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Covalent Intermediate in the Catalytic Mechanism of the Radical S-Adenosyl-L-methionine Methyl Synthase RlmN Trapped by Mutagenesis

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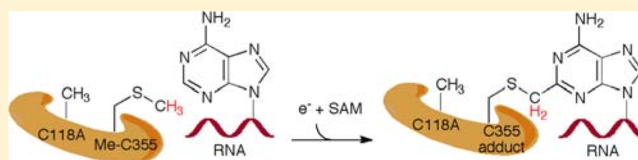
S *Supporting Information*

ABSTRACT: The posttranscriptional modification of ribosomal RNA (rRNA) modulates ribosomal function and confers resistance to antibiotics targeted to the ribosome. The radical S-adenosyl-L-methionine (SAM) methyl synthases, RlmN and Cfr, both methylate A2503 within the peptidyl transferase center of prokaryotic ribosomes, yielding 2-methyl- and 8-methyl-adenosine, respectively. The C2 and C8 positions of adenosine are unusual methylation substrates due to their electrophilicity. To accomplish this reaction, RlmN and Cfr use a shared radical-mediated mechanism. In addition to the radical SAM CX_3CX_2C motif, both RlmN and Cfr contain two conserved cysteine residues required for *in vivo* function, putatively to form (cysteine 355 in RlmN) and resolve (cysteine 118 in RlmN) a covalent intermediate needed to achieve this challenging transformation. Currently, there is no direct evidence for this proposed covalent intermediate. We have further investigated the roles of these conserved cysteines in the mechanism of RlmN. Cysteine 118 mutants of RlmN are unable to resolve the covalent intermediate, either *in vivo* or *in vitro*, enabling us to isolate and characterize this intermediate. Additionally, tandem mass spectrometric analyses of mutant RlmN reveal a methylene-linked adenosine modification at cysteine 355. Employing deuterium-labeled SAM and RNA substrates *in vitro* has allowed us to further clarify the mechanism of formation of this intermediate. Together, these experiments provide compelling evidence for the formation of a covalent intermediate species between RlmN and its rRNA substrate and well as the roles of the conserved cysteine residues in catalysis.



■ INTRODUCTION

The ribosome, containing both ribosomal proteins and ribosomal RNA (rRNA), catalyzes the biosynthesis of proteins critical to cellular function. Within the ribosome, it is the rRNA components that play the predominant structural and functional roles. Owing to its indispensible activity, the bacterial ribosome is a frequent target of antibiotic agents.[1] Unsurprisingly, compounds that interact with the ribosome and interfere with protein synthesis make close contacts with the rRNA located in functionally important regions of the ribosome.[2−5] Consequently, modifications to the rRNA in these regions can abrogate binding of antibiotics, leading to restoration of ribosomal activity and antibiotic resistance. One of the most common rRNA modifications leading to antibiotic resistance is the S-adenosyl-L-methionine (SAM)-dependent installation of a methyl group on a specific nucleobase.[6] The recently discovered, clinically significant resistance enzyme, Cfr, catalyzes the formation of an 8-methyladenosine residue at position 2503 (*Escherichia coli* numbering) of 23S rRNA,[7] which resides within the peptidyl transferase center (PTC) of the bacterial ribosome. This modification confers resistance to five classes of PTC-targeted antibiotics,[8] including the synthetic oxazolidinone linezolid, an agent commonly reserved as a "last line of defense" against resistant pathogens. RlmN, a homologue of Cfr, modifies the same adenosine base as Cfr (A2503),[9] resulting in the formation of 2-methyladenosine.[10] RlmN has recently been demonstrated to methylate adenosine residues at position 37 of several *E. coli* transfer RNAs, indicating a broader substrate specificity and a larger role in fine-tuning interactions critical to translation.[11] While the biological role of Cfr's activity may be more striking,[8] the positioning of the constitutive RlmN modification at the intersection of the PTC and the exit tunnel, as well as the impact of the modified base on ribosomal interactions with the nascent polypeptide, makes this modification no less critical to understand.[12,13]

These two homologous enzymes are members of the radical SAM superfamily and are presumed to employ a shared, radical-based mechanism to modify the target base in rRNA.[10,14,15] Both Cfr and RlmN contain the conserved CX_3CX_2C cysteine-rich motif, which ligates the [4Fe-4S] iron−sulfur cluster

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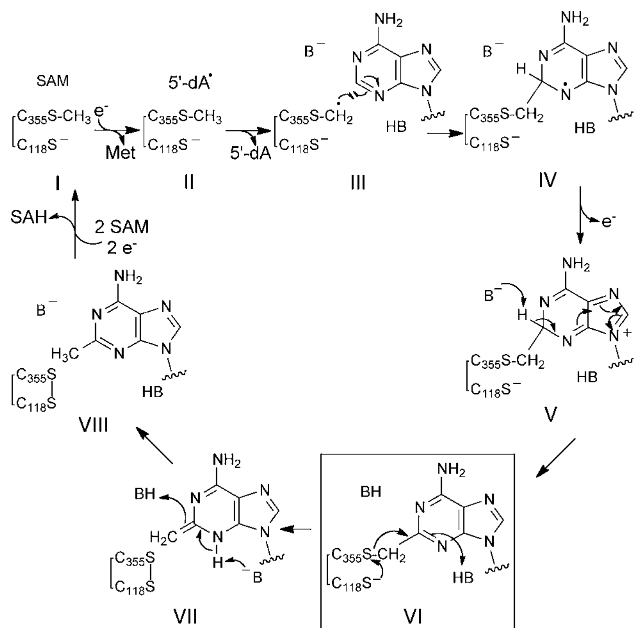
employed by all members of the radical SAM superfamily.[16] Members of this class of enzymes have been implicated in a broad range of pathways and reactions,[17,18] catalyzing amine shifts,[19−23] nucleotide reduction,[24−26] sulfur insertions,[27−32] DNA repair,[33,34] methylthiolation,[35−40] oxidative decarboxylation,[41−43] peptide modifications,[44−47] and complex rearrangements[48] among many others. Within this superfamily, RlmN and Cfr are the only known examples of methyl synthases, and both assemble a methyl group on their products from a hydrogen atom initially present on the substrate and a methylene fragment ultimately derived from SAM.[14,15]

These methyl synthases share significant homology, including two conserved cysteine residues that are not associated with the iron−sulfur cluster: cysteines 105 and 338 in Cfr and cysteines 118 and 355 in RlmN. These residues are proposed to play important roles in catalysis. Prior *in vivo* work indicates that both of these cysteines are crucial to the ability of Cfr to elicit antibiotic resistance.[49] Further, primer extension assays demonstrated that rRNA isolated from C105A mutant strains exhibits a strong stop at A2503, consistent with a modification other than a methyl group—which yields a weaker stop.[49]

In conjunction with the foregoing *in vivo* data, elegant single-turnover experiments[14] and an X-ray crystallographic structure of RlmN[50] point to a pathway initiated by the pre-methylation of cysteine 355 (RlmN numbering), by a first molecule of SAM bound at the [4Fe-4S] cluster, followed by the binding of a second molecule of SAM. With the injection of an electron, the 5′-deoxyadenosyl (5′-dA) radical formed from the second equivalent of SAM activates the thiomethyl group at cysteine 355 by abstracting a hydrogen atom. The resulting thiomethylene radical adds into the adenine ring of A2503. Loss of an electron from this initial adduct (species IV in Scheme 1), possibly to the now oxidized [4Fe-4S] cluster,[51] leads to the formation of a covalent RlmN-RNA thioether species. This adduct is subsequently resolved by the action of cysteine 118 in conjunction with acid/base catalysis (Scheme 1).[14]

Scheme 1. Proposed Mechanism of RlmN-Mediated Methylation of RNA



While this proposed mechanism is consistent with the observed formation of the thiomethyl species on cysteine 355, no conclusive evidence of the putative intermediate species has been provided. To address this uncertainty, we sought to characterize the mechanistically critical RNA-protein adduct (VI in Scheme 1). Herein, we describe a combination of *in vivo* and *in vitro* approaches to characterize the adduct formed between RlmN and RNA as well as its mechanism of formation.

■ EXPERIMENTAL PROCEDURES

General. Anaerobic manipulations were carried out in a glovebox (MBraun) under a 99.997% nitrogen atmosphere containing less than 2 ppm oxygen. All reagents were analytical grade or the highest grade commercially available and used as supplied unless noted otherwise. Iron quantitation,[10,52] preparation of truncated rRNA substrates,[10] RNA digestion,[10] high-performance liquid chromatography (HPLC) separation, and liquid chromatography-mass spectrometry (LC-MS) analysis of nucleotides[15] were performed as previously noted. The RlmN C118S and C118G mutants were generated from the C118A mutant plasmid under conditions analogous to those previously employed, using the oligonucleotide primers noted in the Supporting Information (Table S1), and the DNA sequence was confirmed (Elim Biopharmaceuticals, Inc.).

Expression, Purification, and Reconstitution of Wild-Type and Mutant RlmN. Wild-type (WT) RlmN and C355A RlmN were expressed, purified, and reconstituted as described previously.[10,14] C118 mutants were prepared by modified versions of those previous protocols. Holo-C118A/G/S RlmN was overexpressed and purified by His_6 affinity chromatography with Talon resin. Anion exchange chromatography, employing either HiTrap Q[10] or MonoQ columns (GE) was subsequently used to separate protein covalently modified by RNA *in vivo* from RNA-unmodified protein. These proteins were reconstituted and further purified by size exclusion chromatography prior to use. Apo-C118S RlmN was overexpressed and purified as described previously for WT apoprotein.[53] This protein did not form an RNA-RlmN adduct *in vivo* (see below), so the anion-exchange step noted above was omitted, and the remainder of the protocol was performed as described. RNA-RlmN adduct samples to be analyzed by RNA-digest, gel-shift, or MS methods were subjected to RNase treatment with a mixture of RNases A and T_1 (Thermo) according to the manufacturer's protocol. The in-gel RNase digestion protocol is similar and is described in detail in the Supporting Information.

2-Methyladenosine Production by Wild-Type, C118, and C355 Mutant RlmN. In order to assess the ability of mutant proteins to install methyl groups on RNA substrates, the amount of 2-methyladenosine from hydrolysates of RNA reacted with mutant enzymes was compared to that from WT. As previous work indicated that flavodoxin (FldA)[54] and its cognate reductase (Fpr)[55] were a suitable reducing system for RlmN and Cfr activity assays,[14] these proteins were employed in our assays. 250 μL reactions were performed under the following conditions: 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 500 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 200 μM SAM, 20 μM RNA (rRNA truncation 2496−2582), and 25 μM RlmN WT or mutant enzyme. The reducing system was composed of 20 μM FldA, 2 μM Fpr and 2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and reactions were allowed to proceed for 1 h at 37 °C. The RNA was recovered from the reactions, digested and separated as previously. Fractions corresponding to the elution time of authentic 2-methyladenosine were lyophilized and further analyzed by LC-MS to detect the presence of a species at m/z = 282 corresponding to intact 2-methyladenosine as previously. To determine the threshold of detection of 2-methyladenosine from WT enzyme, the 2-methyladenosine from WT reactions corresponding to between 25 and 500 nmol of recovered RNA were analyzed. Subsequently, samples that would contain any 2-methyladenosine produced in reactions of C355A, C118A, or C118S and corresponding to between 600 and 2000 nmol of RNA were analyzed in an identical fashion. If no 2-methyladenosine can be detected in a mutant sample representing 10

times the RNA of the threshold amount of WT RNA, the mutant was presumed to have less than 10% of WT activity, allowing for threshold activities of mutants to be established.

Generation of Endogenous RlmN-FLAG. A C-terminal DYKDDDDK octapeptide (FLAG) sequence was fused to the genomic version of *rlmN* on the chromosome of *E. coli* using the techniques and constructs first described by Datsenko and Wanner.[56] The C-terminal FLAG sequence was incorporated into the primer pair (Table S1) used to amplify *kan* from pKD4 via polymerase chain reaction (PCR).[56] This PCR product was transformed into *E. coli* strain BW25113 carrying the λ-red system on pKD46,[56] and successful incorporation onto the chromosome was selected through kanamycin resistance. The kanamycin resistance cassette was removed using the FLP expression plasmid pCP20, and the tagged locus was verified by sequencing. These strains were used in subsequent immunoprecipitation experiments.

Immunoprecipitation of Endogenous RlmN-FLAG. An overnight culture of *E. coli* BW25113 in which a C-terminal FLAG sequence was fused to the genomic copy of *rlmN* was diluted 1:100 into fresh Luria−Bertani (LB) medium, grown at 37 °C with agitation at 200 rpm, and aliquots were removed at 30-min intervals. Cell pellets were frozen at −80 °C and all processed after collection of the final time point. Cells were lysed using BugBuster protein extraction reagent according to the manufacturer's instructions, and protein concentrations were determined by the method of Bradford.[57] Ten μg of total protein from each time-point was run on a polyacrylamide gel and the protein was subsequently electroblotted to a PVDF membrane. Proteins bearing a FLAG-tag were detected with an anti-FLAG horseradish peroxidase conjugated antibody and visualized by chemiluminescence.

A 4-L portion of LB medium was inoculated 1:100 from an overnight culture of *E. coli* expressing the FLAG-tagged endogenous RlmN. The cells were harvested after 90 min. The resulting cell pellet was resuspended in 5 mL of Tris-buffered saline containing Tween-20, containing 1 mM ethylenediaminetetraacetic acid and 100 μM phenylmethanesulfonyl fluoride, and lysed by sonication. The resulting lysate was treated with anti-FLAG M2 affinity gel as per the manufacturer's instructions to isolate FLAG fusion proteins. The resulting FLAG fusions were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), and the band exhibiting the expected molecular weight (MW) of RlmN-FLAG (∼45 kDa) was excised from the gel. The protein was subjected to in-gel trypsin digestion and the tryptic peptides were characterized by LC-MS/MS as described in the Supporting Information.

Generation and PAGE Analysis of *in Vitro* Generated RNA-RlmN C118 Mutant Adduct. Reactions to form an RNA-protein adduct from T7-transcribed RNA and purified C118 mutant enzyme were performed with two types of RlmN samples. The first were Fe−S cluster-containing C118 mutant proteins purified away from the *in vivo* RNA-RlmN adduct, exhibiting chromatographic behavior similar to WT and either methylated or unmodified at C355. The second were apo (Fe−S cluster-free) versions of C118 mutant RlmN expressed in minimal media under iron-deficient conditions in the presence of a strong chelating agent. Apo-RlmN had previously been shown to lack methylation at C355,[53] and we further examined whether C118 RlmN mutants expressed under these conditions formed adducts *in vivo* or could form adducts *in vitro*. As such, they were purified and reconstituted as previously indicated. In order to assess the ability of these unmodified mutant proteins to form RNA-RlmN adducts *in vitro*, small scale (50−100 μL) reactions were performed under the following conditions: 200 mM HEPES pH 7.5, 500 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 100 μM SAM (200 μM with apo, reconstituted samples to compensate for the lack of any C355 methylation), 25 μM RlmN mutant enzyme, and 20 μM RNA encompassing residues 2496−2582 of 23S rRNA. The reducing system was composed of 20 μM FldA, 2 μM ferrodoxin:NADPH oxidoreductase and 2 mM NADPH. All assays were initiated by the addition of RlmN. Aliquots of the reaction mixtures were separated by PAGE in gels containing 5% polyacrylamide, 7 M urea, and 0.1% SDS; RNA species were visualized by ethidium bromide staining and imaged using a Typhoon imaging system.

Mass Spectrometry. Tryptic digests were analyzed on an LTQ-Orbitrap XL or VELOS hybrid tandem mass spectrometer (Thermo Scientific) coupled with a nanoACQUITY HPLC system (Waters) in LC-MS/MS mode. Details of the HPLC separation are provided in the Supporting Information. Mass measurements (MS survey) were performed in the Orbitrap with high resolution and mass accuracy; collision-induced dissociation (CID) experiments were performed in the linear ion trap on computer-selected, multiply charged precursor ions properly adjusting the CID conditions to the m/z and z values of the precursor ions.

Data Processing. Peak lists were generated from the raw data files using an in-house peak-picking software, PAVA. With the peak list, a strict database search was performed using the search engine, Protein Prospector (http://prospector.ucsf.edu), against the SwissProt database, with no species specificity, permitting only tryptic cleavages (1 missed cleavage). Cysteine carbamidomethylation was a fixed modification; methionine oxidation, N-terminal glutamine cyclization, and protein acetylation as variable modifications were considered. Mass accuracy was 20 ppm for the precursor ions, and 0.8 Da for the fragments. Once it was established that RlmN was indeed present and at a reasonable purity, a subsequent search was performed targeting unspecified modifications occurring on the protein in general or specifically on cysteine residues.[58] Finally, the CID data supporting the assignments were carefully inspected, and the mass accuracy of the precursor ions was manually calculated. This latter step was necessary because the software only indicates potential mass modification at certain residues, without providing an elemental composition.

■ RESULTS

Mutant Activity Compared to Wild-Type. Prior *in vivo* experiments with Cfr and its cysteine mutants have implicated cysteine 105 and cysteine 338 as necessary to catalyze the 8-methyl modification of A2503.[49] Therefore we anticipated that corresponding mutations to RlmN cysteines 118 and 355, would result in enzymes with virtually no ability to form 2-methyladenosine on RNA substrates. Using known quantities of RNA recovered from *in vitro* WT reactions as a frame of reference, we were able to establish a detection limit for the 2-methyladenosine product to which the products of mutant reactions could be compared. Whereas the 2-methyladenosine from 25 nmol of RNA from a WT reaction was still detectable (Figure S1), no signal was evident from between 600 and 2000 nmol of RNA from mutant reactions under identical conditions. From these results, it was determined that C355A, C118A and C118S exhibit ≤1.3, 3.1, and 4.2% of WT activity, respectively. These low activities agree well with the putatively indispensible roles of the conserved cysteine residues implicated in prior *in vivo* experiments, as well as the proposed chemical mechanism (Scheme 1).

Methylation of Endogenous RlmN. Previously, overexpressed RlmN has been shown to be methylated at cysteine 355 by both X-ray crystallographic analysis[50] and protease digestion followed by LC-MS/MS.[14] To evaluate the methylation state of the protein during active rRNA modification and ribosome assembly, the genomic copy of *rlmN* was altered to encode a C-terminal FLAG sequence to ensure that all experiments were performed under conditions where RlmN was expressed at its normal cellular level. Time-dependent expression of RlmN-FLAG was evaluated by Western blotting for the RlmN-FLAG in lysates at various time points (Figure S2). RlmN-FLAG is maximally expressed ∼90 min after dilution of a saturated culture 1:100 into fresh LB medium, corresponding to an OD_{600} of ∼0.25. This timing is consistent with the growth rate-dependent control observed for ribosome biosynthesis,[59]

suggesting that RlmN produced under these conditions is the catalytically relevant resting state of the enzyme. RlmN-FLAG was immunoprecipitated from the 90-min time point and the isolated protein was characterized by tryptic digestion, LC-MS/MS similar to prior experiments.[14] This analysis revealed the peptide encompassing residues 348−365 yields an $m/z(2+)$ = 909.4149 species consistent with methylated peptide (calculated value = 909.4203, mass difference of 6 ppm). Fragmentation of this precursor ion yielded b/y series ions that confirmed the site of the methyl modification as cysteine 355,[60] indicating modification of the enzyme under physiological conditions (Figure S3).

Characterization of RNA-RlmN C118 Mutant Adduct Formation *in Vivo*. Prior *in vivo* work with C105A Cfr demonstrated a strong stop at A2503 of the isolated rRNA in primer extension assays, indicating a significant perturbation of the rRNA at this position, although the precise nature of this change was not assessed.[49] These observations are consistent with the attachment of a large substituent to A2503 and possibly the formation of a covalent adduct between Cfr and rRNA at A2503 *in vivo*. C118A RlmN was also shown to exhibit a shift in its λ_{max} from 278 to 265 nm in addition to the release of modified RNA nucleotides upon RNase and phosphatase treatment; however, no direct evidence of covalent attachment or the putative points of attachment between C118A RlmN and RNA was provided.[14] To further investigate the possibility that a covalent adduct between RNA and RlmN cysteine 118 mutant enzymes forms *in vivo*, the C118A, C118G, and C118S mutants of RlmN were overexpressed and purified by Talon chromatography. The various mutants were examined to determine if the nature of the residue at position 118 might influence the relative amounts of RNA-adducted and unmodified protein. When the isolated His-tagged proteins were analyzed by SDS-PAGE, the samples exhibited two distinct bands, one at the native MW of RlmN, 43 kDa, and the second at ~65 kDa (Figure 1a,b). From this analysis, it appeared that the C118S RlmN formed the greatest proportion of adduct *in vivo*, so it was employed in subsequent *in vitro* experiments (see below). In the case of C118A RlmN, when this heterogeneous material was purified by anion exchange chromatography, the two species were resolved, with the lower MW species eluting at a much lower salt concentration than the higher MW species (Figure S4). This observation is indicative of the higher MW species being much more anionic in character at pH 7.5−8. This higher MW species also exhibits a much greater absorbance at 260 nm than the lower MW species. Together, these results are consistent with the addition of the polyanionic phosphate backbone of an RNA molecule to the protein. Further, when the putative RNA-RlmN adduct purified by anion exchange was treated with a mixture of RNases A and T_1, the resulting protein exhibited native-like MW (Figure 1c). This native-like, RNase-treated material was analyzed by tryptic digestion, LC-MS/MS, revealing the peptide containing cysteine 355 is modified by a 147 Da species (Figure 2). The modified peptide was chromatographically resolved from the unmodified or *S*-methylated peptide, and the precursor ion bearing the +147 Da modification was subjected to CID analysis. In this instance, the fragment ions were measured in the Orbitrap with high resolution and mass accuracy, and the main sequence ions unambiguously indicate that the modification resides on C355 (Figure 2). The observed mass of 147 Da is consistent with a methylene-linked adenine adduct analogous to the covalent

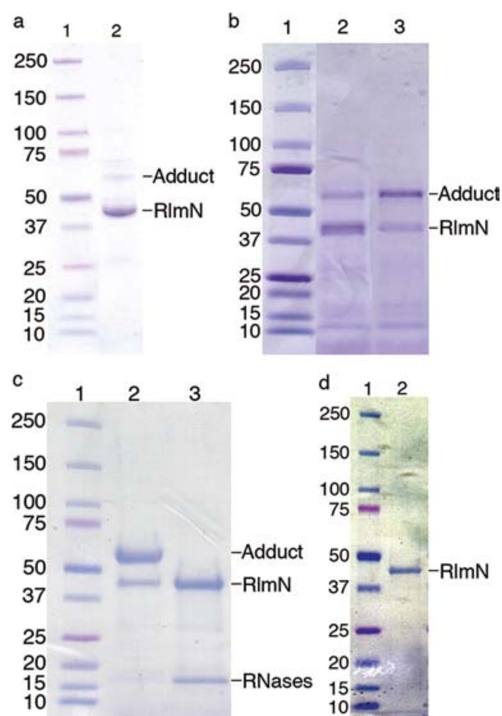
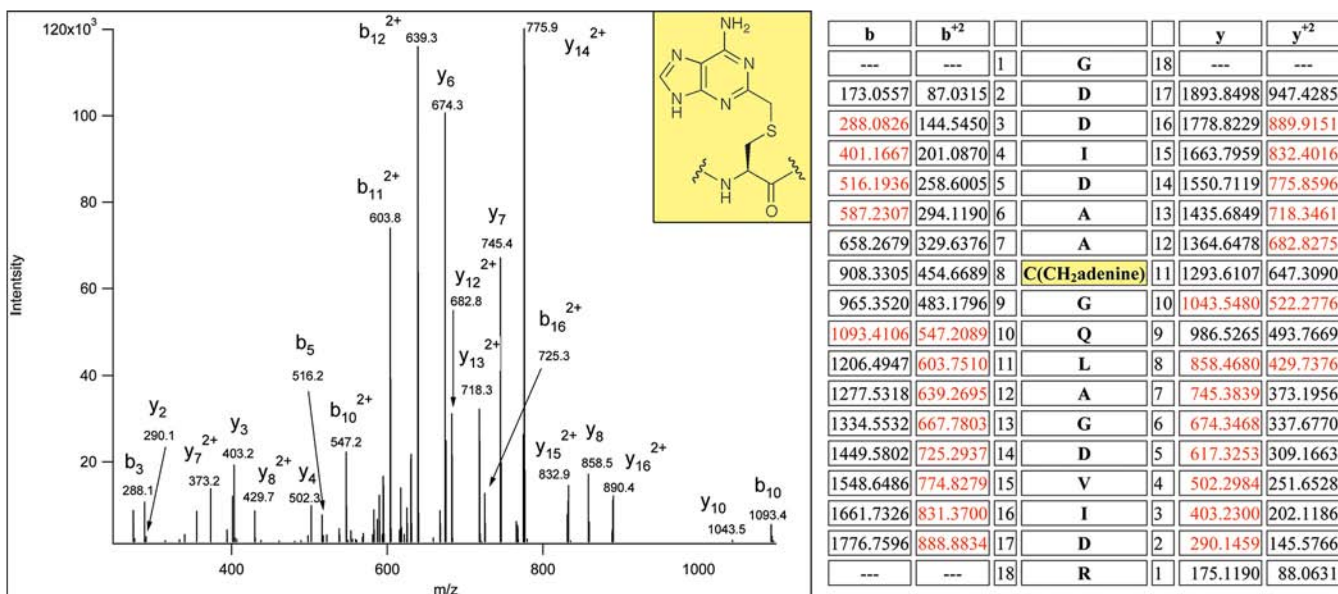


Figure 1. SDS-PAGE analysis of RlmN C118 mutant proteins. Protein markers are in lane 1 of all gels. (a) C118A RlmN purified by immobilized metal ion affinity chromatography (lane 2). (b) C118G RlmN (lane 2) and C118S RlmN (lane 3) purified by immobilized metal ion affinity chromatography. (c) Untreated C118A RlmN-RNA adduct purified by anion exchange (lane 2). Sample from lane 2, after treatment with RNases A and T_1 (lane 3). (d) C118S RlmN grown under metal-deficient conditions and purified by immobilized metal ion affinity chromatography (lane 2). In this sample, no RNA adduct could be detected.

intermediate species in the proposed mechanism (VI in Scheme 1). Though these samples were not specifically treated to cleave the glycosidic bonds between the RNA nucleobases and ribose, it appears that the linkage is not stable under the experimental conditions. Interestingly, when C118S RlmN was prepared as the apoprotein (lacking the Fe−S cluster), the material exhibited only one band at the native MW of 43 kDa (Figure 1d). This observation is consistent with the previously noted requirement of an intact Fe−S cluster for *S*-methylation,[53] as well as the requirement of the cluster for subsequent radical SAM chemistry and adduct formation.

RNA-RlmN C118 Mutant Adduct Formation *in Vitro*. To investigate whether a covalent adduct between RlmN cysteine 118 mutants and RNA also forms *in vitro*, the recombinant mutant proteins C118A and C118S were incubated with an RNA fragment encompassing residues 2496−2582 of 23S rRNA and the FldA reducing system. In light of the previously observed protein gel-shift of the *in vivo* RNA-modified material (Figure 1a−c), a gel-shift assay monitoring RNA was employed to monitor covalent modification of the RNA fragments *in vitro*. When RNA from these reactions is separated by PAGE and visualized with ethidium bromide, complete reaction mixtures containing C118A or C118S demonstrate two distinct bands (Figure 3, lanes 4 and 5, respectively). The first species is unreacted RNA (87 nt, ~28 kDa); the second species appears at a MW consistent with an RNA-RlmN adduct, expected MW ~71 kDa (mass equivalent to 215 nt). The formation of the adduct



b	b^{+2}				y	y^{+2}
---	---	1	G	18	---	---
173.0557	87.0315	2	D	17	1893.8498	947.4285
288.0826	144.5450	3	D	16	1778.8229	889.9151
401.1667	201.0870	4	I	15	1663.7959	832.4016
516.1936	258.6005	5	D	14	1550.7119	775.8596
587.2307	294.1190	6	A	13	1435.6849	718.3461
658.2679	329.6376	7	A	12	1364.6478	682.8275
908.3305	454.6689	8	C(CH_2adenine)	11	1293.6107	647.3090
965.3520	483.1796	9	G	10	1043.5480	522.2776
1093.4106	547.2089	10	Q	9	986.5265	493.7669
1206.4947	603.7510	11	L	8	858.4680	429.7376
1277.5318	639.2695	12	A	7	745.3839	373.1956
1334.5532	667.7803	13	G	6	674.3468	337.6770
1449.5802	725.2937	14	D	5	617.3253	309.1663
1548.6486	774.8279	15	V	4	502.2984	251.6528
1661.7326	831.3700	16	I	3	403.2300	202.1186
1776.7596	888.8834	17	D	2	290.1459	145.5766
---	---	18	R	1	175.1190	88.0631

Figure 2. Ion trap CID data of ^{348}GDDIDAAC(CH_2adenine)GQLAGDVIDR^{375} formed from RlmN C118A protein *in vivo* and subsequently RNase treated. The precursor mass was $m/z(3+) = 650.9631$, within ~1 ppm of the calculated mass of 650.9623 (3+ ion of RlmN 348−375 peptide +CH_2adenine). The table at right lists all main sequence ions detected within 20 ppm of the calculated value. In comparison to the methyl derivative (Figure S3), the higher charge state of the precursor ion yielding the best CID data as well as the presence of doubly charged N- and C-terminal fragments clearly indicate the incorporation of a basic modification. The site of modification within the peptide and the deduced structure of the modification are highlighted in yellow.

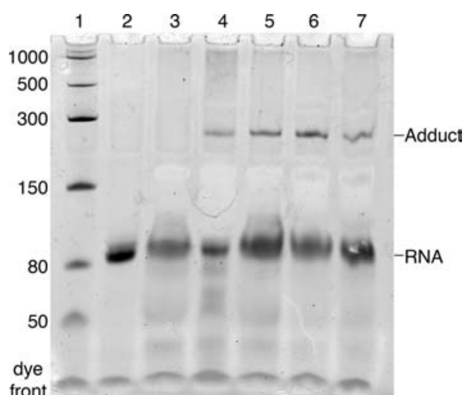
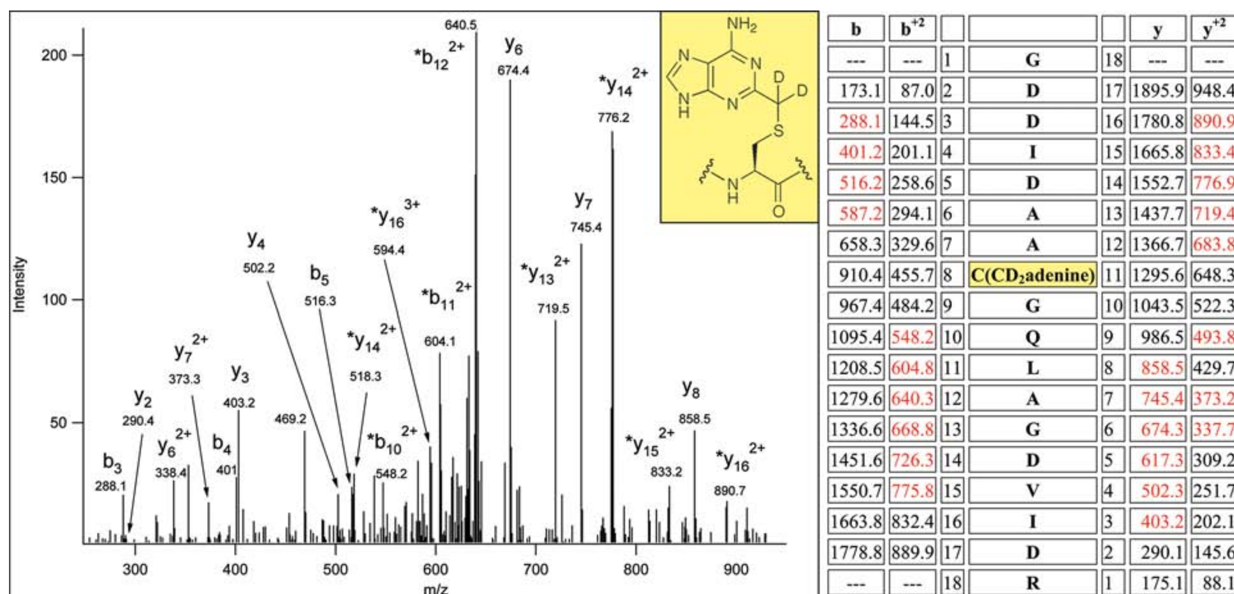


Figure 3. *In vitro* gel-shift assay of RlmN C118 mutants. RlmN mutant proteins were incubated with RNA fragments as indicated, and RNA was separated by denaturing PAGE and visualized with ethidium bromide. Lane 1, RNA markers of the indicated size, in nt; lane 2, only the 87 nt RNA fragment used in assays; lane 3, a negative control lacking NADPH; lane 4, reaction with C118A RlmN, SAM, and RNA; lane 5, reaction with C118S RlmN, SAM, and RNA; lane 6, reaction with C118S RlmN, SAM, and D-RNA; lane 7, reaction with C118S RlmN, [*methyl*-$^2H_3$]-SAM, and RNA.

species is dependent on the presence of a complete reducing system, as when NADPH is omitted, no gel-shifted species appears (Figure 3, lane 3). This finding is consistent with the role of NADPH as the ultimate electron source in the FldA/Fpr (flavodoxin and reductase) system that leads to the reductive cleavage of SAM, resulting in the formation of the critical 5′-dA radical that initiates the subsequent radical-based reactions. When the adduct species was excised from the gel and subjected to in-gel RNase A and T_1 and trypsin digestion, subsequent LC-MS/MS experiments analogous to those above for the *in vivo* adduct revealed an identical 147 Da modification on cysteine 355 of C118S mutant protein exposed to rRNA substrate (Figure S5), again consistent with the proposed methylene-linked adenosine intermediate (VI in Scheme 1). These *in vitro* experiments with defined RNA substrate demonstrate that a covalent adduct is formed between RlmN cysteine 118 mutant enzyme and rRNA substrate.

To further interrogate the mechanism of formation of this intermediate arrested in mutant enzymes, the incorporation of deuterium into the protein-RNA adduct was examined. RlmN C118S mutant protein was expressed in minimal media in the presence of a strong chelator, resulting in protein lacking both the iron−sulfur cluster and methylation at cysteine 355.[53] The Fe−S cluster of this apoprotein was then chemically reconstituted, resulting in [4Fe-4S]-containing RlmN C118S enzyme with a free thiol at cysteine 355. This protein was then incubated with the same RNA fragment as above and [*methyl*-$^2H_3$]-SAM. In a separate experiment, C118S RlmN (either free thiol or *S*-methyl at C355) was incubated with unlabeled SAM and D-RNA (i.e., substrate in which all adenosine residues have been replaced with [2-^{2}H]-adenosine; similar deuterium substituted rRNA has previously been demonstrated as a substrate for RlmN[15]). The resulting RNA-RlmN C118S adducts (Figure 3, lanes 6 and 7) were digested with RNases and trypsin as above. The presence and location of deuterium in the modified cysteine 355 peptides was assessed by LC-MS/MS and compared to the spectra of the same species formed from completely unlabeled reactants. In agreement with the proposed formation of the covalent adduct via addition of the thiomethylene radical into the substrate adenine (Scheme 1), the species generated from [*methyl*-$^2H_3$]-SAM and unlabeled RNA incorporates two deuterium atoms into the group modifying cysteine 355, consistent with a CD_2-methylene linkage between the cysteine 355 sulfur and the adenine ring (Figure 4). Reactions with unlabeled SAM and D-RNA yielded MS and MS/MS data (Figure S6) indistinguishable from those generated by reactions with completely



b	b+2				y	y+2
---	---	1	G	18	---	---
173.1	87.0	2	D	17	1895.9	948.4
288.1	144.5	3	D	16	1780.8	890.9
401.2	201.1	4	I	15	1665.8	833.4
516.2	258.6	5	D	14	1552.7	776.9
587.2	294.1	6	A	13	1437.7	719.4
658.3	329.6	7	A	12	1366.7	683.8
910.4	455.7	8	C(CD_2adenine)	11	1295.6	648.3
967.4	484.2	9	G	10	1043.5	522.3
1095.4	548.2	10	Q	9	986.5	493.8
1208.5	604.8	11	L	8	858.5	429.7
1279.6	640.3	12	A	7	745.4	373.2
1336.6	668.8	13	G	6	674.3	337.7
1451.6	726.3	14	D	5	617.3	309.2
1550.7	775.8	15	V	4	502.3	251.7
1663.8	832.4	16	I	3	403.2	202.1
1778.8	889.9	17	D	2	290.1	145.6
---	---	18	R	1	175.1	88.1

Figure 4. Ion trap CID data of ^{348}GDDIDAAC(CD_2adenine)GQLAGDVIDR^{375} formed from RlmN C118S protein *in vitro* in the presence of [*methyl*-$^2H_3$]-SAM. The precursor mass was $m/z(3+) = 651.6353$, within 3 ppm of the calculated mass of 651.6332 (3+ ion of RlmN 348–375 peptide +CD_2adenine). Fragments that showed the 2 Da shift due to deuterium incorporation are labeled with asterisks. Main sequence ions detected are shown in red in the table. The site of modification within the peptide and the deduced structure of the modification are highlighted in yellow.

unlabeled precursors (Figure S5), consistent with the loss of the amidine proton from the RNA-protein adduct to an adjacent, solvent shielded, general base. This result agrees with the previously observed retention of deuterium label in product 2-methyladenosine,[15] as the shielded proton is returned to product only subsequent to resolution of the adduct. Such retention in the product of a proton abstracted from substrate has previously been observed.[61,62]

■ DISCUSSION

The radical SAM methyl synthases, RlmN and Cfr, utilize a novel, methyl group assembly mechanism to achieve methylation of the 2- and 8-amidine carbons of A2503 within 23S rRNA, thereby exerting significant influence on the biological function of the modified ribosomes.[8,13] Multiple mechanistic scenarios have been suggested to account for the experimental findings.[14,15] In this work, we provide strong evidence for the mechanism invoking methylation of a cysteine residue in the protein and subsequent formation of a covalent RNA-protein adduct as originally proposed by Grove et al.[14] Importantly, our mutagenesis-enabled trapping of the covalent intermediate provides the first direct evidence of any of the proposed reactive intermediates occurring subsequent to the initial electron insertion.

Crucial to this mechanism are cysteine residues 118 and 355 in *E. coli* RlmN, which are conserved among RlmN and Cfr homologues across species.[49] We have investigated the roles of both cysteines in this study. For cysteine 355, we examined methylation status under endogenous conditions. We showed that protein produced from the genomic copy of *rlmN* under the native promoter at the time of maximal protein expression and ribosome biosynthesis exhibits methylation at C355. The fact that this modification is observed during optimal growth strongly implies that this modification is involved in the *in vivo* rRNA methylation catalyzed by RlmN, confirming its catalytic relevance. Interestingly, methylation of a reactive cysteine in the active site of the DNA methyltransferase Dnmt3a has recently been observed and attributed to either spurious methylation of a nucleophilic cysteine residue or a regulatory mechanism inactivating excess enzyme in the absence of substrate.[63] While either of these possibilities are conceivable for RlmN, the above argument suggests that, for RlmN, this methylation is directly involved in catalysis.

For cysteine 118, the resolving cysteine, we examined C118A and C118S mutant RlmN to obtain insight into the covalent RlmN-RNA species formed both *in vivo* and *in vitro*. Previous studies showed that purified C118A RlmN exhibited an absorbance maximum at 265 nm vs 278 nm for WT,[14] and that ribonuclease and phosphatase treatment of these samples released nucleotides including pseudouridine.[14] Our observation of the significant increase in anionic character of this species, as revealed by ion exchange chromatography, as well as the gel shift upon ribonuclease treatment together strongly argue that this species is a covalent adduct between RNA and RlmN. Significantly, LC-MS/MS experiments localize the site of RNA modification to cysteine 355 on RlmN. This cysteine exhibits a +147 modification consistent with a methylene-linked adenosine covalent intermediate in the RlmN mechanism.

The *in vivo* formation of a protein-RNA adduct with cysteine 118 mutant enzymes can be recapitulated *in vitro*, with the formation dependent on the putative *in vivo* reducing system, FldA and ferrodoxin:NADPH oxidoreductase. Further, the incorporation of two deuterium atoms into the *in vitro* adduct from [*methyl*-$^2H_3$]-SAM, as determined by LC-MS/MS, supports the addition of the cysteine 355-derived thiomethylene radical into the substrate adenosine in RNA. The lack of deuterium in the intermediate formed from D-RNA is consistent with the one electron oxidation of the initial adduct (IV in scheme 1) and subsequent deprotonation, leading to re-aromatization of adenine and the formation of the methylene-linked RNA-cysteine 355 adduct. The observed incorporation of deuterium from labeled substrates into the covalent adduct is

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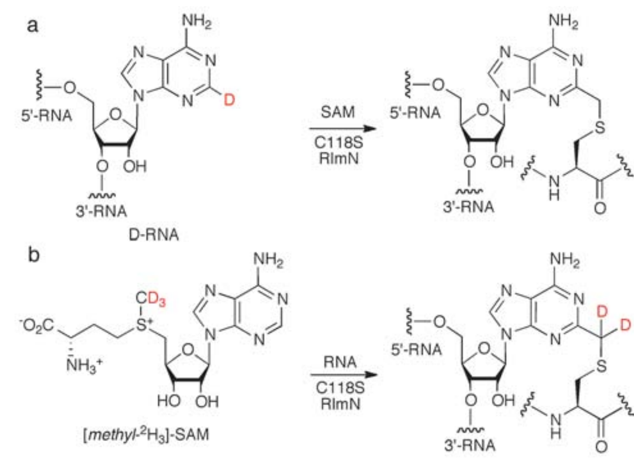
indicated in Scheme 2. Our results demonstrate the obligatory role of cysteine 118 in product formation, specifically by acting

Scheme 2. Deuterium Incorporation from Substrates into Covalent Adduct between C118 Mutants of RlmN and RNA



subsequent to the formation of the covalent intermediate. These observed critical roles for both cysteines 118 and 355 agree well with the prior *in vivo* observations that Cfr C105A and C338A mutants are not resistant to florfenicol or tiamulin, and short polynucleotides, encompassing A2503, isolated from the rRNA of these mutant strains exhibit no methylation by MALDI-MS analysis.[49]

We also note that the adduct formed by cysteine 118 mutants both *in vivo* and *in vitro* remains relatively stable in the presence of high concentrations of thiol reducing agents. The *in vivo* adducts were purified in the presence of 10 mM β-mercaptoethanol or 5 mM DTT depending on the purification step. Despite this exposure, the covalent RNA adduct is not resolved during purification. The *in vitro* adducts were formed in the presence of 1 mM DTT, indicating that low concentrations of this reagent do not perturb this intermediate. Further, when the *in vivo* adduct is prepared for SDS-PAGE, it is exposed to ∼350 mM β-mercaptoethanol under denaturing conditions, yet the RNA-modified band is still evident. Cumulatively, these observations indicate that a sulfur nucleophile is necessary, though not sufficient, to resolve the covalent RNA-protein adduct. This may be due to conformational constraints on the approach of the nucleophile, possibly concomitant with protonation of the intermediate, which is unlikely to be readily accomplished with externally supplied thiol.

CONCLUSION

In summary, the findings presented here clearly support the existence and the intermediacy of the covalent adduct between RlmN and rRNA substrates and highlight the crucial roles for the two conserved cysteines unassociated with the [4Fe-4S] cluster. These results lend experimental evidence to the proposed mechanism[14] that combines two canonical roles of the common metabolite *S*-adenosylmethionine: methylation and radical activation. The unique methylations catalyzed by RlmN and Cfr at aromatic amidine positions of a critically positioned nucleotide enable the modification of complex RNA substrates with significant biological impact.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the generation of FldA and Fpr expression plasmids, purification of FldA and Fpr and in-gel RNase and tryptic digests of RlmN; sequences of oligonucleotide primers used to generate FldA and Fpr PCR products, C118S RlmN and endogenous RlmN-FLAG (Table S1); LC-MS analysis of 2-methyladenosine from WT and mutant RlmN reactions (Figure S1); Western blot analysis of endogenous RlmN-FLAG (Figure S2); CID spectra of endogenous RlmN-FLAG C355-Me peptide (Figure S3); Anion exchange chromatograph of C118A mutant RlmN (Figure S4); CID spectra of the *in vitro* generated C355 methyleneadenine-modified peptide from C118S RlmN (Figure S5); CID spectra of the *in vitro* generated C355 methyleneadenine-modified peptide from C118S RlmN produced using D-RNA substrate (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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