A Radically Different Mechanism for *S*-Adenosylmethionine–Dependent Methyltransferases

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Methylation of small molecules and macromolecules is crucial in metabolism, cell signaling, and epigenetic programming and is most often achieved by *S*-adenosylmethionine (SAM)–dependent methyltransferases. Most employ an S_N2 mechanism to methylate nucleophilic sites on their substrates, but recently, radical SAM enzymes have been identified that methylate carbon atoms that are not inherently nucleophilic via the intermediacy of a 5′-deoxyadenosyl 5′-radical. We have determined the mechanisms of two such reactions targeting the sp^2-hybridized carbons at positions 2 and 8 of adenosine 2503 in 23*S* ribosomal RNA, catalyzed by RlmN and Cfr, respectively. In neither case is a methyl group transferred directly from SAM to the RNA; rather, both reactions proceed by a ping-pong mechanism involving intermediate methylation of a conserved cysteine residue.

A number of different posttranscriptional and posttranslational modifications adorn protein and RNA components of the ribosome. Although the functions of most of these modifications are currently unknown, many are believed to confer stability and translational fidelity and to direct proper ribosomal assembly (*1*–*3*). Among the most common modifications is a methyl group derived from *S*-adenosylmethionine (SAM) that is appended to specific atoms in amino acid side chains or nucleotide bases or sugars. Most of these groups are added by S_N2 displacement mechanisms, involving attack of a nucleophile on the methyl group of SAM with concomitant release of *S*-adenosyl-L-homocysteine (*4*, *5*). Intriguingly, two SAM-dependent methylations of 23*S* ribosomal RNA (rRNA) of bacterial ribosomes involve electrophilic rather than nucleophilic sp^2-hybridized carbons (Fig. 1) (*6*–*8*); the enzymes RlmN (YfgB) and Cfr methylate C-2 and C-8 of adenosine 2503 (A2503), respectively. Moreover, the poor acidity of the protons attached to C-2 and C-8 precludes their removal by typical polar (acid/base) processes that are mediated by the side chains of the 20 common amino acids (*9*, *10*).

About half of all antibiotics currently in use target the bacterial ribosome, with most binding to sites on the large (50*S*) subunit, disrupting functions associated with guanosine 5′-triphosphate hydrolysis, peptide-bond formation, and exit of the nascent polypeptide (*1*, *11*). The latter two functions are inhibited by several classes of antibiotics, which bind to overlapping regions. Thus, bacterial interventions to resist an antibiotic that targets this region often result in resistance to multiple drugs (*1*, *12*). A2503 of 23*S* rRNA is in the peptidyltransferase center of the 50*S* subunit near the entrance to the exit tunnel for the nascent peptide (*13*–*16*). Modification of C-2 is thought to play a housekeeping function (*6*, *17*). Although the *yfgB* gene is not essential, *Escherichia coli yfgB*-null strains lose to wild-type (WT) strains in cogrowth competition experiments (*17*). Modification of C-8 of A2503 confers resistance to several classes of antibiotics, including phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A (*8*, *18*–*20*). The *cfr* gene, which encodes this activity, was first identified on a plasmid isolated from *Staphylococcus sciuri*, an animal pathogen (*19*). More recently, it has been found on the chromosome of a methicillin-resistant strain of *S. aureus* (MRSA) obtained from a hospital isolate along with the *ermB* gene, which encodes a 23*S* A2058 dimethylase (*21*). Coexpression of these genes renders the bacterium resistant to all currently used antibiotics that target the 50*S* subunit (*21*). Bioinformatics analysis indicates that RlmN is widespread throughout eubacteria and that Cfr evolved directly from it as an antibiotic-resistance mechanism (*22*). In fact, Cfr, which shares 33% sequence identity with *E. coli* RlmN, also catalyzes methylation at C-2 of A2503, although it is not the preferred target (*7*).

RlmN and Cfr belong to a class of SAM-dependent enzymes designated radical SAM (RS), which reductively cleave SAM to give a 5′-deoxyadenosyl 5′-radical (5′-dA•) rather than to mediate alkylation reactions (using polar mechanisms) (*23*). The 5′-dA• intermediate is used to abstract key hydrogen atoms from the substrate; however, the transformations that follow differ widely among RS reactions (*24*–*26*). All RS enzymes contain at least one [4Fe-4S] cluster—typically coordinated by the Cys residues of a CxxxCxxC motif—which supplies the electron for the reductive cleavage of SAM. In *E. coli*, the iron-sulfur (Fe/S) cluster is reduced by an electron derived from the flavodoxin/flavodoxin reductase (Flv/Flx) reducing system, whereas in vitro it can also be supplied by low-potential artificial reductants such as dithionite or illuminated deazaflavin (*27*).

In vitro studies by Yan *et al.* (*28*) showed that in RlmN and Cfr, SAM acts as both a radical

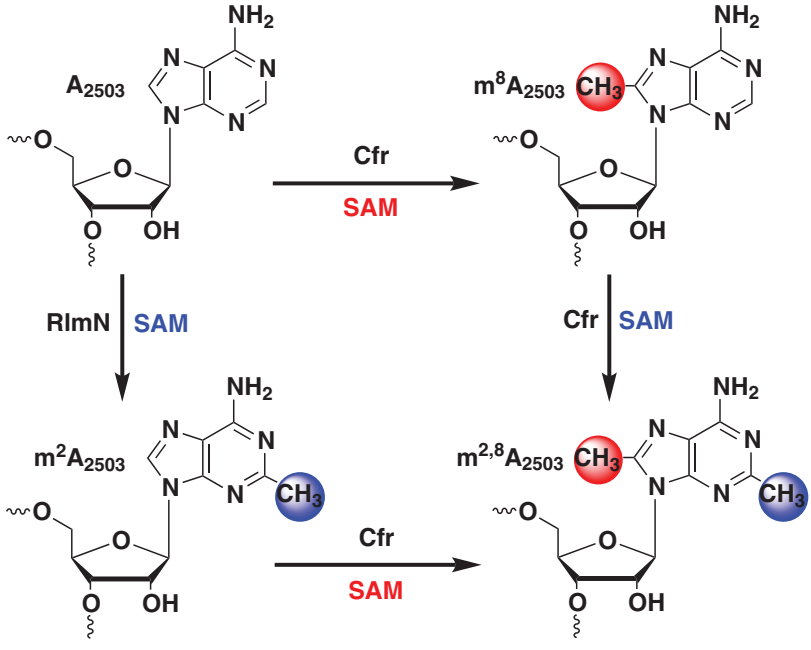

Fig. 1. Reactions catalyzed by RlmN and Cfr. RlmN catalyzes uniquely methylation at C-2, whereas Cfr catalyzes methylation at C-8 and C-2, although C-8 is the preferred target.

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generator and as the source of the appended methyl group. Moreover, these studies showed that both enzymes act preferentially on naked 23*S* rRNA, of which helices 89 and 90 to 92 in domain V are critical for substrate recognition and turnover (*28*). To gain further insight into the mechanism of catalysis by RlmN and Cfr, we initiated activity determinations using a seven-nucleotide oligomer (7-mer) spanning positions 2500 to 2506 on rRNA, which included the naturally occurring pseudouridine modification at position 2504. Figure 2 shows mass spectra of the resulting methylated adenosine (CH_3-Ad) products of a reaction containing 500 μM WT RlmN ($RlmN_{WT}$), 1.5 mM SAM, and 250 μM RNA substrate (*29*). A chromatographic peak at retention time 6.85 min (Fig. 2, red trace) exhibits a mass spectrometry (MS) peak at mass/charge ratio (*m/z*) = 282.1 (Fig. 2, inset, red trace) and a fragmentation pattern consistent with that of methylation at the 2 position of adenosine (m^2A) (*7*), indicating that the 7-mer can indeed be methylated by RlmN. MS peaks at 283.1 and 284.1 *m/z* (Fig. 2, inset) derive from natural-abundance ^{13}C in the product. Use of the in vivo reducing system (Flv/Flx/NADPH, where NADPH is the reduced form of nicotinamide adenine dinucleotide phosphate) in place of dithionite does not give turnover, even after 18 hours of incubation. To confirm that the methyl group derives from SAM, as had been shown previously by Yan *et al*. (*28*), we incubated both $RlmN_{WT}$ and Cfr as described above, with the exception that SAM was replaced with *S*-adenosyl-L-[methyl-d_3] methionine (d_3-SAM). Unexpectedly, the RNA product of the $RlmN_{WT}$ reaction gives the same *m/z* and isotopic distribution (Fig. 2, inset, solid black trace) as the product of the reaction with unlabeled SAM, suggesting that the methyl group transferred under single-turnover conditions (protein in excess of substrate) does not reflect the isotopic composition of SAM added to the assay mixture. To test whether the methyl group could derive from a SAM molecule that bound tightly to the enzyme during its production in *E. coli*, a 200 μM sample of $RlmN_{WT}$ was quenched in acid and analyzed (*29*); SAM was not detected (fig. S2). We obtained similar results when Cfr (500 μM) was incubated with d_3-SAM (1.5 mM) and the 7-mer RNA substrate (250 μM) under single-turnover conditions (Fig. 2, dotted black trace). In this case, the product's retention time is slightly less (6.8 min) than that of the product from the RlmN reaction but identical to that of a synthetic m^8A standard (figs. S1 and S3). In addition, its *m/z* value and fragmentation pattern are also identical to those of the synthetic m^8A standard and consistent with published results (*7*). Importantly, the methyl group transferred to the adenosine corresponding to A2503 is again found to lack deuterium from d_3-SAM. Thus, the same surprising isotope-tracing result is found for Cfr.

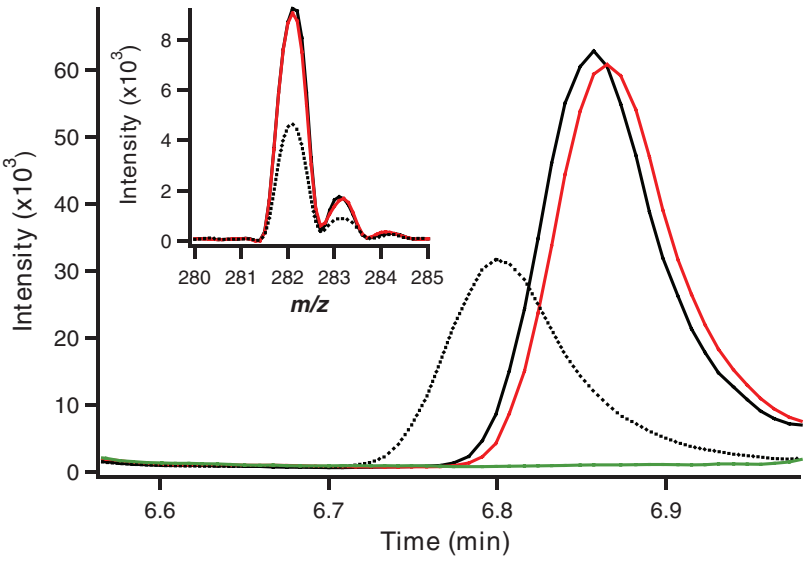

Fig. 2. Liquid chromatography–MS analyses of the methylated products of the RlmN and Cfr reactions. Single-turnover experiment in the presence of 250 μM 7-mer RNA substrate using 500 μM $RlmN_{WT}$ and 1 mM SAM (red solid trace), 500 μM $RlmN_{WT}$ and 1 mM d_3-SAM (black solid trace), or 500 μM Cfr and 1 mM d_3-SAM (black dotted trace). The peak at 6.8 min (black dotted trace) corresponds to m^8A, with its associated mass spectrum in the above inset (black dotted trace). The peak at 6.85 min corresponds to m^2A generated in the presence of SAM or d_3-SAM with their associated mass spectra in the above inset (red and black solid traces, respectively). The green trace is a control corresponding to the above reactions in the absence of Cfr or $RlmN_{WT}$.

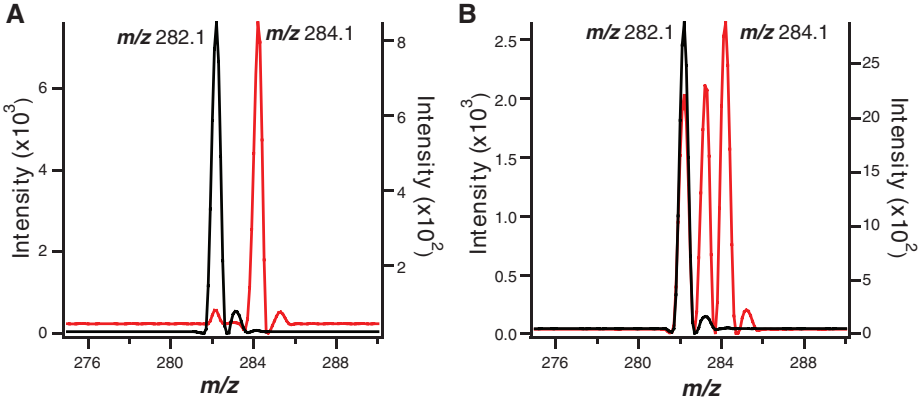

Fig. 3. Mass spectra of CH_3-Ad products derived from $RlmN_{WT}$ and Cfr produced in and isolated from *E. coli* grown in the presence or absence of d_3-Met. Each experiment contains 250 μM 7-mer RNA substrate with (**A**) 400 μM $RlmN_{WT}$ and 1 mM d_3-SAM (black trace) or 400 μM $RlmN_{WT}$ (d_3-Met) with 1 mM SAM (red trace) or (**B**) 300 μM Cfr and 1 mM d_3-SAM (black trace) or 300 μM Cfr (d_3-Met) with 1 mM SAM (red trace). Peaks at *m/z* 282.1 correspond to m^2A ($RlmN_{WT}$) or m^8A (Cfr) with no deuterium enrichment, whereas peaks at *m/z* 284.1 correspond to m^2A ($RlmN_{WT}$) or m^8A (Cfr) with two deuterium atoms. No major peaks at *m/z* 285.1 are observed, which would correspond to m^2A ($RlmN_{WT}$) or m^8A (Cfr) with three deuterium atoms.

To test the possibility that the methyl group transferred in the first turnover derives from the protein, both $RlmN_{WT}$ and Cfr were produced in and isolated from an *E. coli* methionine auxotroph cultured in the presence of [methyl-d_3] methionine (d_3-Met) and then used in subsequent activity determinations (*30*). Figure 3 shows mass spectra of the associated products. As described above, when unlabeled $RlmN_{WT}$ (Fig. 3A) or Cfr (Fig. 3B) is incubated with d_3-SAM (black traces), the CH_3-Ad products are found to lack deuterium (*m/z* 282.1). In contrast, when $RlmN_{WT}$ (Fig. 3A) or Cfr (Fig. 3B) isolated from *E. coli* supplemented with d_3-Met during growth [RlmN (d_3-Met) and Cfr (d_3-Met), respectively] is incubated in the presence of unlabeled SAM (red traces), the CH_3-Ad products exhibit peaks corresponding to *m/z* = 284.1, two mass units higher. This result implies that the transferred methyl group contains two deuteriums and thus does derive from the protein. The finding that a peak for *m/z* = 285.1 (corresponding to three deuteriums) is not observed implies that methyl transfer takes

place with the loss of one deuterium atom. This result stands in contrast to the accepted mechanism of SAM-dependent methyl transfer, in which the methyl group is transferred without exchange of any of its three hydrogens (*4*, *5*). The ladder of *m/z* values (284.1, 283.1, and 282.1) seen with Cfr (d_3-Met) (Fig. 3B, red trace) suggests the presence of a step that effects hydrogen exchange with solvent either before or after methyl transfer.

In all RS enzymes characterized to date, SAM is reductively cleaved to a 5′-dA• intermediate. Many RS enzymes catalyze abortive cleavage of SAM in the presence of dithionite, which can complicate efforts to assess the role of the 5′-dA• (*31*, *32*). As has been shown previously, $RlmN_{WT}$ and Cfr both cleave SAM abortively, even in the absence of substrate (fig. S4) (*28*, *33*). Studies on other RS enzymes have shown that abortive cleavage is reduced substantially—though not always completely (fig. S4)—when dithionite is replaced by the in vivo reducing system (Flv/Flx/NADPH) (*31*, *34*, *35*). However, as described above, the in vivo reducing system does not support turnover of the 7-mer RNA substrate. Studies by Yan *et al*. have shown that a segment of 23*S* rRNA spanning nucleotides 2018 to 2788 supports turnover at least as well as the entire 23*S* rRNA (*28*). Therefore, the corresponding 771-nucleotide RNA substrate was synthesized by in vitro transcription and used in single-turnover activity determinations (*29*). Figure 4 shows mass spectra of 5′-dA analyzed from reactions conducted with RlmN (d_3-Met) (Fig. 4A) and Cfr (d_3-Met) (Fig. 4B) in the presence of unlabeled SAM. The dotted red line in both panels corresponds to a 5′-dA standard at natural abundance, which displays the expected *m/z* = 252.1. The solid black line in both panels corresponds to 5′-dA generated during turnover. Astonishingly, the results demonstrate substantial deuterium enrichment in 5′-dA, implying that the role of the 5′-dA• is not to abstract a hydrogen atom from C-2 or C-8 of the substrate as previously suggested (*28*), but instead to abstract a hydrogen atom from a protein-bound methyl group (*36*).

To identify the amino acid that donates a methyl group, we performed MS on tryptic digests of $RlmN_{WT}$ that had been reduced and alkylated with iodoacetamide (*29*). One peptide (GDDIDAAC^{355}GQLAGDVIDR) (*37*) contained a methylated Cys residue (*m/z* 909.42017, 45 spectra, 76%) or was alkylated by iodoacetamide (*m/z* 930.92358, 14 spectra, 24%). The masses of the intact precursors are within 3 parts per million of the theoretical values, and both the *b*- and *y*-ion series produced by fragmentation unambiguously define the site of the methyl modification as Cys^{355} (fig. S5).

A mechanism for catalysis by $RlmN_{WT}$ that is consistent with observations is proposed in Fig. 5. In the initial, priming step, a methyl group from the first molecule of SAM is transferred to

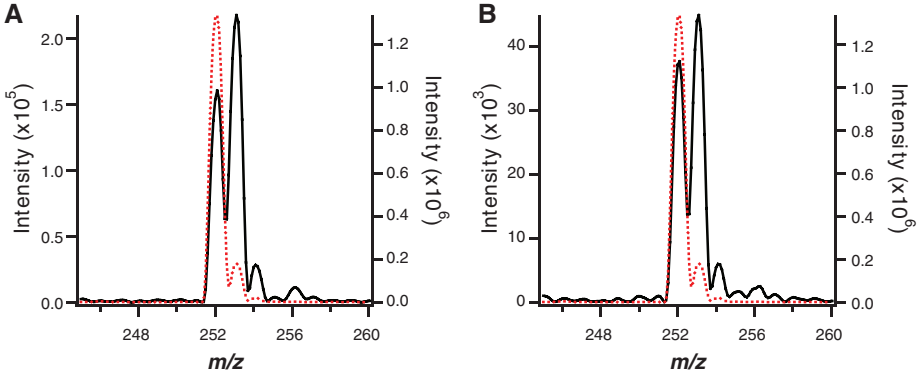

Fig. 4. Analysis of 5′-dA isolated from reactions conducted with RlmN (d_3-Met) and Cfr (d_3-Met) in the presence of unlabeled SAM, (**A**) 150 μM RlmN (d_3-Met), and (**B**) 150 μM Cfr (d_3-Met). Both reactions contained 1.5 mM SAM (black trace), 20 μM 771-mer RNA substrate, and the Flv/Flx reducing system. The red dotted traces correspond to the mass spectrum of commercially available 5′-dA.

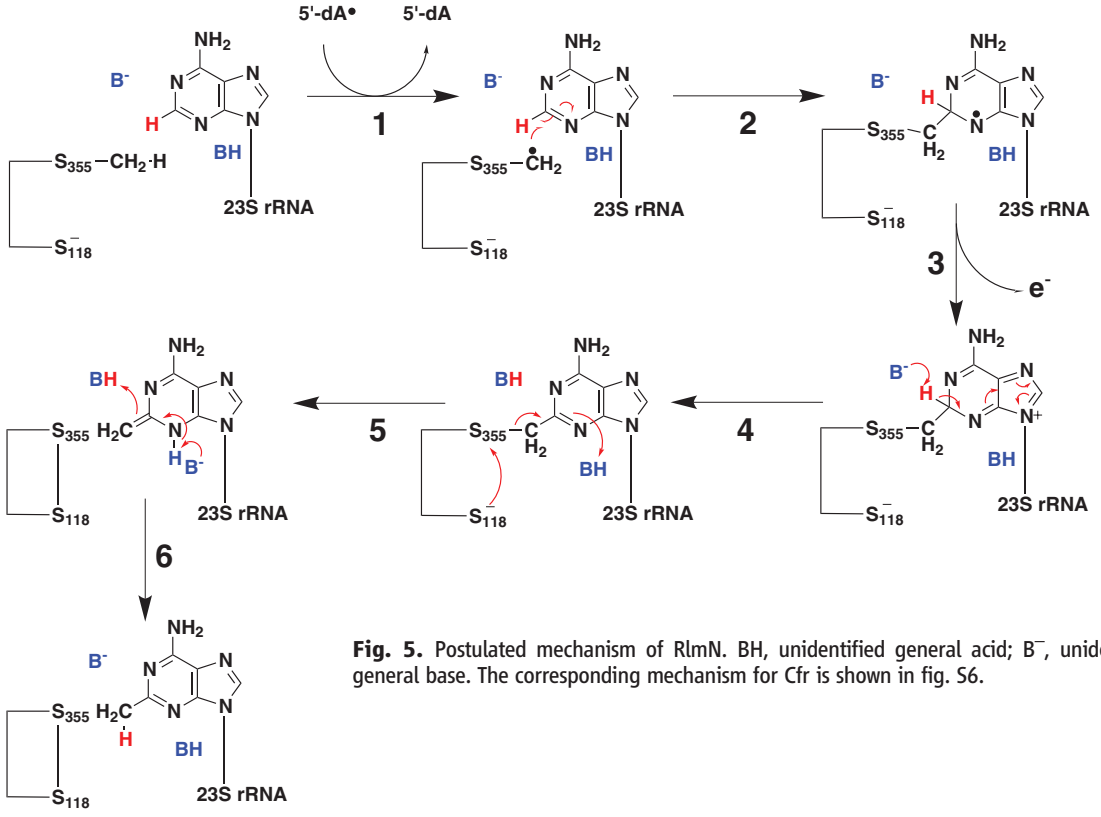

Fig. 5. Postulated mechanism of RlmN. BH, unidentified general acid; B$^-$, unidentified general base. The corresponding mechanism for Cfr is shown in fig. S6.

Cys^{355} by a typical S_N2 displacement mechanism. Reductive cleavage of a second SAM molecule gives the 5′-dA• radical, which, in the subsequent step of the mechanism (**1**), abstracts a hydrogen atom from the protein-bound methyl group to yield a neutral, carbon-centered radical. Attack of the carbon-centered radical on sp^2-hybridized C-2 of A2503 in 23*S* rRNA results in formation of a carbon–carbon bond and generation of a resonance-delocalized radical on the nucleotide base (**2**). Loss of an electron (**3**)—perhaps back to the Fe/S cluster—and abstraction of the proton from C-2 give the alkylated product cross-linked to the protein via Cys^{355} (**4**). This cross-link is resolved by attack of the Cys^{118} thiolate (vide infra) onto Cys^{355} to yield a disulfide bond and an enamine (**5**), which collapses to the methylated product upon tautomerization and acquisition of a proton (**6**). An analogous mechanism for Cfr-catalyzed methylation of C-8 is depicted in fig. S6. The mechanism provides rationale for the exchange of solvent hydrons into the product via reversible imine/enamine tautomerization initiated by cleavage of the thioether linkage upon disulfide formation.

The mechanism also allows rationalization of previously published results that were incompletely understood (*22*). Both RlmN and Cfr contain five conserved Cys residues, of which three are in the canonical C^{112}xxxC^{116}xxC^{119} (*S. aureus* Cfr numbering) RS motif. In vitro mutagenesis studies by Yan *et al*. showed, as expected, that substitution of any of these Cys residues with Ala residues abrogates turnover (*28*), consistent with in vivo studies by Giessing *et al*. and Kaminska *et al*. (*7*, *22*). Further studies by Kaminska *et al*. showed that the remaining two conserved Cys residues (Cys^{105} and Cys^{338}) are also absolutely required for generation of m^8A by Cfr. The Cys^{105}→Ala^{105} (C105A) substitution resulted in a stop in a reverse transcription reaction used to assess modification of the RNA substrate, suggesting that some type of modification took place. However, failure of the C105A variant to elicit resistance to florfenicol and tiamulin in vivo suggested that the 23*S* rRNA did not contain the m^8A modification (*22*). $RlmN_{C118A}$ used in our studies displays a maximum wavelength at 265 nm (fig. S7B) rather than the 280 nm that is observed for $RlmN_{WT}$ (fig. S7A), suggesting that it contains bound nucleic acid. Figure S8 depicts an elution profile of $RlmN_{C118A}$ subjected to digestion with P1 nuclease and alkaline phosphatase, which shows not only the canonical RNA bases but also pseudouridine and other modified bases, confirming the presence of RNA. These characteristics are not observed for WT Cfr (fig. S7D), $RlmN_{WT}$ (fig. S7A), or $RlmN_{C355A}$ (fig. S7C), consistent with a model in which Cys^{118} is required to resolve the covalently bound intermediate.

This strategy of generating a covalent methylcysteinyl intermediate obviates a potential steric clash between the hydrogen-abstracting species and the methyl-donating species, given that both would need to occupy the same space for proper orbital alignment for transfer to or from an sp^2-hybridized carbon. Moreover, there is no precedent in enzymology for abstracting a hydrogen atom from an sp^2-hybridized carbon. Furthermore, a Cys residue is an energetically favorable methyl carrier for radical addition, because adjacent sulfur atoms stabilize carbon-centered radicals and therefore decrease the homolytic bond-dissociation energy associated with their generation (*38*). The ability of Cys residues to form reversible disulfide bonds allows for reductive cleavage of the otherwise stable thioether covalent adduct.

Although the proposed mechanisms of RlmN and Cfr are similar, there are a few differences. Cfr appears to have evolved directly from RlmN using the same mechanistic strategy to methylate C-8 of A2503, but still retaining some ability to methylate C-2. Cfr's bifunctional activity suggests that its substrate-binding pocket is less rigid, allowing for at least two different conformations of A2503 that would put either C-2 or C-8 in resonance with the enzyme's catalytic machinery. This flexibility might be accompanied by access of solvent to the active site, which manifests itself in exchange of solvent-derived hydrons with the product. Or, in the absence of solvent access to the active site, isotope exchange in the case of Cfr might result from a lysyl residue acting as a general acid/base, whereas in the case of RlmN it would be a monoprotic residue.

A number of other RS enzymes have been predicted to catalyze methyltransfer to unactivated carbon atoms in pathways that involve the biosynthesis of a number of secondary metabolites (*39*), as well as specific bacteriochlorophylls (*40*). These enzymes are all annotated as cobalamin-binding proteins, and cobalamin is believed to be the source of the appended methyl group (*41*, *42*). Our study shows how such a reaction might be accomplished without this additional complex cofactor.

References and Notes

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29. Details and methods are provided in the supporting material on *Science* Online.
30. The expression of the *yfgB* or *cfr* genes in an *E. coli* methionine auxotroph cultured in the presence of [methyl-d_3]methionine results in RlmN and Cfr containing deuterated methionine residues. More importantly, any methylated residue would bear a deuterated methyl group, because methionine is used biosynthetically to make SAM, which donates the methyl group in vivo during production of the protein.
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36. The peak at *m/z* = 252.1 in the black traces (RlmN and Cfr reactions) derives predominantly from abortive cleavage of SAM (fig. S4), in which the 5′-dA• abstracts a hydrogen atom that is ultimately solvent-derived, but also from a fraction of the enzyme that is isolated in its unmethylated state and is remethylated by unlabeled SAM.
37. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Methods
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