Mechanistic Diversity of Radical S-Adenosylmethionine (SAM)-
dependent Methylation

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Radical S-adenosylmethionine (SAM) enzymes use the oxidizing power of a 5′-deoxyadenosyl 5′-radical to initiate an amazing array of transformations, usually through the abstraction of a target substrate hydrogen atom. A common reaction of radical SAM (RS) enzymes is the methylation of unactivated carbon or phosphorous atoms found in numerous primary and secondary metabolites, as well as in proteins, sugars, lipids, and RNA. However, neither the chemical mechanisms by which these unactivated atoms obtain methyl groups nor the actual methyl donors are conserved. In fact, RS methylases have been grouped into three classes based on protein architecture, cofactor requirement, and predicted mechanism of catalysis. Class A methylases use two cysteine residues to methylate sp^2^-hybridized carbon centers. Class B methylases require a cobalamin cofactor to methylate both sp^2^-hybridized and sp^3^-hybridized carbon centers as well as phosphate phosphorous atoms. Class C methylases share significant sequence homology with the RS enzyme, HemN, and may bind two SAM molecules simultaneously to methylate sp^2^-hybridized carbon centers. Lastly, we describe a new class of recently discovered RS methylases. These Class D methylases, unlike Class A, B, and C enzymes, which use SAM as the source of the donated methyl carbon, are proposed to methylate sp^2^-hybridized carbon centers using methylenetetrahydrofolate as the source of the appended methyl carbon.

The prevalence and significance of methylation reactions in biology is well established (1, 2). Methyl groups are appended to a wide array of biological molecules, including numerous small-molecule metabolites and natural products, and various macromolecules, such as proteins, DNA, RNA, carbohydrates, and lipids. In the vast majority of methylation reactions, S-adenosyl-l-methionine (SAM)^2 is the source of the appended methyl group (3, 4). In classical methyltransferase (MTase) reactions, strong nucleophiles such as R-O^- attack the sp^3^-hybridized methyl group of SAM to generate the methylated product with concomitant formation of S-adenosyl-l-homocysteine (SAH) (2, 5, 6). Although less common, weakly nucleophilic, sp^2^-hybridized carbon atoms are also known to react in this manner, but with nucleophilic assistance by an active-site cysteine residue, as in C5 methylation of cytosines by DNA methyltransferases (7–10). Relatively recently, however, it has come to light that SAM, and in some instances additional cofactors, can be used to methylate inert carbon or phosphorous atoms via pathways involving radical intermediates. These reactions are catalyzed exclusively by radical SAM (RS) enzymes (11).

RS enzymes (Pfam PF04055), now comprising over 113,000 unique sequences (12), catalyze the reductive cleavage of SAM to a highly oxidizing 5′-deoxyadenosyl 5′-radical (5′-dA), which initiates radical-based transformations by strategically abstracting a substrate hydrogen atom (H^+)(13–15). All of these enzymes require at least one [4Fe-4S] cluster, which supplies the requisite electron for reductive cleavage (16). The diversity, as well as in many instances the complexity, of transformations catalyzed by RS enzymes is astonishing (14, 15, 17). Of the 113,000 recognized or suspected RS proteins, at least 10,000 are known or predicted to catalyze methylation reactions, although this number is likely to be an underestimation due to the large number of sequences with unknown function. Several review articles on RS methylases have been published in the recent past (11, 18–21). One key review by Zhang et al. (11) assigned these enzymes into three classes (Class A, B, and C) that are distinguished by protein architecture, cofactor requirement, and predicted reaction mechanism. Class A enzymes use two strictly conserved cysteines to catalyze methylation of sp^2^-hybridized carbon centers, whereas Class B enzymes use a cobalamin cofactor to catalyze methylation of phosphinate phosphorous atoms and sp^2^- and sp^3^-hybridized carbon centers. Lastly, Class C enzymes exhibit sequence homology to the RS protein HemN, and may bind two molecules of SAM simultaneously to effect methylation of sp^2^-hybridized carbon centers (11). Herein, we recapitulate some of the most salient features of that review, update current understanding and theory about RS methylases, and describe a new class (Class D) of RS methylases that, unlike Classes A, B, and C, are proposed to use methyltetrahydrofolate rather than SAM as the source of the appended methyl carbon (22).

Class A RS Methylases

Class A RS methylases are represented by RlmN and Cfr, and are the best characterized of these enzymes (Table 1). RlmN catalyzes methylation of C2 of adenosine 2503 (A2503) in 23S rRNA as well as C2 of adenosine 37 (A37) in several Escherichia coli tRNAs and most likely tRNAs in other organisms (23–25). RlmN is ubiquitous or nearly ubiquitous in bacteria, and is also found in limited occurrences in archaea and lower eukaryota (26). It is a nonessential protein in E. coli, but enhances translational fidelity and fitness of the organism (27). Cfr, which is homologous to RlmN, preferentially catalyzes methylation of...
C8 of the exact same nucleotide (A2503) but will also catalyze C2 methylation (28). Cfr-catalyzed C8 methylation confers resistance to at least five classes of currently used antibiotics that target the 50 S subunit of the bacterial ribosome (29–32).

In addition to the three cysteines that ligate the iron-sulfur (Fe/S) cluster cofactor, RlmN and Cfr contain two additional strictly conserved cysteines that are essential for catalysis (33–35). These additional cysteines distinguish Class A enzymes from other RS methylases and contribute to their unique reaction mechanism (11, 33, 35). Initial in vitro characterization of RlmN and Cfr by Fujimori and co-workers (36) demonstrated that both SAH and 5′-dA are produced during the reaction, suggesting that SAM is used both as a radical initiator and as the source of the methyl group. Subsequent studies by the same group showed that only two deuteriums are incorporated in the methylated products when [methyl-3H]SAM is used in the RlmN reaction, indicating that an intact methyl group is not transferred directly from SAM to the RNA substrate (37). Initial biochemical studies showed that the Fe/S cluster is indeed required for methyltransfer from SAM to Cys-355 (38, 39).

Further characterization of the RlmN and Cfr reactions under single turnover conditions suggested that they proceed via a ping-pong mechanism rather than one in which two molecules of SAM and the RNA substrate are bound simultaneously (33). Studies by Booker and co-workers (33, 38, 39) showed that during the first turnover of RlmN and Cfr, when overproduced and purified with the [4Fe-4S] cluster intact, exogenous SAM is not the source of the methyl group. When RlmN and Cfr reactions were run in the presence of [methyl-3H]SAM and a 7-nucleotide RNA substrate, the methylated products were not enriched with deuterium. However, when RlmN and Cfr were overproduced in a methionine auxotroph of E. coli cultured in medium supplemented with [methyl-3H]methionine, the methylated products contained up to two deuteriums when the enzymes were incubated under turnover conditions in the presence of unlabeled SAM. Therefore, in the first turnover, the incorporated methyl group reflected, albeit not fully, the isotopic composition of SAM in the cells. Subsequent high-resolution mass spectrometric analysis of purified RlmN revealed the presence of a methyl moiety appended to Cys-355 of the protein (Cys-338 in Cfr), which was later observed in the x-ray crystal structure of RlmN with bound SAM (33, 38). The x-ray crystal structure of RlmN in the presence of SAM provided strong evidence that the unique iron-ion of the Fe/S cluster serves as the binding site both for the SAM molecule that donates a methyl group to Cys-355 (Cys-338 in Cfr) and for the SAM molecule that is cleaved to the 5′-dA. The structure shows coordination of SAM to the unique iron ion of the [4Fe-4S] cluster, as is typical for RS enzymes, with the sulfur atom of Cys-355 located within 6 Å of the methyl and 5′-carbons of SAM. Further Mössbauer spectroscopic and biochemical studies showed that the Fe/S cluster is indeed required for methyltransfer from SAM to Cys-355 (38, 39).

Based on the above experimental observations as well as those from other laboratories (40), a mechanism was proposed that accounted for all published data and that highlighted the unique roles of the two active-site cysteines of RlmN and Cfr (supplemental Fig. S1) (33). To initiate catalysis, the methyl group of one molecule of SAM is transferred to one of the conserved cysteines via a polar SN2 mechanism, resulting in formation of an S-methylcysteine residue and SAH. After SAH is released, a second molecule of SAM binds and is reductively cleaved to form the 5′-dA, which abstracts a hydrogen atom (H) from a second, simultaneously bound molecule of SAM (37).

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Evidence for the cross-linked intermediate was initially provided in studies in which an RlmN C118A variant, overproduced in and purified from *E. coli*, contained covalently bound RNA (33). In subsequent studies of a similar RlmN variant (C118S), it was shown unequivocally by high-resolution mass spectrometry that Cys-355 was cross-linked to an adenine base via a methylene bridge. EPR spectroscopic studies of the WT Cfr reaction were also used to provide evidence for the cross-linked species and to show both its chemical and its kinetic competence in advancing to the products. When EPR samples containing Cfr, a 155-nucleotide rRNA substrate analog, and dithionite (a low potential reductant used to initiate reductive cleavage of SAM) were recorded, a paramagnetic signal characteristic of an organic radical strongly coupled to a single proton was observed (41). Additional studies using specific isotopically labeled substrates in concert with electron nuclear double resonance spectroscopy and density functional theory showed that the radical was delocalized throughout the adenine ring but had maximum spin density at N7 (41). Moreover, the radical-containing species was shown to be chemically and kinetically competent using rapid-freeze-quench EPR in concert with mass spectrometry to monitor the formation of product, wherein the rate of formation of the m8A product was consistent with the formation and decay rates of the radical species. Therefore, the radical species most likely represents a mechanistically relevant intermediate and provides a launching pad for probing downstream steps of the mechanism (41).

The cognate radical is not observed during turnover of WT RlmN, suggesting that its rate of decay is faster than its rate of formation. Interestingly, the radical is observed in RlmN C118A or C118S variants (35). The EPR spectrum obtained with the C118S variant is consistent with the proposed radical species observed in the Cfr reaction, except the radical is more strongly coupled to C2 of the adenine ring rather than C8, and the majority of spin density is located on N3 rather than N7. Although the radical intermediate forms and decays on the time scale of product formation in the WT Cfr reaction, the radical species obtained with RlmN C118S, or a similar variant in Cfr (C105A), accumulates but does not decay to any significant extent (35). This observation suggests that this second conserved cysteine is necessary for decay of the radical intermediate in both the RlmN and Cfr reactions, implicating it in a role other than, or in addition to, formation of a disulfide bond as initially proposed. Based on these results, three alternate mechanisms were proposed for decay of the paramagnetic species and resolution of the protein-RNA cross-linked intermediate, each involving initial deprotonation at C2 (C8 in Cfr) followed by resolution of the cross-link by a radical fragmentation mechanism. In these scenarios, Cys-118 of RlmN (Cys-105 of Cfr) may (i) participate in a disulfide radical species with Cys-335 after deprotonation at C2 and radical fragmentation; (ii) act as the base that abstracts the C2 proton; or (iii) act in both capacities. The last step would be acquisition of an electron by the resulting thyl radical or disulfide radical anion to regenerate the Cys-355 thiolate (35). Scheme 1 depicts the scenario that we currently favor, in which Cys-118 acts as the base that abstracts the C2 proton.

**Class B RS Methylases**

Class B methylases currently represent the largest and most versatile class of RS methylases (11, 12). In contrast to Class A enzymes, which can only methylate sp²-hybridized carbon atoms, Class B enzymes methylate both sp²-hybridized and sp³-hybridized carbon atoms or phosphinate phosphorous atoms found in a diverse set of substrates, including enzyme cofactors and natural products that exhibit antitumor, antibiotic, or herbicide behavior (Table 1) (11). They are distinguished in architecture from other classes of RS methylases by an N-terminal cobalamin-binding domain in addition to the core RS domain. Although predicted cobalamin-containing methylases were the first RS methylases to be recognized (14), their characterization has lagged considerably behind Class A enzymes due to the difficulty associated with their heterologous overproduction in a soluble form (42–44). Of the four Class B enzymes that have been studied *in vitro* (TsrM, GenK, Fom3, and PhpK), only TsrM was not purified from insoluble inclusion bodies and refolded before reconstitution of its Fe/S cluster (45).

PhpK was the first Class B RS methylase to be purified and characterized *in vitro* (42). It catalyzes the methylation of the phosphinate group of 2-acetylamino-4-hydroxypyrophosphinylbutanoate (NAcDMPT) to produce 2-acetylamino-4-hydroxymethylphosphinylbutanoate (NAcPT), representing the so-called P-methylase family of RS enzymes (11, 46, 47). NAcPT is also known commonly as γ-phosphothreonin or glufosinate, an analog of glutamate that inhibits bacterial and plant glutamine synthetases and serves as the active ingredient in a number of herbicides (48). When Wang and co-workers (42) overproduced PhpK from *Kitasatospora phosalacinea* in *E. coli*, it was found solely in inclusion bodies. The protein was purified from inclusion bodies and refolded, and its Fe/S cluster was reconstituted.
Two-dimensional $^1$H–$^{31}$P gradient heteronuclear single-quantum correlation NMR was used to assess whether the reconstituted enzyme could catalyze the intended reaction. Reactions containing NAcDMPT, SAM, methylcobalamin, and dithionite resulted in formation of a new $^4$H–$^{31}$P cross-peak corresponding to a methyl group attached to phosphorus atom. This cross-peak was not observed in reactions lacking dithionite, suggesting that methylation takes place via a radical-dependent mechanism. When $[^{13}$C]$\text{methyl}$cobalamin was used in PhpK reactions, new cross-peaks were observed corresponding to a methyl group attached to the phosphorus atom of NAcPT (42).

Class B RS methylases also methylate $sp^3$-hybridized carbon centers, as is observed in the reactions catalyzed by Fom3 and GenK. Fom3, one of the first recognized RS methylases, catalyzes the stereoselective methylation of 2-hydroxyethylphosphonate (HEP) to afford $S$-2-hydroxypropylphosphonate (HPP), the penultimate step in the biosynthesis of the broad spectrum antibiotic fosfomycin (47, 49). Early feeding studies using a mutant of 

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\text{Streptomyces wedmorensis}
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that was deficient in cobalamin biosynthesis showed that the organism could no longer produce fosfomycin unless its growth medium was supplemented with hydroxocobalamin. When hydroxocobalamin was replaced with $[^{13}$C]$\text{methyl}$cobalamin, $^{13}$C-labeled fosfomycin was produced, suggesting that methylcobalamin was the source of the appended methyl group (50). More recently, Fom3 was characterized in vitro by Wang and co-workers (43). Like PhpK, when Fom3 was overproduced in 

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\text{E. coli}
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or 

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\text{Streptomyces lividans}
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it was found exclusively in the form of insoluble aggregates. Again, after purifying the protein from inclusion bodies, refolding it, and reconstituting its Fe/S cluster, NMR ($^{13}$P) was used to assess its activity. When Fom3 was incubated overnight with HEP, SAM, dithionite, and methylcobalamin, a new peak was observed that was consistent with HPP. The new peak was not observed in control reactions, in which Fom3, dithionite, SAM, or methylcobalamin was omitted, indicating enzyme dependence and suggesting a radical-mediated transformation (43). A mechanism for Fom3 has been advanced by van der Donk and co-workers (49), in which a 5’-dA abstracts the pro-R H’ from HEP, with attack of the resulting organic substrate radical onto the methyl moiety of methylcobalamin, affording cob(II)alamin and the HPP product.

GenK, from 

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\text{Micromonospora echinospora}
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is responsible for the methylation of C-6’ of gentamicin $X_2$, an intermediate in the biosynthesis of the aminoglycoside antibiotic gentamicin C1 (Table 1). Efforts from the Liu laboratory (44) led to the first in vitro characterization of the protein. Because of its insolubility upon heterologous expression in 

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\text{E. coli}
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GenK was isolated from inclusion bodies and refolded before its Fe/S cluster was reconstituted. Reconstituted GenK was capable of converting the substrate, gentamicin $X_2$, into the product, G418, but only when reaction mixtures contained SAM, reductant, and either methylcobalamin or hydroxocobalamin. Moreover, it was observed that G418, 5’-dA, and SAH were produced in near equal quantities, suggesting that two equivalents of SAM are required to synthesize one equivalent of G418. When reactions were conducted in the presence of $[^{13}$C]$\text{methyl}$-cobalamin, suggesting that a methyl group from SAM is transferred initially to cobalamin before being appended onto the substrate.

Two mechanisms that are consistent with experimental observations have been proposed for the GenK reaction (44). In one mechanism, the 5’-dA ‘abstracts a C-6’ H’, and a methyl radical is transferred from methylcobalamin to afford cob(I)-alamin and the G418 product. Methylcobalamin is then regenerated in situ upon reduction of cob(II)alamin to cob(I)-alamin with subsequent transfer of a methyl group from SAM to this highly nucleophilic species (Scheme 2, pathway A). Alternatively, upon abstraction of a C-6’ H’, the proton from the C-6’-hydroxyl group is lost, affording a ketyl radical, a resonance form of which contains a carbanion and an unpaired electron on the oxygen atom. A subsequent nucleophilic attack of the carbanion onto the methyl group of methylcobalamin affords cob(I)alamin and an alkoxy product radical, which is converted into the G418 product upon receiving an electron and a proton (Scheme 2, pathway B) (44).

Unlike Fom3 and GenK, TsrM uses cobalamin to catalyze the methylation of an $sp^3$-hybridized carbon center, C2 of the indole ring of tryptophan (Trp). This reaction is an early step in the formation of the quinaldic acid moiety of thiostrepton A, a thiopetide natural product that possess antitumor and antibiotic activity (Table 1) (51, 52). Early metabolic feeding studies using chirally labeled methionine showed that the methyl
group is appended on the tryptophan substrate with net retention of configuration, consistent with a transformation involving two $\text{S}_{\text{N}}2$-based steps that each take place with inversion of stereochemistry (53). Berteau and co-workers (45) purified TsrM by affinity chromatography as a Strep-tag fusion. When the protein was incubated with Trp and methylcobalamin under turnover conditions, 2-methyltryptophan (mTrp) was produced. However, in contrast to the GenK-catalyzed reaction, no 5‘-dA was detected, although greater than 900 $\mu$M mTrp was formed in the reaction. By contrast, SAH was formed in concentrations that were near stoichiometric with mTrp, suggesting that SAM is the source of the appended methyl group. When SAM was replaced with [methyl-$^3$H$_2$]SAM, both [methyl-$^3$H$_2$]cobalamin and [methyl-$^3$H$_2$]Trp were isolated, suggesting a mechanism in which cobalamin acts as an intermediate methyl carrier. A mechanism in which methylcobalamin undergoes homolysis to generate cob(II)alamin and a methyl radical that adds to C2 of Trp was suggested (45). The loss of an electron, presumably back to the cob(II)alamin species, and a proton would result in formation of mTrp and cob(I)alamin, which could acquire a methyl group from SAM via nucleophilic displacement (54). The inability to detect 5‘-dA, as well as the lack of a strong low potential reduciant needed for turnover, might suggest that TsrM evolved an RS domain only to catalyze a non-radical reaction.

**Class C RS Methylases**

Class C RS methylases catalyze the methylation of sp$^2$-hybridized carbon centers, but do not contain the two conserved cysteines required for methylation via a Class A mechanism. They are annotated as having significant sequence similarity with coproporphyrinogen III oxidase (HemN), an RS enzyme that catalyzes the conversion of coproporphyrinogen III to protoporphyrinogen IX in one of the pathways for heme biosynthesis. This reaction involves two oxidative decarboxylations of the propionate side chains of rings A and B of the substrate to vinyl groups, with transfer of an electron to an undefined source (55). A 2.07 Å x-ray crystal structure of HemN unexpectedly revealed electron density corresponding to two simultaneously bound SAM molecules. One SAM molecule was coordinated to the unique iron ion of the RS cluster, whereas the second SAM molecule was bound immediately adjacent to the first (56). The presence of two simultaneously bound SAM molecules in the structure of HemN suggests that this binding arrangement may be operative in RS Class C methylases, wherein the SAM molecule bound to the Fe/S cluster is the precursor to the 5‘-dA, whereas the second SAM molecule is the source of the methyl group. Given that Class C RS methylases appear to methylate only sp$^2$-hybridized carbon centers, they may operate via abstraction of a H from the methyl group of SAM by the 5‘-dA with subsequent radical addition onto the carbon center of the substrate undergoing methylation.

To date, all Class C methylases appear to be involved in the biosynthesis of complex secondary metabolites that exhibit antitumor and antibiotic behavior (Table 1). As such, *in vitro* characterization of these reactions has been hampered by substrate availability and/or a lack of knowledge of the step along the biosynthetic pathway in which the methylase acts. Nevertheless, there may exist some level of mechanistic diversity within Class C methylases, given that some of the natural products in which they play a biosynthetic role contain other C1-derived moieties, such as cyclopropane rings (57, 58).

Recently, a Class C RS methylase was identified from a cluster of genes involved in the biosynthesis of yatakemycin (YTM), a DNA alkylating agent produced by *Streptomyces* sp. TP-A0356 that exhibits antitumor and antibiotic activity (57). YTM is composed of three distinct polycyclic domains that are separated by amide bonds (59, 60). The central domain contains a spirocyclopropane ring essential for its biological activity, and previous metabolic feeding studies showed that the methylene carbon of the cyclopropane ring is derived from SAM (61). When the ytkT gene of the YTM biosynthetic cluster was inactivated by gene replacement, a new metabolite (YTM-T) appeared that was similar to YTM, but which lacked the cyclopropane ring. After heterologous overproduction of the protein in *E. coli*, YtkT and its presumed [4Fe-4S] cluster were reconstituted artificially. Upon incubation of reconstituted YtkT under turnover conditions with YTM-T, a new species (MeYTM-T) was formed whose structure was consistent with a methylated derivative of YTM-T (Scheme 3A) (57). How MeYTM-T is converted into the cyclopropane derivative in YTM is still unknown.

Recently, the biosynthetic gene cluster of the antifungal agent jawsamycin was identified. This natural product, produced by *Streptovercillium fervens* HP-891, consists of a 5’-amino-5’-deoxy-5,6-dihydouridine moiety connected in an amide linkage to a polycyclicpanpanated fatty acid. Metabolic feeding studies suggested that the fatty acid backbone of the molecule derives from a polyketide biosynthetic pathway, whereas the methylene groups of the cyclopropane rings derive from 1-methionine (62, 63). One open reading frame in the gene cluster, Jaw5, was observed to encode an RS enzyme containing a C$_x$C$_x$C$_x$C$_x$C$_x$ motif, and its gene product, Jaw5, exhibits significant sequence similarity to HemN and other Class C RS methylases. Based on this information and the assignment of all other open reading frames in the cluster to other functions in the biosynthetic pathway, Jaw5 was suggested to be responsible for the cyclopropane modification. Two possible mechanisms were proposed, both of which involve two simultaneously bound SAM molecules and an H abstraction from the methyl group of one SAM molecule by a 5‘-dA derived from a reductive cleavage of the other (Scheme 3B). In one instance (i), the SAM methylene radical adds to the ene-one of the substrate to yield a substrate radical, which attacks the bridging methylene carbon of SAM to yield the cyclopropane substituent. The co-product, the radical cation of SAH, then acquires an electron to afford a complete valence shell. In the second instance (ii), the SAM methylene radical acquires an electron to yield an ylide, which attacks the ene-one to generate a substrate carbanion. Subsequent attack of the carbanion onto the bridging methylene carbon of SAM yields the cyclopropane substituent and SAH.
Finally, a new class of RS methylases, designated Class D, has recently been described (22). The prototype, MJ0619 from Methanocaldococcus jannaschii, is hypothesized to be involved in the biosynthesis of methanopterin (MPT), a coenzyme used in C1 metabolism during methanogenesis and methylotrophy in certain archaea and bacteria. The cofactor contains a pterin ring similar to that in folate, but with methyl groups appended at C7 and C9. In some organisms that make MPT, the homologs of the mj0619 gene are found in the neighborhood of the gene encoding /H9252-ribofuranosylaminobenzene 5-phosphate synthase, which is known to be involved in MPT biosynthesis. To determine whether MJ0619 catalyzes methylation of pterin or a pterin precursor during formation of MPT, White and co-workers (22) used its gene to transform E. coli, which does not naturally synthesize MPT, and the presence of methylated pterins in crude extracts from the bacteria was assessed by mass spectrometry after appropriate oxidation of the precursor molecules. Both 7-methylpterin and 6-ethyl-7-methylpterin, a marker for methylation at C7 and C9, were observed. Further studies suggested that the natural substrate for MJ0619 is 6-hydroxymethyl-dihydropterin.

MJ0619 contains two Cx,Cx,C motifs comprising Cys-73, Cys-77, Cys-80, Cys-98, Cys-102, and Cys-105. When Cys-77 and Cys-102 were individually changed to alanyl residues, the C77A variant still produced 7-methylpterin in E. coli crude cell extract, whereas the C102A variant produced no observable methylated product. This experiment suggests that only the cysteines in the latter motif coordinate the [4Fe-4S] cluster to which SAM associates, or that the cluster bound by Cys-73, Cys-77, and Cys-80 is associated with C9 methylation, whereas the cluster bound by Cys-98, Cys-102, and Cys-105 is associated with C7 methylation, and that C7 methylation must precede C9 methylation.

Subsequent metabolic feeding studies showed that the activity of MJ0619 is not stimulated by cobalamin, suggesting that cobalamin is not required for MJ0619-catalyzed methylation. Moreover, the appended methyl group was found not to derive from l-methionine, given that 7-methylpterin isolated from E. coli cultured in the presence of [methyl-2H3]methionine contained no deuterium enrichment. By contrast, when E. coli was cultured on [2H3]acetate, isolated 7-methylpterin was enriched in deuterium. The labeling pattern of deuterium incorporation was inconsistent with the methyl group deriving from methyltetrahydrofolate, but consistent with it deriving from N5,N10-methylenetetrahydrofolate (CH2THF). In the proposed mechanism, the role of the 5’-dA is to abstract an H from C7 of the substrate, which is followed by radical addition of the substrate onto CH2THF. After injection of an electron and a proton, elimination of tetrahydrofolate (THF) affords a substrate containing a C7 exocyclic methylene group. Hydride transfer from THF to the exocyclic methylene results in product formation, as is observed in thymidylate synthase (supplemental Fig. S2) (64).

**Conclusion**

Nature has devised a number of strategies for catalyzing the methylation of completely unactivated carbon atoms or phosphinate phosphorous atoms. In all instances, these reactions proceed through radical intermediates and are catalyzed exclu-
sively by members of the RS superfamily. Although they presumably all involve the intermediacy of a 5'-dA’, the mechanisms by which the methyl groups are appended are quite divergent. Given the importance of biological methylation and the large number of unannotated sequences within the RS superfamily, it is likely that additional strategies await to be elucidated.

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