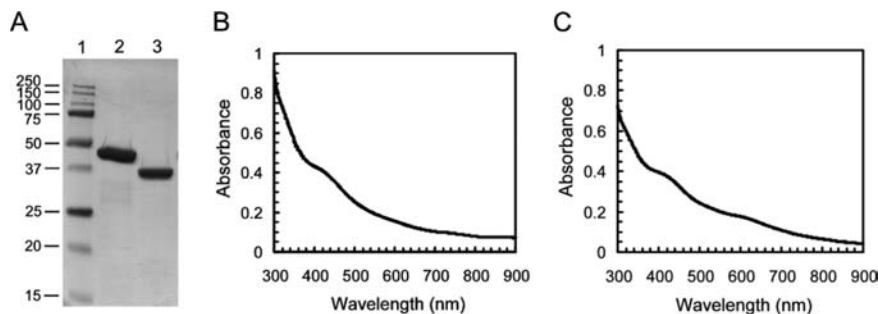
Figure 1. Gel analysis and spectral properties of methyltransferases. (A) SDS-PAGE of purified RlmN and Cfr: lane 1, protein markers; lane 2, RlmN; lane 3, Cfr. (B, C) UV−vis absorption spectra of fully reconstituted (B) RlmN, 48 μM and (C) Cfr, 55 μM. The spectra were recorded in 50 mM Tris-HCl buffer, pH 8.0.
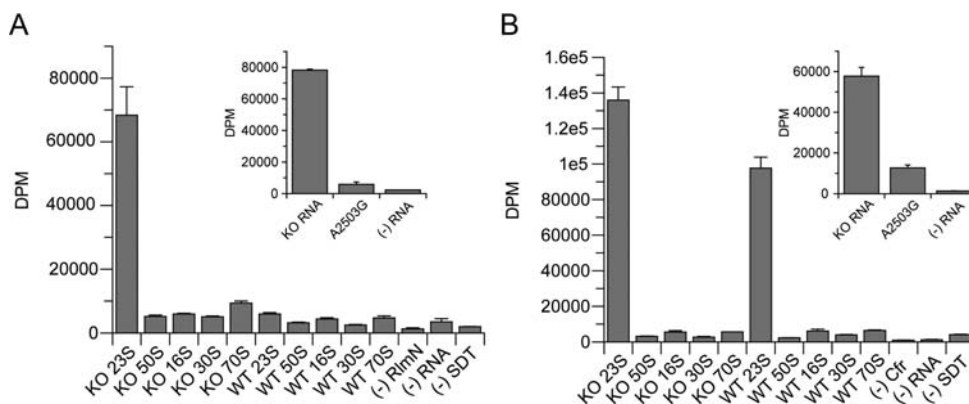
Figure 2. Activity of RlmN (A) and Cfr (B) toward ribosomes and their subunits. Candidate substrates isolated from the *rlmN* knockout strain of *E. coli* are prefaced with KO, while those isolated from the wild type strain are labeled as WT. SDT, sodium dithionite. Inserts show a substantial reduction of methyl transfer to rRNA isolated from the A2503G mutant strain.

**In Vitro Activity and rRNA Substrates of RlmN and Cfr.** To determine whether RlmN and Cfr display methyltransferase activity *in vitro*, a series of rRNAs were incubated with each of the methyltransferases. In a typical assay, the enzyme was incubated under anaerobic conditions with purified rRNA (Supporting Information) in the presence of sodium dithionite as a source of the electron required for the reduction of the [4Fe-4S] cluster to the +1 oxidation state and *S*-adenosyl-L-[methyl-^{3}H]methionine ([^{3}H-methyl]-SAM) as a methyl donor. The intact 70S *E. coli* ribosome, the large (50S) and small (30S) ribosomal subunits, and the rRNA components of both of these subunits, 23S rRNA (with 5S rRNA) and 16S rRNA, were tested as candidate substrates for RlmN and Cfr. These candidate substrates were prepared either from the wild type (WT) - *E. coli* or from the *rlmN* knockout strain. In the wild type 23S rRNA, A2503 is supposed to be C2 methylated by the action of endogenous RlmN, whereas A2503 remains unmodified in 23S rRNA from the *rlmN* knockout strain. Following the incubation with the methyltransferase and [^{3}H-methyl]-SAM, rRNA was recovered from the reaction and analyzed for the presence of tritium-derived radioactivity. These experiments established 23S rRNA from the *rlmN* knockout strain as the only substrate for RlmN, while both the wild type and *rlmN* knockout 23S rRNA were substrates for Cfr (Figure 2A, B). Our findings are in agreement with previous *in vivo* studies that pointed to 23S rRNA as the target of these enzymes.[4,5,11] Only protein-free rRNA, but not large ribosomal subunits or 70S ribosomes, could serve as RlmN or Cfr substrates (Figure 2A, B). The inability of Cfr or RlmN to modify these latter substrates is likely a consequence of steric hindrance precluding the access of the enzymes to A2503 in the context of the mature 50S

subunit. To better establish the specificity of both enzymes, we prepared rRNA with an A2503 to G mutation (Supporting Information). Neither Cfr nor RlmN could catalyze transfer of the label from [^{3}H-methyl]-SAM to this mutant rRNA (Figure 2, inserts), indicating that the activity of both methyltransferases is targeted toward A2503.

In 23S rRNA, A2503 is located in a single-stranded region flanked by helices 89 and 90−92 (Figure 3A−C). To further define rRNA substrate requirements for the RlmN- and Cfr-catalyzed methyl transfer, several fragments of 23S rRNA were prepared by *in vitro* transcription and tested in methylation assays with purified enzymes. 23S rRNA fragments encompassing parts of domain V (pos. 2018−2788, 2018−2625, and 2447−2625) were readily used by RlmN and Cfr as substrates for methylation (Figure 3D−F). Removal of a larger part of domain V did not prevent RlmN or Cfr from modifying their RNA target as long as the helix structure H90−H92, which includes the functionally important A-loop, was present in the substrate construct. The activity of both enzymes was somewhat attenuated with shorter constructs (Figure 3G−K), suggesting that some of the deleted rRNA regions may stabilize the conformation of the RNA substrate recognized by the methyltransferases. The construct that contained helix 89 but lacked helices 90−92 was inactive (Figure 3I), underscoring the importance of the H90−H92 segment as the RlmN and Cfr recognition element.

Establishing the Nature of Adenosine Modification by RlmN and Cfr. To establish the exact pattern of modification of the adenosine target of the Radical SAM methyltransferases, the *in vitro* methylated 23S rRNA was purified and enzymatically digested to single nucleosides. The resulting hydrolysate
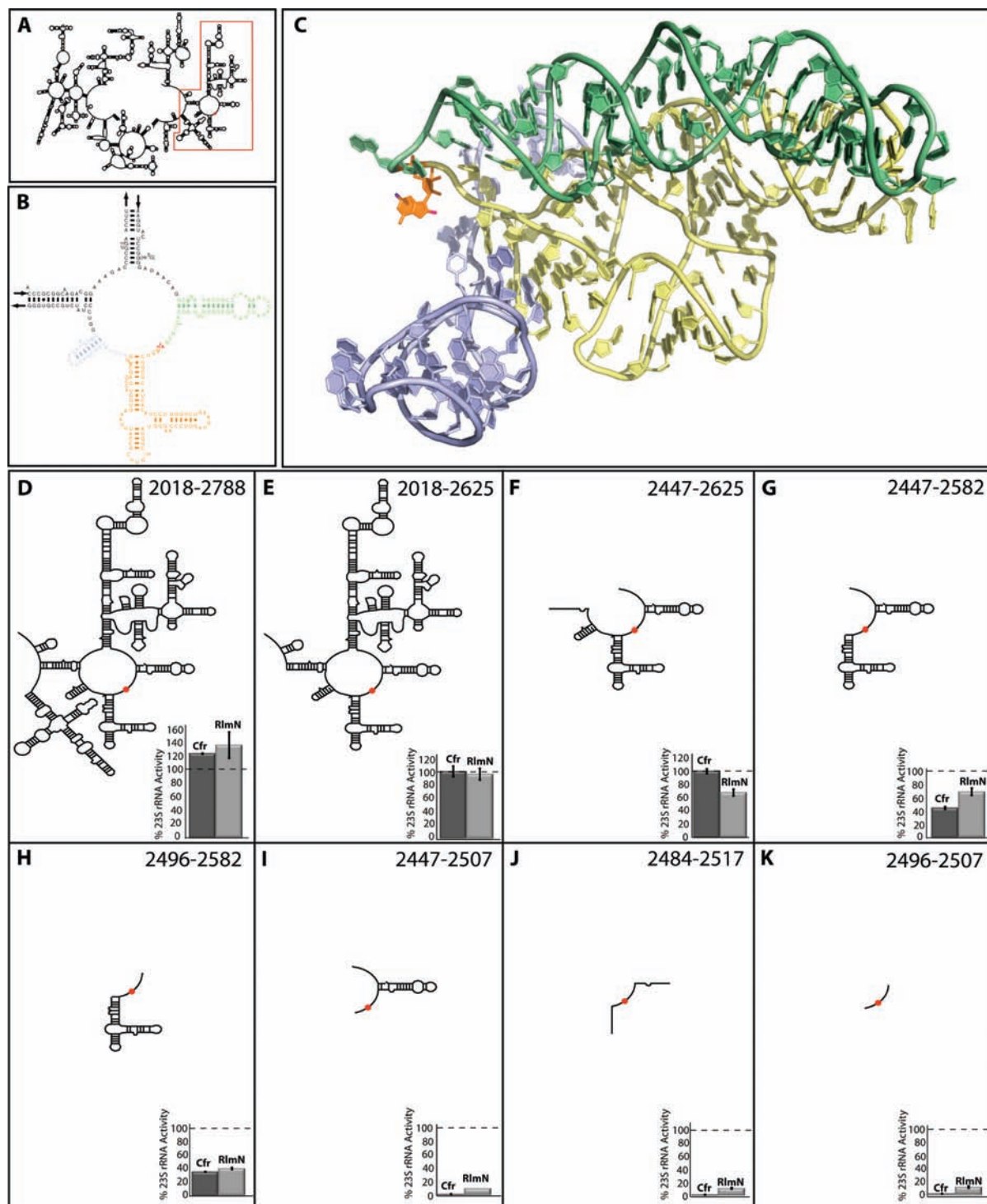
Figure 3. Activity of RlmN and Cfr toward truncated fragments of 23S rRNA. (A) Schematic representation of 23S rRNA secondary structure. The position of A2503 is shown as an orange dot, and the region of 23S rRNA used in the truncations below is boxed in red. (B) Close-up of the 23S secondary structure containing m²A2503, which is highlighted in orange. Helix 89 is pale green, helices 90−92 are yellow, and helix 93 is violet. (C) 3-D schematic of A2503 in relation to helix 89 (pale green), helices 90−92 (yellow), and helix 93 (violet). A2503 is shown in orange, with the methyl groups of C2 and C8 colored as dark purple and magenta, respectively. (D−K) Activities of Cfr (dark gray) and RlmN (light gray) for the specific truncations. The enzyme activities are graphed relative to the activity for full 23S rRNA as the substrate, with 100% delineated with a dashed line. The location of A2503 is again represented by an orange dot.

was analyzed by HPLC, and the retention time of the radiolabeled product was compared with synthetic standards of predicted methylated products, 2-methyladenosine (RlmN), 8-methyladenosine, and 2,8-dimethyladenosine (Cfr). The incubation of 23S rRNA isolated from the *rlmN* knockout strain with RlmN and the subsequent enzymatic digestion to nucleosides resulted in the formation of a radioactive product that coeluted with 2-methyladenosine (Figure 4A). As mentioned earlier, no modification of the wild type 23S rRNA with this enzyme was observed (Figure 4B). When Cfr-treated rRNA from the *rlmN* knockout strain was isolated and digested in the same manner, the appearance of two new products was noted:
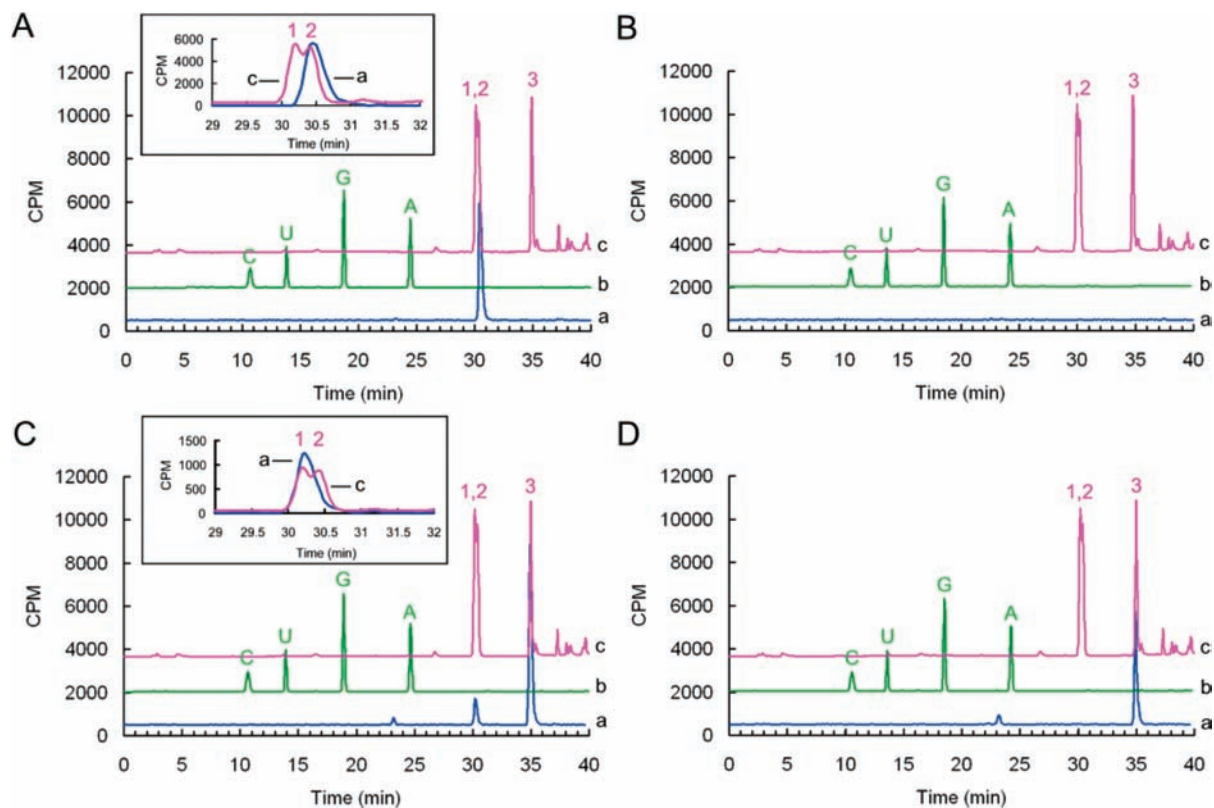
Figure 4. HPLC analysis of methylation products of RlmN and Cfr. (A) RlmN + KO total rRNA; (B) RlmN + WT total rRNA; (C) Cfr + KO total rRNA; (D) Cfr + WT total rRNA. (a) ^{3}H radioactive chromatogram of digested RNA; (b) UV−vis chromatogram of digested RNA at 256 nm; (c) UV−vis chromatogram of synthetic methyladenosine standards at 256 nm. 1. Me^{8}A; 2. Me^{2}A; 3. Me^{2}Me^{8}A. KO: rRNA isolated from *rlmN* knockout *E. coli* strain. WT: rRNA isolated from the wild type *E. coli*.
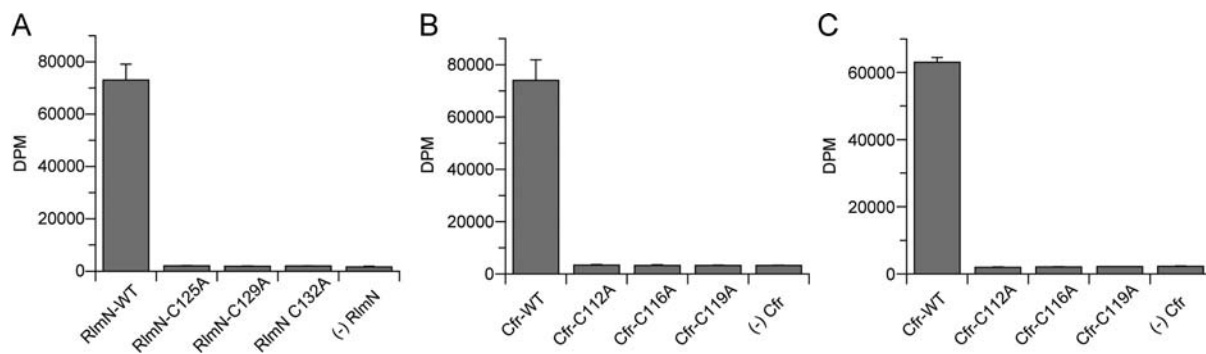
Figure 5. Activity of cysteine to alanine mutants of RlmN toward total rRNA from *rlmN* knockout *E. coli* strain (A), and Cfr mutants toward total rRNA from *rlmN* knockout (B) and wild type *E. coli* strains (C).

8-methyladenosine and 2,8-dimethyladenosine (Figure 4C). Incubation of the same methyltransferase with the wild type 23S rRNA, already modified at the C2 position by the endogenous RlmN, provided 2,8-dimethyladenosine as the sole radiolabeled product (Figure 4D). Our *in vitro* analysis is in agreement with the observed regioselectivity of methylation noted in *in vivo* studies and shows that the purified enzymes retain their native activities.[5,11]

Role of the Cysteine-Rich Motifs in RlmN and Cfr. The CX$_3$CX$_2$C motif, found in both of the methyltransferases, is a characteristic feature of Radical SAM enzymes where it ligates an iron−sulfur cluster. To investigate the importance of this motif in RlmN and Cfr catalysis, we prepared a series of mutants in which cysteines in the CX$_3$CX$_2$C motifs were individually replaced with alanines. The C125A, C129A, and C132A mutants of RlmN as well as the C112A, C116A, and C119A mutants of

Cfr were overproduced, purified, and reconstituted in the same manner as that for the wild type enzymes. All mutants provided soluble proteins (Figure S1), although a substantial decrease in stability as compared to the wild type enzymes was observed during their purification. None of the mutants were active toward their respective 23S rRNA substrates (Figure 5), strongly implicating a crucial role for the cysteine-rich motif and the associated iron−sulfur cluster in the methyl transfer reactions.

Methionine, 5′-deoxyadenosine (5′-dA), and *S*-adenosylhomocysteine (SAH) are SAM-derived byproducts of RlmN and Cfr-catalyzed methyl transfer. Despite the diverse overall outcomes, Radical SAM enzymes utilize a 5′-deoxyadenosyl radical, generated by the reductive cleavage of SAM, to initiate the enzymatic transformations that they catalyze. The formation of this reactive species is accompanied by the formation of methionine. Subsequent 5′-deoxyadenosyl radical-promoted
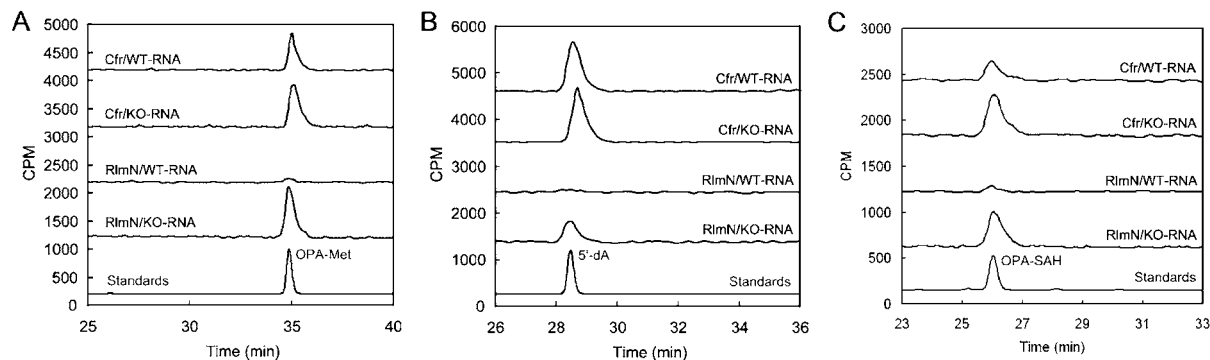
Figure 6. RlmN- and Cfr-catalyzed methylations are accompanied by reductive cleavage of SAM and *S*-adenosylhomocysteine formation. (A) Detection of methionine in the reaction of methyltransferases with [³H-methyl]-SAM. (B) Detection of 5′-deoxyadenosine production in the reaction of methyltransferases with [2,8-³H-adenosyl]-SAM. (C) Detection of *S*-adenosylhomocysteine production in the reaction of methyltransferases with [2,8-³H-adenosyl]-SAM.

hydrogen atom abstraction from the substrate generates 5′-deoxyadenosine. Therefore, the formation of methionine and 5′-deoxyadenosine is a mechanistic hallmark of the reductive cleavage of SAM. To investigate if RlmN- and Cfr-catalyzed methyl transfer is accompanied by the reductive cleavage of SAM, we analyzed reaction mixtures for the formation of methionine and 5′-deoxyadenosine. Following the enzymatic methylation of rRNA with [³H-methyl]-SAM, the reaction mixtures were treated with an *ortho*-phthalaldehyde (OPA) derivatization reagent, and the retention time of the products formed in the reaction mixture were compared with that of OPA-derivatized methionine (Figure 6A). A ³H-labeled peak at 35 min, which coeluted with the OPA-derivatized methionine standard, was detected in the reaction of RlmN with the *rlmN*-knockout RNA, as well as the reactions of Cfr with both the *rlmN*-knockout and the wild type RNA substrates. In contrast, only a trace amount of methionine was formed when RlmN was incubated with the wild type RNA substrate, where pre-existing modification of A2503 precludes the methyl transfer. No methionine production or its formation in only trace amounts was noted when the reactions were performed in the absence of sodium dithionite (Figure S2A) or when substrate or enzyme was omitted from the reaction (Figure S2B).

To determine if 5′-deoxyadenosine production accompanies methylation of the rRNA substrate, we prepared [2,8-³H-adenosyl]-SAM, a SAM analogue tritiated at the adenosine portion of the molecule. This compound was prepared via enzymatic synthesis from [2,8-³H]ATP, as described in Material and Methods. Incubation of RlmN with the *rlmN*-knockout 23S rRNA in the presence of [2,8-³H-adenosyl]-SAM resulted in the formation of 5′-deoxyadenosine (Figure 6B). No formation of this product was noted in the reaction of RlmN with the wild type rRNA. However, reaction of both rRNA substrates with Cfr and [2,8-³H-adenosyl]-SAM led to the formation of 5′-deoxyadenosine, the production of which is dependent on the presence of sodium dithionite (Figure S3A). Similarly, the omission of enzyme or substrate from the incubation mixtures led to the formation of only trace amounts or complete abrogation of the production of 5′-deoxyadenosine (Figure S3B). The formation of the trace amounts of methionine and 5′-deoxyadenosine in the control reactions is attributed to reductive cleavage of SAM uncoupled from the product formation. Together, the detection of methionine and 5′-deoxyadenosine support a radical-based mechanism for RlmN- and Cfr-catalyzed methylations, involving the reductive cleavage of SAM and subsequent hydrogen-atom abstraction from the substrate.

As noted above, transfer of the ³H-labeled methyl group from SAM to RNA clearly established that SAM is the methyl donor in the methylation reactions (Figure 2). These findings suggest formation of *S*-adenosylhomocysteine (SAH) as a byproduct of the methyl transfer.[53] Having synthesized [2,8-³H-adenosyl]-SAM, we were able to monitor SAH formation. Following incubation of RlmN and Cfr with both the wild type and the knockout rRNA, the reaction mixture was OPA-derivatized as described in Materials and Methods, and SAH formation was assessed by coelution of the OPA-derived reaction mixture with the authentic OPA-derivatized SAH standard (Figure 6C). SAH production was detected in the reaction of RlmN with the *rlmN*-knockout RNA and the reaction of Cfr with both the wild type and the knockout RNA substrates. Small amounts of SAH were noted in the reaction of RlmN with the wild type rRNA and are likely a consequence of SAM hydrolysis uncoupled to the product formation. No SAH formation was noted in any of the negative control reactions (Figure S4). Together, our data indicate that Radical SAM methyltransferases RlmN and Cfr utilize two SAM molecules per each introduced methyl mark: the first one to generate the 5′-deoxyadenosyl radical and initiate catalysis, and the second as the methyl donor.

To confirm that the formation of methionine, 5′-deoxyadenosine, and SAH is coupled to the methylation of the substrate, the formation of these SAM-derived byproducts was monitored over time and correlated to the time-dependent formation of the methylated product (Figure 7). For these studies we chose the RlmN-catalyzed methylation, as this enzyme introduces a single methyl mark. The incubation of the substrate with [³H-methyl]-SAM demonstrated that the product formation correlates well to the release of methionine (Figure 7A), providing strong evidence in support of the reductive cleavage of SAM as a productive pathway-related transformation. Furthermore, the time-dependent correlation between the formation of 5′-deoxyadenosine and SAH in the reaction with [2,8-³H-adenosyl]-SAM (Figure 7B) lends further support for the coupling of the homolytic cleavage of SAM (5′-deoxyadenosyl radical and 5′-deoxyadenosine formation; substrate activation) and substrate methylation (SAH formation). The absence of strict correlation between RNA methylation and formation of SAM-derived byproducts is likely a consequence of the abortive cleavage of SAM, evident in control experiments performed in the absence of the substrate (Figures S2 and S3, Supporting Information).

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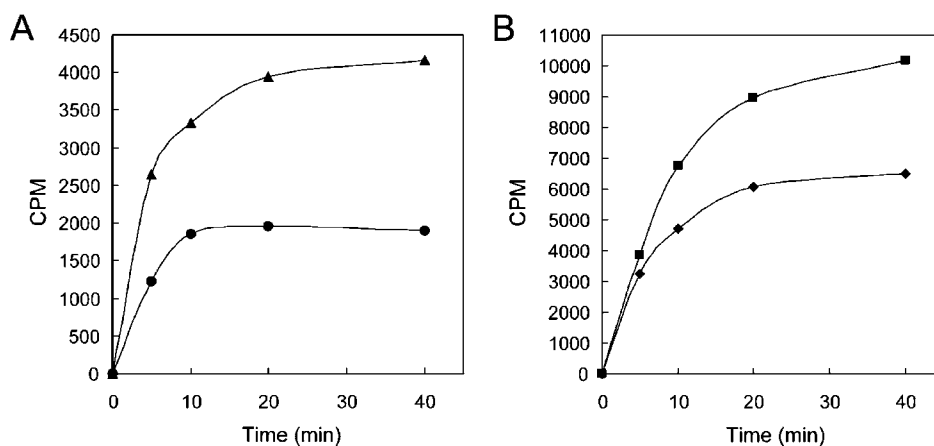
Figure 7. Time-dependent formation of methyladenosine (●), methionine (▲), 5′-deoxyadenosine (■), and S-adenosylhomocysteine (◆) in the RlmN-catalyzed methylation. (A) Formation of methyladenosine and methionine was monitored in the reaction of RlmN with [³H-methyl]-SAM. (B) Formation of 5′-deoxyadenosine and S-adenosylhomocysteine was monitored in the reaction of RlmN with [2,8-³H-adenosyl]-SAM.

Scheme 3. 23S rRNA Modifications Catalyzed by RlmN and Cfr
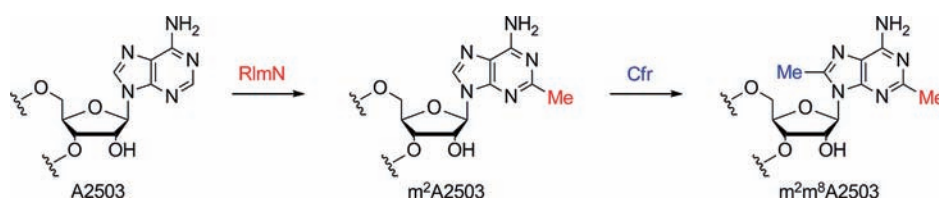
Discussion

In this study, we purified and analyzed enzymatic activities of two Radical SAM methyltransferases that operate upon the same nucleotide residue in a 3000 nucleotide-long RNA. RlmN introduces a "native" C2 methylation of A2503 in the bacterial 23S rRNA, whereas Cfr methylates the C8 position and confers antibiotic resistance. Unlike the methylation of other RNA bases via a polar mechanism (for example, enzymatic mono- and dimethylation of A2058 in 23S rRNA that leads to macrolide resistance[54]), the modifications of A2503 are unique as they are carried out on amidine carbons of the substrate. These carbon–carbon bond forming methyl transfer reactions are catalyzed by two methyl transferases, RlmN and Cfr, both members of the Radical SAM enzyme superfamily (Scheme 3). While members of this family have been shown to catalyze a number of transformations, to date no methyltransferase activity has been demonstrated _in vitro_. By showing that purified and _in vitro_ reconstituted RlmN and Cfr catalyze the methylation of 23S rRNA and by obtaining experimental evidence consistent with the radical mechanism, we now add C-methyltransferase activity to the inventory of transformations catalyzed by Radical SAM enzymes.

In this study, RlmN and Cfr were cloned and expressed as His-tagged fusions. Both enzymes were purified under anaerobic conditions and their iron–sulfur clusters reconstituted. Both enzymes were capable of methylation of 23S rRNA. The nature of the enzymatic products was established by coelution of these products, digested to single nucleosides, with synthetic standards. These experiments showed that while RlmN catalyzes methylation at position C2 of the substrate, Cfr could catalyze methylation at two distinct positions of the substrate adenine nucleotide (C2 and C8), with the physiologically relevant C8

as the preferred substrate. This observation also indicates that RlmN action is not a prerequisite for Cfr activity and that the two enzymes may even compete for the unmethylated rRNA substrate during ribosomal assembly (see below). Our observation that the C2 methylation is catalyzed by RlmN, while the C8 methylation is performed by Cfr, is in agreement with _in vivo_ studies in which the nature of modification was determined by TLC analysis and mass spectrometry.[5,11] In addition, our studies unambiguously established that SAM is a methyl donor for both enzymes, as radioactivity was transferred to the RNA substrate when [³H-methyl]-SAM was used in the reaction.

The most intriguing aspect of the RlmN- and Cfr-catalyzed methylations is the ability of the enzymes to append a methyl group to aromatic carbon centers. Such a reaction requires high activation energy, as the homolytic bond dissociation energy for hydrogen atom abstraction from the C2 carbon of adenosine is approximately 98 kcal·mol⁻¹, among the highest known for anaerobic C–H bond cleavage.[18,55] These enzymes catalyze adenosine methylation by using a radical mechanism for substrate activation. The radical chemistry is enabled by the [4Fe–4S] cluster, the presence of which was established by UV–vis spectroscopy and the iron content determination of the purified and reconstituted proteins. Mutation of any of the conserved cysteines within the characteristic CX₃CX₂C abolished methyltransferase activity for both RlmN and Cfr. This is consistent with the _in vivo_ observation that mutants of the iron–sulfur cluster-coordinating cysteine residues in Cfr fail to protect bacterial cells from antibiotic action.[5] We observed that the enzymatic methylation requires the presence of sodium dithionite, a common one-electron donor in the reactions catalyzed by Radical SAM enzymes and a surrogate for the physiological reductant, likely necessary for the reduction of

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Scheme 4. Proposed Mechanism for Methylation Catalyzed by the Radical SAM Methyltransferase RlmN
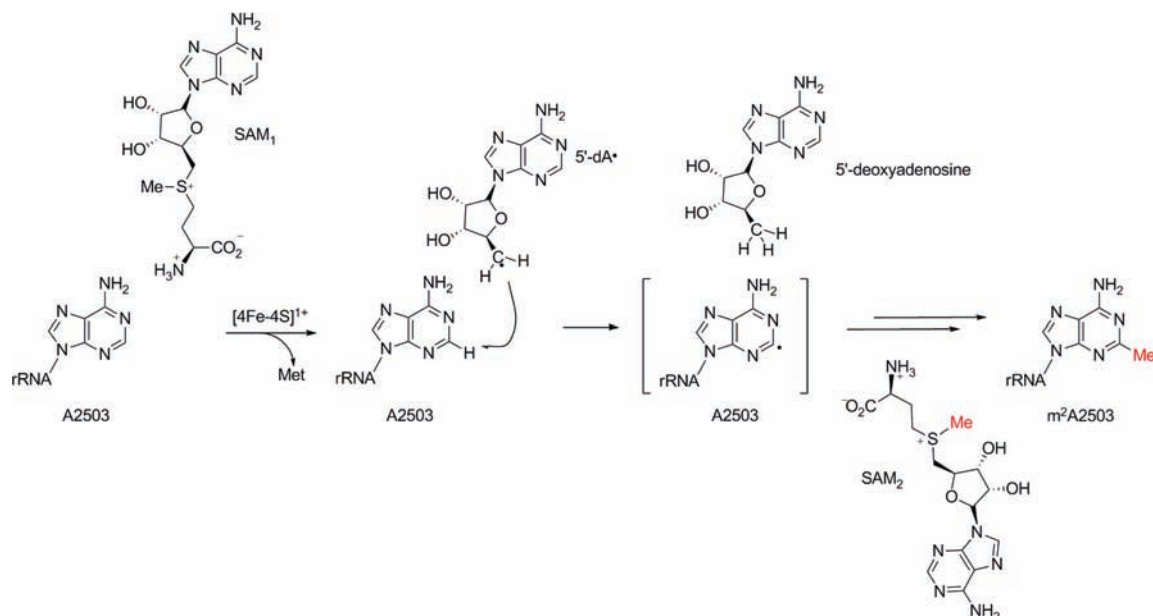
the iron−sulfur cluster to the +1 oxidation state, and initiation of the reaction cycle (Scheme 1).

In addition to the involvement of the [4Fe−4S] cluster, a hallmark of Radical SAM enzymes is the reductive cleavage of the cofactor SAM to generate methionine and a 5′-deoxy-adenosyl radical. Substrate activation is achieved by 5′-deoxyadenosyl radical-mediated hydrogen atom abstraction, leading to generation of 5′-deoxyadenosine as one of the reaction products (Scheme 1).[16−18] By using [³H-methyl]-SAM and [2,8-³H-adenosyl]-SAM, we demonstrated that RlmN- and Cfr-catalyzed methyl transfer reactions are accompanied by the production of methionine and 5′-deoxyadenosine, suggesting the role of the 5′-deoxyadenosyl radical in hydrogen atom abstraction. In addition to 5′-deoxyadenosine, RlmN- and Cfr-catalyzed methylations also generate *S*-adenosylhomocysteine, suggesting that these methyltransferases consume two molecules of SAM per every methyl group introduced. The evidence that methionine, 5′-deoxyadenosine, and SAH are formed on the productive pathway was obtained by monitoring their time-dependent formation in the RlmN-catalyzed reaction, which indicated a strong correlation between the formation of 2-methyladenosine and methionine, as well as 5′-deoxyadenosine and SAH. As methionine and 5′-deoxyadenosine are both formed as a result of the homolytic cleavage of SAM, these experiments provided a link between the introduction of the methyl group into the RNA and consumption of two molecules of SAM.

Based on these findings, we postulate a methylation mechanism depicted in Scheme 4. In the first step, the [4Fe−4S]¹⁺ cluster donates one electron to SAM₁ to form the 5′-deoxyadenosyl radical. This highly reactive radical then abstracts a hydrogen atom from the substrate (the C2 or C8 position of the adenosine) to generate a substrate-centered radical. In the subsequent steps, a methyl group is transferred to the substrate from SAM₂, forming the methylated product. The exact nature of the substrate-derived species that is subject to methylation is unknown, and its identification is hampered by the tremendous complexity of the rRNA substrate. Binding of two molecules of SAM per each molecule of the methyltransferase is in agreement with the recent structural modeling of Cfr.[56] Given their requirement for two distinct roles of SAM in catalysis, as

a source of the hydrogen atom abstracting 5′-deoxyadenosyl radical and as a donor of the methyl group, RlmN and Cfr resemble MiaB and RimO, members of the methylthiotransferase subclass of the Radical SAM superfamily.[36−39] Further similarity between MiaB and the methyltransferases described in this study is evident in the conserved first catalytic step, where all three enzymes likely use the 5′-deoxyadenosyl radical to abstract a hydrogen atom from an sp²-hybridized carbon center of an adenosine-derivative nucleotide in the substrate rRNA.

We have determined that neither enzyme can act on the assembled 50S subunit or the 70S ribosome, but protein-free 23S rRNA proved to be a good substrate. This observation is fully compatible with the location of A2503 deep in the peptidyl-transferase cavity of the mature large ribosomal subunit, where it is poorly accessible to the modification enzymes. Thus, RlmN- and Cfr-catalyzed methylation of A2503 most likely takes place during ribosome assembly.[57] Many intermediate assembly steps separate naked 23S rRNA and the mature 50S subunit in the assembly pathway, thus limiting the knowledge of the precise ribosomal assembly step at which the two enzymes may act. However, the observation that the enzymes are not functional with the mature 50S subunit substrate indicates that there is only a narrow time frame during the course of the subunit assembly when A2503 can be modified. This conclusion has important ramifications for the Cfr-mediated mechanism of antibiotic resistance because the extent of modification (and thus, the extent of resistance) may critically depend on the rate of ribosomal assembly. Other factors, such as pretreatment of cells with antibiotics that inhibit ribosome biogenesis or the activity of other modification enzymes that utilize adjacent segments of rRNA in the heavily modified peptidyl transferase center, may influence the window of opportunity for the Cfr and RlmN enzymes to act. The environmental conditions and growth phase of the cell can also have effects on the extent of A2503 modification, since ribosomes need to be actively assembling in order for Cfr and RlmN to methylate the rRNA.

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Following this logic, it is also possible that organisms with different rates of ribosomal biogenesis may then be modified at A2503 to different extents. Association of *cfr* with mobile genetic elements such as plasmids and transposons makes it potentially prone to rapid horizontal transfer between bacterial species.[8,58] Investigation of the species-specific variation of Cfr-mediated A2503 modification and its correlation with pretreatment with other antibiotics may pave the way for better antibiotic regimens and new approaches for combating antibiotic resistance.

Studies of the RNA substrate of RlmN and Cfr suggest that the 23S rRNA segment required for moderate methylation of A2503 is limited to helix system H90−H92 and the adjacent single-stranded stretch of RNA that includes A2503 (Figure 3). Removal of helices 90−92 from the RNA substrate precluded both RlmN and Cfr from modifying A2503, indicating that this helical structure is the key recognition element for both enzymes. At the late steps of ribosomal assembly, proper folding of this structure may be assisted by an RNA helicase DbpA, which directly interacts with helix 92.[59,60] Therefore, depending on the sequence of events, DbpA could also influence the extent of A2503 modification by RlmN and/or Cfr. Nevertheless, we did not observe any difference in resistance to florfenicol when Cfr was expressed in wild type or in *dbpA⁻* cells (LaMarre and Mankin, unpublished) and thus concluded that either Cfr (and probably RlmN) acts upon RNA prior to DbpA action or the 90−92 helical element of 23S rRNA can be recognized by the methyltransferases irrespective of its DbpA-mediated transformation. Knowledge of the minimal RNA substrate of the RlmN and Cfr radical SAM methyltransferases should facilitate subsequent structural and kinetic studies of both enzymes.

The role of the RlmN-catalyzed C2 methylation of A2503 in the process of translation is unknown. Previous work has demonstrated that while growth rates of wild type and ∆*rlmN* cells are comparable, cells lacking the RlmN methyltransferase slowly lose in growth competition with wild type cells.[11] The *rlmN* gene is present in many bacterial species, and its homologues are found in some single-celled eukaryotes and archaea, indicating that the A2503 modification may assist ribosome functions in all three major evolutionary domains. A2503 is located at the junction between the peptidyl transferase center and the nascent peptide exit tunnel and is apparently critical for the ability of the ribosome to sense and respond to specific nascent peptide sequences (Vazquez-Laslop and Mankin, in preparation). Posttranscriptional modification of A2503 may be required to optimize this function. In addition, H92,

which is located close to A2503 and is a part of an rRNA element recognized by RlmN, contains the highly conserved A-loop, which makes critical contacts with the A-site tRNA.[60] It is therefore possible that the A2503 C2 methylation serves as one of the check points of proper ribosome assembly and gives a stamp of approval for the subsequent assembly steps (possibly involving DbpA-mediated refolding of helix 92). The role of posttranscriptional modifications as indicators of correct assembly has been proposed in the case of some modifying enzymes such as KsgA and RluD, highlighting the possibility that the endogenous C2 methylation might play a similar function.[61,62]

In summary, we have successfully reconstituted two methylation events of an adenosine nucleotide in the complex 23S rRNA substrate. These methyl transfer reactions are catalyzed by the Radical SAM methyltransferases RlmN and Cfr. We demonstrate that these enzymes require an intact [4Fe−4S] cluster for catalysis and that SAM serves as both a source of the 5′-deoxyadenosyl radical and the methyl donor. We further demonstrated the RNA substrate requirements of both enzymes. To our knowledge, this study represents the first *in vitro* description of a methyl transfer catalyzed by a member of the Radical SAM superfamily and adds a new catalytic function to this diverse enzyme class. Our work represents a starting point for future studies into the mechanism of the Radical SAM enzyme-mediated methyl transfer reactions.

Supporting Information Available: Experimental procedures for preparation of ribosomes and rRNAs, sequences of the oligonucleotide primers used for cloning, mutagenesis and *in vitro* transcription (Tables S1−S3), SDS-PAGE analysis of purified RlmN and Cfr mutants (Figure S1), and negative controls for HPLC detections of methionine (Figure S2), 5′-deoxyadenosine (Figure S3), and *S*-adenosylhomocysteine (Figure S4) in RlmN and Cfr assay mixtures. This material is available free of charge via the Internet at http://pubs.acs.org.

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