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Cfr and RlmN Contain a Single [4Fe-4S] Cluster, which Directs Two Distinct Reactivities for *S*-Adenosylmethionine: Methyl Transfer by S_N2 Displacement and Radical Generation

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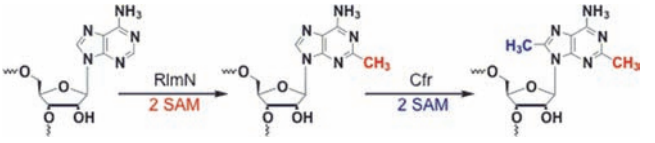
Supporting Information

ABSTRACT: The radical SAM (RS) proteins RlmN and Cfr catalyze methylation of carbons 2 and 8, respectively, of adenosine 2503 in 23*S* rRNA. Both reactions are similar in scope, entailing the *synthesis* of a methyl group partially derived from *S*-adenosylmethionine (SAM) onto electrophilic sp^2-hybridized carbon atoms via the intermediacy of a protein *S*-methylcysteinyl (mCys) residue. Both proteins contain five conserved Cys residues, each required for turnover. Three cysteines lie in a canonical RS CxxxCxxC motif and coordinate a [4Fe-4S]-cluster cofactor; the remaining two are at opposite ends of the polypeptide. Here we show that each protein contains only the one "radical SAM" [4Fe-4S] cluster and the two remaining conserved cysteines do not coordinate additional iron-containing species. In addition, we show that, while wild-type RlmN bears the C355 mCys residue in its as-isolated state, RlmN that is either engineered to lack the [4Fe-4S] cluster by substitution of the coordinating cysteines or isolated from *Escherichia coli* cultured under iron-limiting conditions does not bear a C355 mCys residue. Reconstitution of the [4Fe-4S] cluster on wild-type apo RlmN followed by addition of SAM results in rapid production of *S*-adenosylhomocysteine (SAH) and the mCys residue, while treatment of apo RlmN with SAM affords no observable reaction. These results indicate that in Cfr and RlmN, SAM bound to the unique iron of the [4Fe-4S] cluster displays two reactivities. It serves to methylate C355 of RlmN (C338 of Cfr), or to generate the 5′-deoxyadenosyl 5′-radical, required for substrate-dependent methyl synthase activity.

The addition of a methyl group to an acceptor molecule is among the most critical of cellular reactions. Although methyl groups can emanate from several complex cofactors (e.g., methylcobalamin and methylene- and methyltetrahydrofolate), the vast majority derive from the more understated molecule, *S*-adenosyl-L-methionine (SAM).[1−3] Seminal model and enzymatic studies on SAM-dependent methyl-transfer reactions argue for a polar process, in which an appropriate nucleophile attacks the methyl group of SAM with concomitant elimination of *S*-adenosyl-L-homocysteine (SAH).[4−6] Until recently, a direct S_N2 displacement was the only means by which methyl groups derived from SAM were thought to be appended onto acceptor atoms.[7,8] However, RlmN and Cfr catalyze methyl transfer as well as methyl *synthesis* via radical-dependent *methylene* transfer to electrophilic carbon atoms.[7,8]

Scheme 1. Reactions Catalyzed by RlmN and Cfr in Vivo



Escherichia coli (*Ec*) RlmN and *Staphylococcus aureus* (*Sa*) Cfr share 33% sequence identity and modify the same nucleotide in 23*S* rRNA, adenosine 2503 (A2503). Although RlmN and Cfr act preferentially on naked rRNA,[9] A2503 resides ultimately in the peptidyltransferase center of the 50*S* subunit of the bacterial ribosome.[10−13] Methylation of C2 by RlmN is found throughout eubacteria (Scheme 1).[14] Although this activity is nonessential, *Ec* mutants that lack it lose to wild-type (wt) *Ec* in cogrowth competition experiments.[15] Cfr, which is evolutionarily related to RlmN, also can catalyze methylation of C2 of A2503; however, C8 is its preferred target (Scheme 1).[16] Methylation of C8 confers bacterial resistance to multiple classes of antibiotics, including phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A, as well as the macrolides josamycin and spiramycin.[17]

Cfr and RlmN are members of the radical SAM (RS) superfamily, enzymes that cleave SAM reductively to a 5′-deoxyadenosyl 5′-radical (5′-dA•).[18−21] The common feature of RS enzymes is the use of the 5′-dA• to abstract key substrate hydrogen atoms. Cleavage of SAM requires an electron, which is supplied by a reduced $[4Fe\text{-}4S]^+$ cluster. The Cys residues that coordinate this essential [4Fe-4S] cluster typically reside in a CxxxCxxC motif,[21] although exceptions have been reported.[22−24]

Our recent mechanistic studies of RlmN and Cfr have unraveled an unprecedented strategy for SAM-dependent methylation of an sp^2-hybridized carbon atom.[7] When RlmN or Cfr was incubated with *S*-adenosyl-L-[*methyl*-d_3]-methionine (d_3-SAM) or unlabeled SAM under single-turnover conditions, the isotopic composition of the methyl group incorporated at C2 (RlmN) or C8 (Cfr) of the target adenosine nucleotide did not always reflect that of the methyl donor in the reaction, but rather the isotopic composition of L-methionine in the growth media of the *Ec* used for overproducing the protein. Consistent with this observation, analysis of RlmN by high-resolution mass spectrometry[7] and X-ray crystallography[25] showed the as-isolated (AI) protein to

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bear a methylcysteinyl (mCys) residue at C355. Moreover, methylation always involved incorporation of one hydrogen atom from the mCys residue into 5′-dA, indicating that the 5′-dA• activates the methyl group for radical addition rather than abstracts a hydrogen atom from the nucleotide to be modified. Cleavage of an ensuing methylene-bridged protein−nucleic acid cross-link was proposed to be catalyzed by disulfide-bond formation—with participation of a second absolutely conserved Cys residue (C118 in RlmN; C105 in Cfr), as shown in Scheme S1.[7]

A related study by Yan et al. also found that the 5′-dA• radical does not abstract a hydrogen atom from the substrate nucleotide. In that study, however, the authors were unaware of the initial transfer of a methyl group from SAM to C355 of RlmN (C338 of Cfr), and therefore proposed that the 5′-dA• abstracts a hydrogen atom from the methyl group of a second, simultaneously bound, SAM molecule, activating it for radical addition to the target nucleotide.[8]

The recent X-ray crystal structure of RlmN with SAM bound (2.05 Å)[25] is consistent with the mechanism proposed by Grove et al.[7] The structure shows C355 to reside in a flexible loop containing residues 350−358, which are strictly conserved. This loop is disordered in the absence of SAM but is visible in the RlmN+SAM structure. Only one SAM-binding site was identified in the structure, wherein SAM is coordinated to the unique iron of the [4Fe-4S] cluster via its α-amino and α-carboxy groups, as is observed for other structurally characterized RS proteins.[26] Although it was speculated that the related protein, Cfr, contains two Fe/S clusters,[27] this stoichiometry is not supported by the structure of RlmN. The structure shows C355 to be *S*-methylated, and to be located ∼6 Å from the side chain of C118 and C5′ of SAM. This arrangement is consistent with disulfide-bond formation between C355 and C118 to resolve the protein−nucleic acid covalent adduct as well as abstraction of a hydrogen atom from the C355 mCys by the 5′-dA• as proposed by Grove et al.[7] The absence of any other obvious SAM binding site led to the proposal that SAM coordinated to the [4Fe-4S] must exhibit dual functionality; it acts as both a methylating agent and as the source of the 5′-dA• intermediate.[25] In this work we use Mössbauer spectroscopy in concert with quantitative analyses of iron and sulfide to show that both RlmN and Cfr contain only *one* [4Fe-4S] cluster, which is coordinated by cysteines in the canonical CxxxCxxC motif. This finding implies that the two remaining conserved Cys residues do not coordinate Fe/S species and are free to participate in other modes of catalysis. In addition, we overproduce RlmN in *Ec* under iron-limiting conditions and show that the AI protein is not *S*-methylated at C355 and does not catalyze methylation of C355 when incubated with SAM. Reconstitution of the protein with iron and sulfide followed by addition of SAM results in rapid methylation of C355, consistent with the proposal that SAM coordinated to the [4Fe-4S] cluster has a dual function.

To determine the stoichiometry and configuration of iron-containing species associated with Cfr and RlmN, both proteins were purified from *Ec* cultured in minimal media supplemented with ^{57}Fe, and then analyzed by Mössbauer spectroscopy. The UV−visible spectra of both AI proteins are consistent with the presence of [4Fe-4S] clusters (Figures S1 and S2), in agreement with previous studies.[9,27] The 4.2-K/53-mT spectrum of as-isolated (AI) wild-type (wt) RlmN ($RlmN_{wt}$) (Figure 1A) is dominated by a broad quadrupole doublet with parameters typical of $[4Fe\text{-}4S]^{2+}$ clusters [isomer shift (δ) of 0.44 mm/s and quadrupole splitting parameter (ΔE_Q) of 1.14 mm/s], and accounts for 93% of the total intensity. Together with the ratio of 4.0 Fe per polypeptide, determined by quantitative analyses of acid-labile iron and sulfide (Table S2), the Mössbauer spectrum reveals a stoichiometry of 0.9 [4Fe-4S] clusters per AI $RlmN_{wt}$. Reconstitution of AI $RlmN_{wt}$ with additional Fe and sulfide (RCN $RlmN_{wt}$) does not result in an increase of Fe and sulfide associated with the protein (4.1 Fe per polypeptide). Consistent with this observation, the 4.2-K/53-mT Mössbauer spectrum of RCN $RlmN_{wt}$ (Figure 1B) is virtually identical to that of AI $RlmN_{wt}$ (95% of total intensity attributable to the above quadrupole doublet) and reveals that RCN $RlmN_{wt}$ harbors 1.0 [4Fe-4S] cluster, which is presumably coordinated by the cysteines of the canonical CxxxCxxC RS motif: C125, C129, and C132. Consistent with this hypothesis, the variant, in which C125, C129, and C132 have been replaced with non-coordinating Ala residues, harbors less than 0.02 [4Fe-4S] clusters per protein (Figures S3−S5).

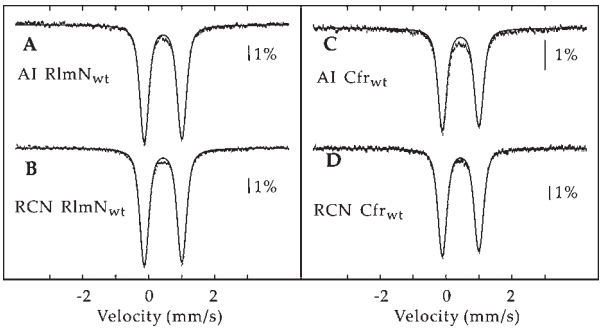


Figure 1. 4.2-K/53-mT Mössbauer spectra of RlmN and Cfr (vertical bars) and quadrupole doublet simulations with parameters quoted in the text (solid lines).

In addition to C125, C129, and C132, RlmN has two additional, strictly conserved, cysteines that are absolutely required for complete turnover: C118 and C355.[28] To show that they do not ligate Fe/S species, Cys→Ala variants of those residues ($RlmN_{C118A}$ and $RlmN_{C355A}$) were engineered and studied with a combination of biochemical and spectroscopic methods. $RlmN_{C118A}$ and $RlmN_{C355A}$ contain 4.0−4.6 Fe per polypeptide in both their AI and RCN forms (Table S2), and display spectroscopic features and cluster stoichiometries that are virtually identical to those of $RlmN_{wt}$, supporting the presence of one [4Fe-4S] cluster per polypeptide (Figures S6 and S7) ligated by C125, C129, and C132.

The 4.2-K/53-mT spectrum of AI Cfr_{wt} (Figure 1C) is dominated by a broad quadrupole doublet indicative of $[4Fe\text{-}4S]^{2+}$ clusters (δ = 0.44 mm/s and ΔE_Q = 1.10 mm/s), which accounts for 86% of the total intensity. The broad and poorly resolved absorption extending from -2 to 2 mm/s (15% of total Fe) is assigned to unspecifically bound iron, because an identical sample does not display EPR features associated with Fe/S clusters with S = 1/2 ground state (Figure S5). Together with the ratio of 4.5 Fe per polypeptide (Table S2), the Mössbauer spectrum reveals a stoichiometry of 1.0 [4Fe-4S] cluster per AI Cfr_{wt}. Reconstitution of AI Cfr_{wt} with additional Fe and sulfide followed by purification by anaerobic molecular-sieve chromatography (RCN Cfr_{wt}) results in a slight decrease of Fe. The 4.2-K/53-mT Mössbauer spectrum of RCN Cfr_{wt} (Figure 1D) can be simulated with a quadrupole doublet with identical parameters, and accounts for 98% of the total intensity. Thus, the results suggest that RCN Cfr_{wt} harbors 1.0 [4Fe-4S] cluster, which is coordinated by C112, C116, and C119. We therefore conclude, that in contrast to radical SAM proteins that catalyze

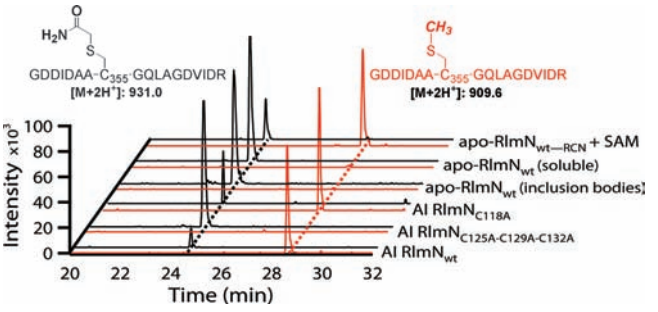


Figure 2. Extracted ion chromatograms (EIC) for the C355-containing peptide from trypsin digests of RlmN. Black traces are EIC of m/z 931.0 [M + 2H], indicative of carbamidomethyl modification to C355. Red traces are EIC of m/z 909.6 [M + 2H], indicative of a methyl modification of C355. Traces correspond to (A) AI RlmN$_{wt}$; (B) AI RlmN$_{C125A-C129A-C132A}$; (C) AI RlmN$_{C118A}$; (D) apo RlmN$_{wt}$, inclusion bodies; (E) apo RlmN$_{wt}$, soluble fraction; (F) apo RlmN$_{wt\rightarrow RCN}$ after addition of 1.5 mM SAM. Spectral pair intensities are normalized to the most abundant EIC.

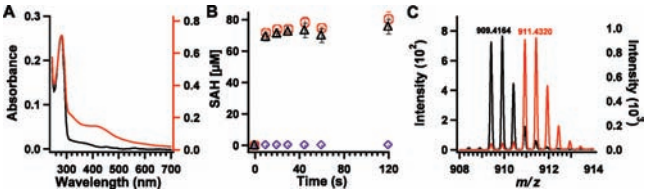


Figure 3. (A) UV−vis traces of apo RlmN$_{wt}$ (5 μM, solid black line) and apo RlmN$_{wt\rightarrow RCN}$ (12 μM, solid red line). (B) Methyl transfer catalyzed by apo RlmN$_{wt}$ (150 μM) or apo RlmN$_{wt\rightarrow RCN}$ (150 μM). Production of SAH by apo RlmN$_{wt}$ (purple diamonds) or apo RlmN$_{wt\rightarrow RCN}$ (red circles) in the presence of 1.5 mM SAM; or apo RlmN$_{wt\rightarrow RCN}$ (150 μM) in the presence of 1.5 mM d_3-SAM (black triangles). Error bars indicate one standard deviation from the average of three assays. (C) Q-Tof MS analysis of tryptic peptides from apo RlmN$_{wt\rightarrow RCN}$ after incubation in the presence of SAM (black trace) or d_3-SAM (red trace). Indicated m/z values correspond to the +2 charge state.

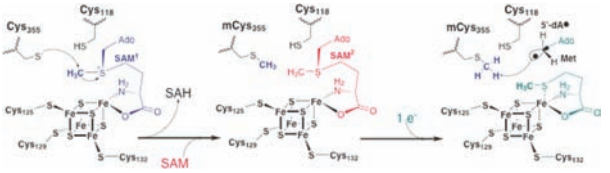
methylthiolation, which contain two distinct Fe/S clusters,[18,29,30] RlmN and Cfr only contain the cluster housed in the canonical CxxxCxxC motif.

While previous studies have firmly established one role for the [4Fe-4S] cluster in the unique methylation reactions catalyzed by RlmN and Cfr[7−9,25] (reductive cleavage of SAM to generate the 5′-dA• intermediate), the role of the [4Fe-4S] cluster, if any, in the first step of the reaction, methylation of a conserved cysteine (C355 in RlmN and C338 in Cfr), has not been addressed. Interestingly, when the RlmN$_{C125A-C129A-C132A}$ triple variant was analyzed by ESI$^+$-MS after alkylation with iodoacetamide and digestion with trypsin, the peptide containing C355 was found exclusively carbamidomethylated, indicating that it did not bear a mCys residue (Figure 2B). This observation contrasts with results obtained for RlmN$_{wt}$, wherein almost 90% of the AI protein contained a mCys residue, and very little of it was alkylated by iodoacetamide during preparation for analysis by mass spectrometry (Figure 2A).[7] To assess the effect of the iron−sulfur (Fe/S) cluster on generation of the mCys residue in RlmN, apo RlmN$_{wt}$ was produced by expression of the *Ec yfgB* gene, which encodes RlmN, in a modified M9 minimal medium containing 75 μM *o*-phenanthroline to chelate available iron (see Supporting Information for experimental details).[31] A large fraction of RlmN was produced in inclusion bodies when expression was carried out in this manner. The soluble fraction was isolated anaerobically under conditions identical to those for isolation of holo RlmN, and a portion was set aside for reconstitution of its [4Fe-4S] cluster with Fe and sulfide by standard methods.[32] Figure 3A shows UV−vis spectra of apo RlmN$_{wt}$ isolated from *Ec* cultured in *o*-phenanthroline-containing medium (solid black line), and RlmN$_{wt}$ reconstituted with iron and sulfide (apo RlmN$_{wt\rightarrow RCN}$) (solid red line). As can be observed, the telltale features of [4Fe-4S] clusters are significantly diminished in apo RlmN$_{wt}$, consistent with the finding of 0.04 Fe and 0.07 sulfides per polypeptide (Table S2), but reappear in the apo RlmN$_{wt\rightarrow RCN}$ sample. Analysis of trypsin-digested apo RlmN$_{wt}$ by LC−MS showed that it did not bear a C355 mCys modification, consistent with the premise that SAM bound to the [4Fe-4S] cluster acts as a methylating agent (Figure 2E). To ensure that in vivo methylation of apo RlmN$_{wt}$ was not the factor that distinguished soluble protein from insoluble protein, apo RlmN$_{wt}$ inclusion bodies were also analyzed by LC−MS; fragments obtained from trypsin digests of the inclusion body fraction did not bear a C355 mCys modification (Figure 2D).

Reconstitution of apo RlmN$_{wt}$ followed by further purification by size-exclusion chromatography afforded a protein containing 2.2 irons and 1.2 sulfides, respectively, per polypeptide (Table S2). This stoichiometry implies that 50% of the RCN protein contained a [4Fe-4S] cluster, given that its UV−vis spectrum does not contain telltale signatures of [2Fe-2S] clusters, and most, if not all, of the adventitiously bound iron was removed by molecular-sieve chromatography. When SAM was added to apo RlmN$_{wt}$, methyl transfer to C355 did not occur, as evidenced by no significant production of SAH (Figure 3B, purple diamonds). By contrast, when SAM or d_3-SAM was added to apo RlmN$_{wt\rightarrow RCN}$, ~0.5 equiv of SAH was produced within the first time point (15 s). Subsequent time points did not show increased amounts of SAH, suggesting that 50% of apo RlmN$_{wt}$ was reconstituted to its holo form (Figure 3B, red circles). Importantly, the quantity of SAH produced was directly proportional to the RlmN cluster stoichiometry, providing further evidence that the [4Fe-4S] cluster is required for methyl transfer. In addition, apo RlmN$_{wt\rightarrow RCN}$ was capable of catalyzing time-dependent methylation of the target nucleotide in a 771-base synthetic RNA substrate (Figure S10). Analysis of apo RlmN$_{wt\rightarrow RCN}$ by LC−MS after incubating it with SAM reveals a peak exhibiting a mass-to-charge ratio (m/z) of 909.6 (+2 charge state) for the C355-containing peptide fragment obtained after trypsin digestion, indicating that it bears a methyl group (Figure 2F, red and black traces). Moreover, the peak at m/z = 909.6 shifts to 911.2 (+2 charge state; thus the shift is ~1.5 units instead of the expected 3 for the +1 charge state) when apo RlmN$_{wt\rightarrow RCN}$ is incubated with d_3-SAM and similarly analyzed (Figure 3C red trace). This modification is specific to C355 of RlmN, given that MS/MS analysis of the peptides shows that the y^+ ion series is shifted by +15 for fragments that bear C355 ($y_{11}{}^{+1}$ and above) (Figure S8).[7]

The effect of C118, another Cys residue that plays a key role in RlmN catalysis, on methylation of C355 was also assessed. As observed in Figure 2C, a large fraction of the AI C118A variant contains a mCys modification, consistent with this residue playing an insignificant role in SAM binding and methylation of C355. In addition, the inclusion of the flavodoxin/flavodoxin reductase/NADPH reducing system had no effect on C355 methylation, indicating that methyl transfer to C355 does not require a reduced [4Fe−4S] cluster (Figure S9), and therefore

Scheme 2. SAM Bound to the [4Fe-4S] Cluster of RlmN or Cfr Is Activated toward Two Distinct Reactivities



most likely proceeds by a polar nucleophilic displacement, as do all characterized SAM-dependent methyltransferases.[1]

Herein we have shown that RlmN and Cfr catalyze two separate and distinct reactions, exploiting both polar and radical reactivities of SAM within a single polypeptide (Scheme 2). Indeed, these proteins both possess methyltransferase and methylsynthase activities. They contain only *one* [4Fe-4S] cluster (ligated by cysteines in CxxxCxxC motifs) to which SAM binds via its α-amino and carboxy groups.[25] In this conformation, methyl transfer to unmodified C355 of RlmN (C338 of Cfr) takes place rapidly. Release of the product, SAH, permits binding of a second molecule of SAM to the exact same site; however, methyl transfer to the reactive cysteine is now blocked. Upon binding of the RNA substrate and the addition of an electron to the $[4Fe\text{-}4S]^{2+}$ cluster, SAM is now induced to fragment into methionine and the 5′-dA•, the latter initiating catalysis by abstracting a hydrogen atom from the mCys residue. Our studies show that methyl transfer to the target Cys residue does not require the presence of substrate. Whether substrate binding accelerates this step is yet to be determined. However, our previous studies showed that substrate binding does indeed effect radical formation when the physiological reducing system is used to supply the requisite electron.[7] Excitingly, this mode of catalysis is a clever twist on the "principle of economy in the evolution of binding sites," wherein Nature evolves only a single substrate-binding site for reactions that involve two or more substrates with similar structural elements, and then mediates transfer of a group from one to the other by means of an intermediate enzyme functional group covalent adduct.[33] The Cfr and RlmN reactions are the first recognized instances in which a single substrate-binding site activates one molecule both for polar and radical-based chemistry.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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