

# Radical-Mediated Enzymatic Methylation: A Tale of Two SAMS

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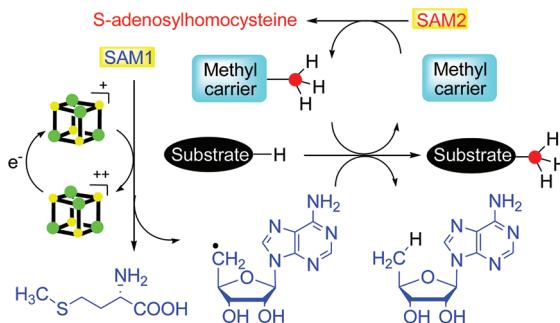
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## CONSPECTUS

Methylation is an essential and ubiquitous reaction that plays an important role in a wide range of biological processes. Most biological methylations use *S*-adenosylmethionine (SAM) as the methyl donor and proceed via an  $S_N2$  displacement mechanism. However, researchers have discovered an increasing number of methylations that involve radical chemistry. The enzymes known to catalyze these reactions all belong to the radical SAM superfamily. This family of enzymes utilizes a specialized [4Fe-4S] cluster for reductive cleavage of SAM to yield a highly reactive 5'-deoxyadenosyl (dAdo) radical. Radical chemistry is then imposed on a variety of organic substrates, leading to a diverse array of transformations. Until recently, researchers had not fully understood how these enzymes employ radical chemistry to mediate a methyl transfer reaction.

Sequence analyses reveal that the currently identified radical SAM methyltransferases (RSMTs) can be grouped into three classes, which appear distinct in protein architecture and mechanism. Class A RSMTs mainly include the rRNA methyltransferases RlmN and Cfr from various origins. As exemplified by *Escherichia coli* RlmN, these proteins have a single canonical radical SAM core domain that includes an  $(\beta\alpha)_6$  partial barrel most similar to that of pyruvate formate lyase-activase. The exciting recent studies on RlmN and Cfr are beginning to provide insights into the intriguing chemistry of class A RSMTs. These enzymes utilize a methylene radical generated on a unique methylated cysteine residue. However, based on the variety of substrates used by the other classes of RSMTs, alternative mechanisms are likely to be discovered. Class B RSMTs contain a proposed N-terminal cobalamin binding domain in addition to a radical SAM domain at the C-terminus. This class of proteins methylates diverse substrates at inert  $sp^3$  carbons, aromatic heterocycles, and phosphinates, possibly involving a cobalamin-mediated methyl transfer process. Class C RSMTs share significant sequence similarity with coproporphyrinogen III oxidase HemN. Despite methylating similar substrates (aromatic heterocycles), class C RSMTs likely employ a mechanism distinct from that of class A because two conserved cysteines that are required for class A are typically not found in class C RSMTs.

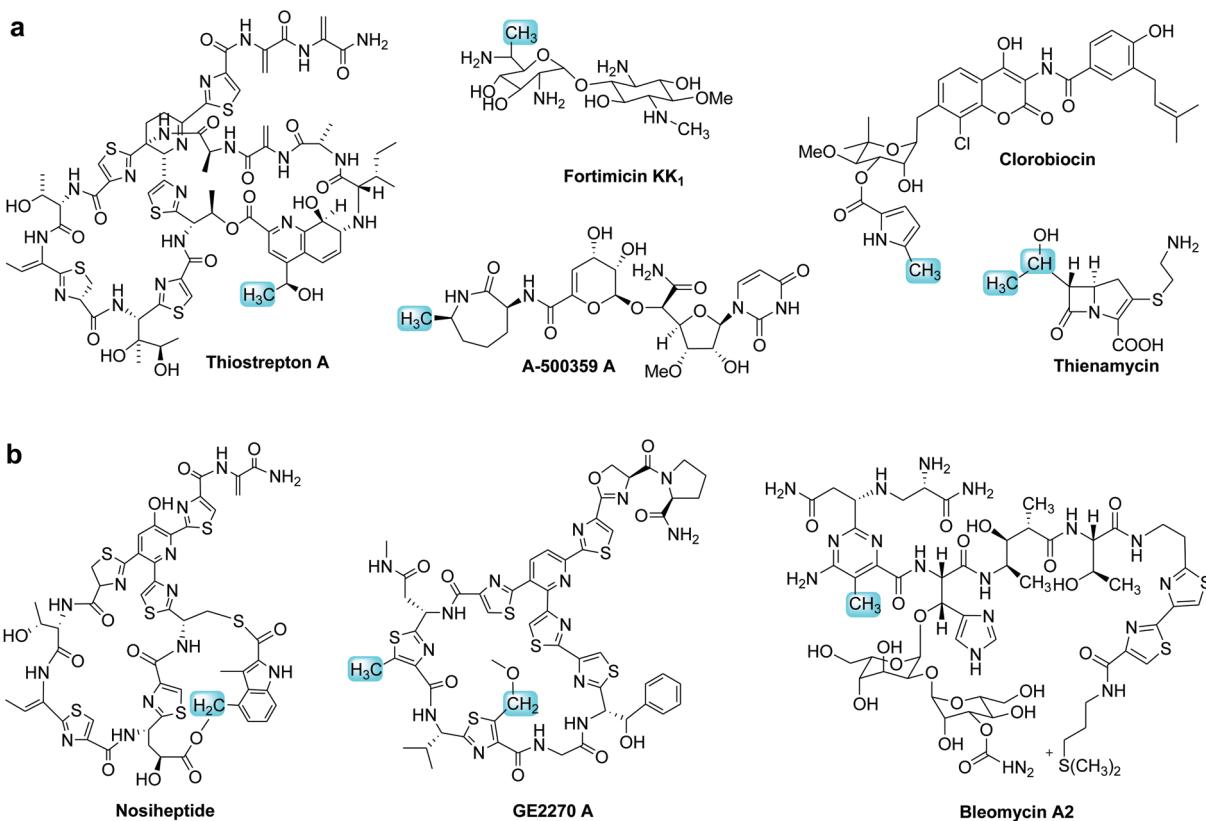
Class A and class B enzymes probably share the use of two molecules of SAM: one to generate a dAdo radical and one to provide the methyl group to the substrate. In class A, a cysteine would act as a conduit of the methyl group whereas in class B cobalamin may serve this purpose. Currently no clues are available regarding the mechanism of class C RSMTs, but the sequence similarities between its members and HemN and the observation that HemN binds two SAM molecules suggest that class C enzymes could use two SAM molecules for catalysis. The diverse strategies for using two SAM molecules reflect the rich chemistry of radical-mediated methylation reactions and the remarkable versatility of the radical SAM superfamily.



## 1. Introduction

Methylation plays an important role in a wide range of biological processes, including gene expression, protein

modification, lipid biosynthesis, and various other metabolic pathways. In most cases, the biological methyl donor is *S*-adenosylmethionine (SAM).<sup>1,2</sup> The electron-deficient



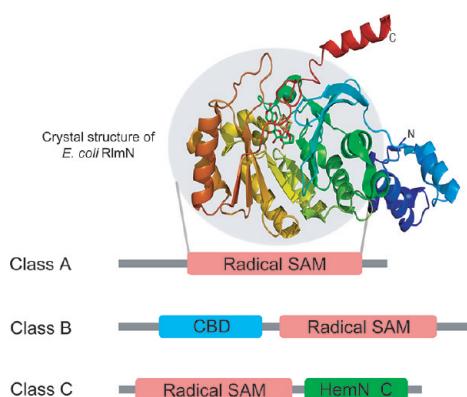
**FIGURE 1.** Selected secondary metabolites whose biosyntheses include RSMTs. Carbon atoms that are introduced by class B RSMTs (a) or class C RSMTs (b) are highlighted by light blue boxes.

nature of the sulfonium group of SAM renders its methyl group electrophilic, such that it reacts with various nucleophiles including oxygen and nitrogen atoms, unsaturated carbons, thiols, and halide ions. The reactions proceed via a typical S<sub>N</sub>2 displacement,<sup>3</sup> resulting in inversion of stereochemistry at the transferred methyl group.<sup>4</sup> The S<sub>N</sub>2 mechanism requires the substrates to be nucleophilic, precluding methylation of saturated unactivated carbon atoms or of electrophilic substrates. To complement the S<sub>N</sub>2 type methylation, nature also employs radical-mediated mechanisms to methylate non-nucleophilic substrates.<sup>5–13</sup>

The enzymes catalyzing radical-mediated methylations known to date all belong to the radical SAM superfamily, a large class of metalloenzymes currently comprising thousands of members across all domains of life.<sup>8,9,12,14–21</sup> Radical SAM proteins contain at least one [4Fe-4S] cluster that is coordinated by three cysteine residues usually within a conserved CxxxCxxC motif. The [4Fe-4S] cluster binds SAM in a bidentate fashion<sup>22–27</sup> and reductively cleaves its carbon–sulfur bond to yield a highly reactive 5'-deoxyadenosyl (dAdo) radical. Radical chemistry is then imposed on a variety of organic substrates, leading to a diverse array of transformations relevant to nucleic acid and protein modification,

and to the biosynthesis of vitamins, coenzymes, and antibiotics. The radical SAM methyltransferases (RSMTs) have also been found in a myriad of pathways, many of which lead to secondary metabolites of potential clinical use (Figure 1). At present, relatively little is known about the catalytic mechanism of most of these enzymes, but the exciting recent studies on the rRNA methyltransferases RlmN and Cfr are beginning to reveal the intriguing chemistry of RSMTs.<sup>5–7,27</sup>

Sequence analyses reveal that the currently identified RSMTs can be grouped into three classes, which based on their sequence appear distinct in protein architecture (Figure 2). Class A RSMTs, which mainly include RlmN and Cfr from various origins, have a single canonical radical SAM domain. The recently solved crystal structure of *E. coli* RlmN<sup>27</sup> revealed that the core of the protein is composed of a partial ( $\beta\alpha$ )<sub>6</sub> barrel (Figure 2) most similar to that of pyruvate formate lyase-activase.<sup>26</sup> Class B RSMTs contain a proposed N-terminal cobalamin binding domain (CBD)<sup>14</sup> in addition to the radical SAM domain found in the C-terminus (Figure 2). The hypothesized substrates of members of this class are highly diverse, and their reactions include methylation of inert sp<sup>3</sup> carbons, aromatic heterocycles, and phosphinates (Table 1). Class C RSMTs share significant sequence similarities with a



**FIGURE 2.** Domain structures of different classes of RSMTs. The radical SAM core in the *E. coli* RlmN crystal structure is highlighted by the gray circle. Structure taken from PDB ID: 3RFA.

relatively well-characterized radical SAM enzyme coproporphyrinogen III oxidase HemN, which contains a separate C-terminal domain (Figure 2) proposed to assist substrate binding.<sup>23</sup> Table 1 lists some representative examples of RSMTs from different classes with their putative substrates. As discussed herein, it appears that different catalytic mechanisms are utilized by the different classes of RSMTs.

## 2. The Novel Chemistry of Class A RSMTs

Class A RSMTs comprise RlmN and Cfr from various organisms, which methylate adenosine 2503 (A2503) of 23S rRNA located in the peptidyl transferase center of the bacterial large ribosomal subunit. Cfr methylates the C8 atom of A2503, which confers bacterial resistance to five classes of antibiotics acting upon the peptidyl transferase center.<sup>24</sup> RlmN, on the other hand, methylates the C2 atom, which is important for translational fidelity and the nascent peptide response.<sup>29,30</sup> In vitro labeling studies performed by two independent laboratories showed that, surprisingly, only two of the three protons on the methyl group of SAM are transferred to the product.<sup>6,7</sup> Furthermore, use of A2503 labeled with deuterium at the C2 position showed that this deuterium ends up in the methyl group.<sup>6</sup> Single-turnover studies<sup>7</sup> and X-ray crystallography<sup>27</sup> provided an explanation for these puzzling results as illustrated in Figure 3.

Catalysis by class A RSMTs requires a priming step involving the methylation of a conserved cysteine residue (Cys355 in RlmN) by SAM to form a methyl thioether group using a typical S<sub>N</sub>2 displacement reaction.<sup>7</sup> A second SAM molecule, thought to bind at the same binding site,<sup>27</sup> is then activated by the reduced [4Fe-4S] cluster to generate a dAdo radical. This radical abstracts a hydrogen atom from the methylthio group to generate a protein-based methylene radical. Addition of this

radical to the adenine ring accompanied by loss of an electron affords an adenine–protein adduct. A nearby Cys118 then initiates reductive cleavage of the thioether covalent adduct, forming a disulfide and releasing the methylated product. This mechanism is strongly supported by the observation of a methylated Cys residue (Cys355) in the RlmN crystal structure.<sup>27</sup> Studies with the RlmN C118A variant, which resulted in a covalent RNA–protein adduct, provide additional evidence for the mechanism.<sup>7</sup> The crystal structure also identified a Glu residue (Glu105), which could facilitate transfer of the proton from C2 of the adenine ring to the methyl group in 2-methyladenosine (Figure 3),<sup>27</sup> consistent with the labeling experiments.<sup>6</sup>

Class A RSMTs have adopted several unique strategies compared to known radical SAM proteins to overcome the low reactivity of their substrates. One is that the hydrogen atom abstraction by the dAdo radical occurs on a methyl group covalently bound to the enzyme rather than on a free substrate. This type of hydrogen abstraction, which produces a protein-based radical located on a carbon that originates from the SAM cosubstrate, is unprecedented in radical SAM enzymology. The closest analogy is found in pyruvate formate lyase activating enzyme and anaerobic ribonucleotide reductase activating enzyme, which abstract hydrogen atoms from Gly residues of their protein substrates.<sup>8</sup> Interestingly, both these Gly residues and the methylated Cys in RlmN are in flexible loops. It was suggested that the flexibility of this arrangement facilitates the dual roles of these residues.<sup>27</sup> For Cys355 in RlmN, these roles are acquisition of the methyl group by nucleophilic attack on SAM and subsequent housing of the methylene radical for attack on the RNA substrate.

The use of SAM for both homolytic and heterolytic chemistry in the same overall transformation is also found in radical SAM methylthiotransferases such as MiaB<sup>31</sup> and RimO.<sup>32,33</sup> These enzymes install a methylthio group on an isopentenylated-adenosine ring of tRNA and on an aspartate residue of the small ribosomal protein S12, respectively. Like the class A RSMTs discussed here, these enzymes use one molecule of SAM for homolytic chemistry and a different molecule of SAM for heterolytic chemistry. The order of homolytic and heterolytic steps is reversed, however, with initial generation of a dAdo radical for sulfur insertion and subsequent S<sub>N</sub>2-like methylation of the sulfur installed in the first step.<sup>31–33</sup>

## 3. Class B RSMTs: Putative Cobalamin-Dependent Enzymes

Class B RSMTs are possibly didomain enzymes consisting of a radical SAM core domain and a putative N-terminal CBD

**TABLE 1.** Representative Examples of RSMTs<sup>a</sup>

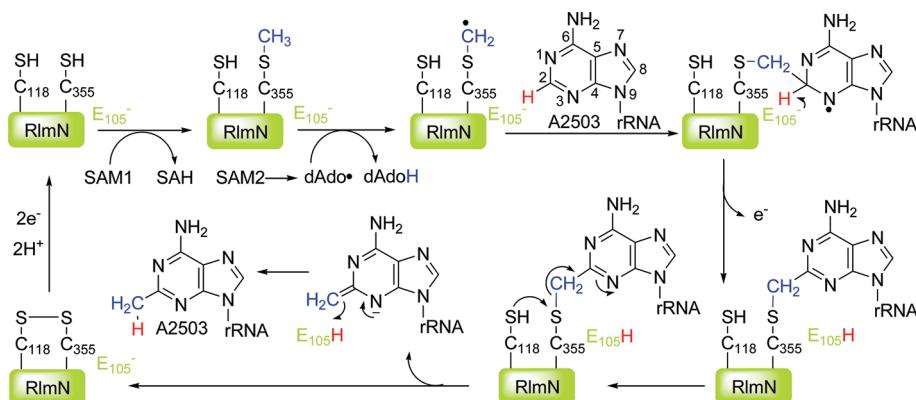
Class	Enzyme	Function	Reference	Substrate
A	RlmN	rRNA modification	5-7	
	Cfr	rRNA modification	5-7	
B	Fom3	Fosfomycin biosynthesis	10, 11	
	Fms7	Fortimicin A biosynthesis	62	
	GntE and K	Gentamicin biosynthesis	63	
	ThnK, L and P	Thienamycin biosynthesis	47	
	PacJ, N and O	Pactamycin biosynthesis	64	(X=H, OH or NHR)
	CndI	Chondrochlorens biosynthesis	65	
	Swb9	Quinomycin biosynthesis	66	
	HpnP	2-methylhopanoid biosynthesis	67	
	BchQ, BchR	Chlorophyll Biosynthesis	41	
C	CapT	A-500359 A biosynthesis	68	
	CloN6	Clorobiocin biosynthesis	34	
	CouN6	Coumermycin biosynthesis	69	
	TsrT(TsrM)	Thiostrepton biosynthesis	35, 36	
	SioT	Siomycin biosynthesis	35	
	BcpD/PhpK	Bialaphos biosynthesis	11, 37-40	
	NosN	Nosiheptide biosynthesis	53	
	NocN	Nocathiacin biosynthesis	54	
	TpdI	Thiomuracin biosynthesis	56	
C	TpdL and U	GE2270 biosynthesis	56	
	Blm-Orf8	Bleomycin biosynthesis	57	
	Tlm-Orf11	Tallysomycin biosynthesis	58	
C	Zbm-Orf26	Zorbamycin biosynthesis	59	

<sup>a</sup>The methylation sites of the substrates are indicated by solid circles.

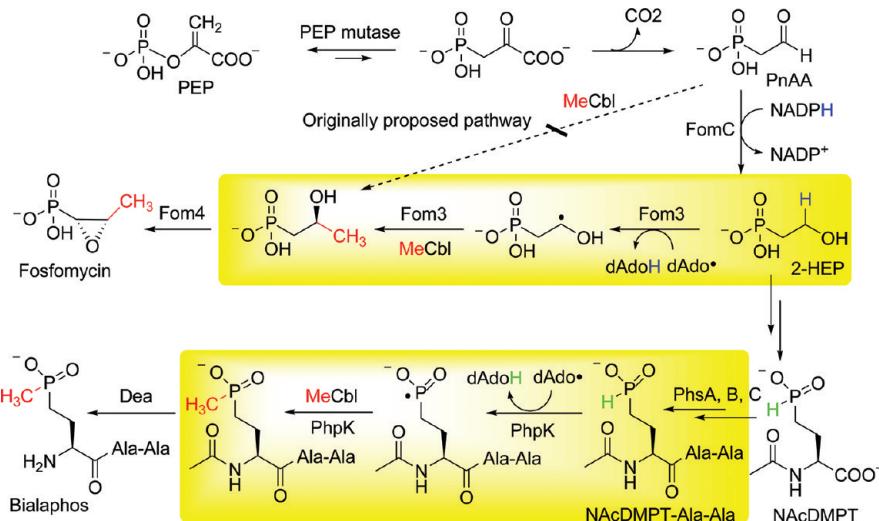
that was initially identified by Sofia et al. using iterative PSI-BLAST searches.<sup>14</sup> The members of this class include enzymes that are hypothesized to methylate unactivated sp<sup>3</sup> carbons, aromatic heterocycles, and phosphinates; examples include Fom3,<sup>10</sup> CloN6<sup>34</sup> and TsrT (also designated TsrM),<sup>35,36</sup> and BcpD<sup>37,38</sup>/PhpK,<sup>39,40</sup> respectively. It is noteworthy that not all CBD-containing radical SAM enzymes are

methyltransferases. BchE, for example, is a radical cyclase involved in chlorophyll biosynthesis,<sup>41</sup> but it is believed to have a similar domain architecture as class B RSMTs based on sequence homology,<sup>14</sup> indicating further potential diversification of the use of these intriguing enzymes.

Several studies have supported the function of select class B members. For example, when CloN6 was inactivated in the



**FIGURE 3.** Proposed mechanism<sup>7</sup> of RNA adenine methylation catalyzed by *E. coli* RlmN, a representative member of class A RSMTs.

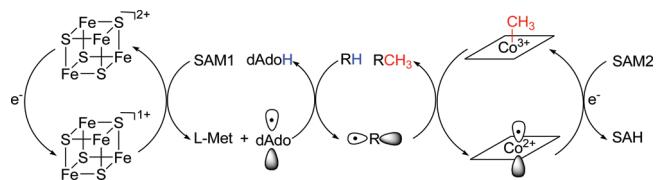


**FIGURE 4.** Biosynthetic pathways of fosfomycin and bialaphos.<sup>43</sup> The proposed catalytic mechanism of the class B RSMTs Fom3 and PhpK are highlighted in the yellow boxes.<sup>10,11</sup> The phosphonate groups are shown as monoanionic but could be dianionic ( $pK_{a,2}$  near 7).

chlorobiocin producer strain, the mutant accumulated a chlorobiocin derivative lacking the 5-methyl group of the pyrrole moiety.<sup>34</sup> This finding strongly supported the role of CloN6 in methylating the pyrrole ring during chlorobiocin biosynthesis. In addition, gene disruption studies combined with feeding experiments suggested that Fom3 methylates 2-hydroxyethylphosphonate (2-HEP) during the biosynthesis of fosfomycin (Figure 4),<sup>10</sup> instead of phosphonoacetaldehyde (PnAA) as proposed originally.<sup>38</sup> Utilization of methylcobalamin (MeCbl) by class B RSMTs has been supported by isotopic labeling studies performed in fosfomycin and bialaphos biosynthesis.<sup>37,38,42</sup> After supplying [<sup>14</sup>CH<sub>3</sub>]-MeCbl and *N*-acetyldeethylphosphinothricin-Ala-Ala (NAcDMPT-Ala-Ala, Figure 4) either to a strain defective in the biosynthesis of cobalamin<sup>42</sup> or to cell free extracts,<sup>37</sup> the radiolabel was incorporated into bialaphos. Very recently, methyltransfer from [<sup>13</sup>CH<sub>3</sub>]-MeCbl to NAcDMPT was also demonstrated

with purified PhpK, establishing the first *in vitro* reconstitution of a class B methyltransferase.<sup>40</sup> Collectively, these experiments provide support for the involvement of MeCbl in the methylations catalyzed by Fom3 and PhpK/BcpD.

The notion that methylation reactions involved in the biosynthesis of some natural products might involve chemistry other than S<sub>N</sub>2 was forecasted by early labeling studies demonstrating that net retention of the methyl configuration occurred, not inversion as expected of a S<sub>N</sub>2 mechanism. These studies focused on C2' methylation of L-Trp during thiostrepton biosynthesis,<sup>44,45</sup> and on methylation in thienamycin biosynthesis<sup>46</sup> (Figure 1). In both cases, methionine, the precursor of SAM, provides the methyl group as established by feeding studies. Several attempts to isolate the L-Trp C2' methyltransferase from the thiostrepton producer strain were unsuccessful.<sup>45</sup> The lifetime of the active enzyme was very short, and the enzyme activity was lost



**FIGURE 5.** Proposed catalytic cycle of class B RSMTs.

after dialysis, ultrafiltration, or any purification steps, all of which are consistent with RSMTs featuring an oxygen-sensitive [4Fe-4S] cluster. Recent characterization of the biosynthetic gene cluster of thiostrepton allowed identification of the class B RSMT TsrT/TsrM as a candidate for 2-methyl-L-Trp formation.<sup>35,36</sup> In addition, the products of three methyltransferases genes *thnK*, *thnL*, and *thnP* found in the thienamycin biosynthetic gene cluster are also members of class B RSMTs.<sup>47</sup> The observed stereochemistry and the discovery of these genes provide further support that the class B RSMTs may use chemistry other than  $S_N2$  to achieve methylation.

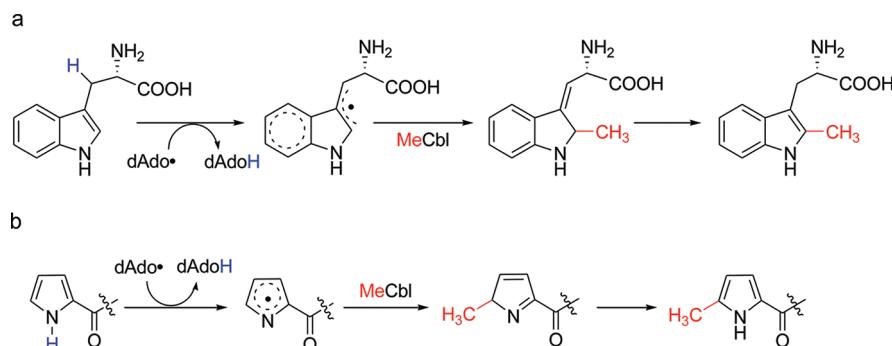
Based on the general chemistry of radical SAM proteins and the net retention of stereochemistry, a plausible mechanism for class B methyl transfer has been proposed (Figure 5).<sup>10,11,43</sup> The dAdo radical produced by reductive cleavage of SAM is postulated to abstract a hydrogen atom from the substrate to generate a radical intermediate. Reaction of this radical with MeCbl is then proposed to result in the methylated product and release of cob(II)alamin. Such transfer of a methyl group from MeCbl to a carbon-based radical has precedent in cobalamin model chemistry.<sup>48</sup> Although the stereochemistry of homolytic methyl transfer is not known, inversion is the likely result. Finally, regeneration of MeCbl is achieved via reductive methylation of cob(II)alamin by SAM, again with inversion of stereochemistry for a net retentive process. Reduced flavodoxin may possibly provide the required electron for this last step, as is observed for methionine synthase.<sup>49,50</sup>

Given the variety of substrates that class B RSMTs are believed to act on, the dAdo radical may abstract a hydrogen atom from an  $sp^3$  carbon resulting in an alkyl radical, or from a phosphinate breaking the relatively weak phosphorus–hydrogen bond and resulting in a phosphinate radical. These types of reactions are illustrated by Fom3 and PhpK involved in fosfomycin and bialaphos biosynthesis, respectively (Figure 4). However, for the methylation of aromatic rings, hydrogen abstraction from an  $sp^2$  carbon lacks precedent in enzymology. One possible solution might be abstraction of a hydrogen atom that would result in a resonance-delocalized radical. For example, methylation of the indole C2 catalyzed by TsrT/TsrM may be initiated by hydrogen

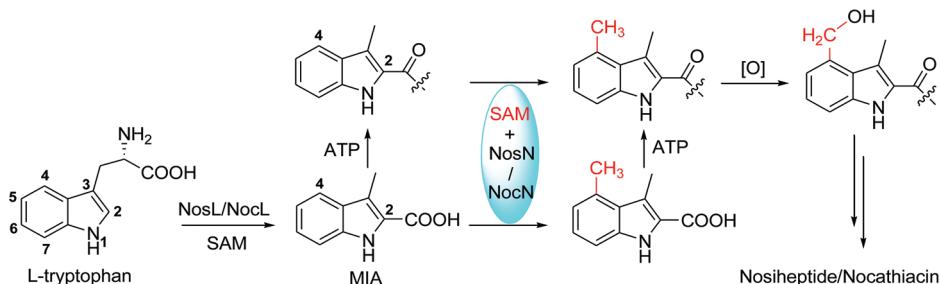
abstraction from the benzylic-like C3 atom of L-Trp (Figure 6a). The pyrrole methylation catalyzed by CloN6 may begin with the breakage of the N1–H bond (Figure 6b), similar to the N–H activation recently proposed for the radical SAM protein NosL.<sup>51</sup> Methylation of the ensuing delocalized radicals with MeCbl would then be followed by rearomatization.

The predicted MeCbl chemistry of class B bears some similarity with that catalyzed by the cobalamin-dependent methionine synthase, MetH, which is a modular enzyme consisting of four distinct domains.<sup>49,50</sup> The C-terminus of MetH comprises a CBD and a SAM binding domain that is reminiscent of the putative architecture of Class B RSMTs. MetH catalyzes methyl transfer from methyltetrahydrofolate (Me-FH<sub>4</sub>) to homocysteine, in which the cobalamin cofactor cycles between MeCbl and cob(II)alamin forms, as it is alternately demethylated by homocysteine and remethylated by Me-FH<sub>4</sub>.<sup>49,50</sup> The SAM binding domain of MetH is involved in the reductive methylation of cob(II)alamin, produced by adventitious oxidation of protein-bound cob(I)alamin by molecular oxygen.<sup>49,50</sup> This repair mechanism for MetH indicates that, in addition to the Me-FH<sub>4</sub> methyl donor used during turnover, SAM can also supply the methyl group to the cobalamin factor under certain conditions. The stereochemical course of the reaction catalyzed by MetH, in which net retention is observed in the overall reaction,<sup>52</sup> is consistent with successive transfers of the methyl group to and from the cobalamin cofactor. The major difference between MetH and class B RSMTs is the predicted mechanism of methyl transfer from MeCbl to the substrate. In MetH, this is achieved by heterolytic chemistry whereas the proposed mechanism for class B RSMTs involves homolytic chemistry.

As discussed above, class A RSMTs use a cysteine residue to mediate methyl group transfer, whereas class B RSMTs appear to use cobalamin for the same function. The cysteine content of class B enzymes is highly variable, and these nonconserved cysteines seem unlikely to take part in the mechanism of methyl transfer used by class A RSMTs. Furthermore, labeling studies for thiostrepton<sup>44</sup> biosynthesis have shown that all three protons of the methyl group of methionine are incorporated into the final product, which also argues against a similar mechanism to that employed by class A RSMTs. Class A and B RSMTs likely have in common the use of two molecules of SAM, one to generate a dAdo radical and one to provide the methyl group to the substrate. However, at present, it cannot be ruled out that, in class B enzymes, MeCbl serves as a stoichiometric cosubstrate rather than a cofactor that is remethylated by SAM.



**FIGURE 6.** Proposed mechanisms of class B RSMTs for methylation of aromatic heterocycles. Hydrogen abstraction by the dAdo radical may occur from a different site on the substrate than where methylation will take place, such as a benzylic-like carbon (a) or the nitrogen of a heterocycle (b).



**FIGURE 7.** Proposed mechanism for indolic acid moiety formation in nosiheptide and nocathiacin biosynthesis.

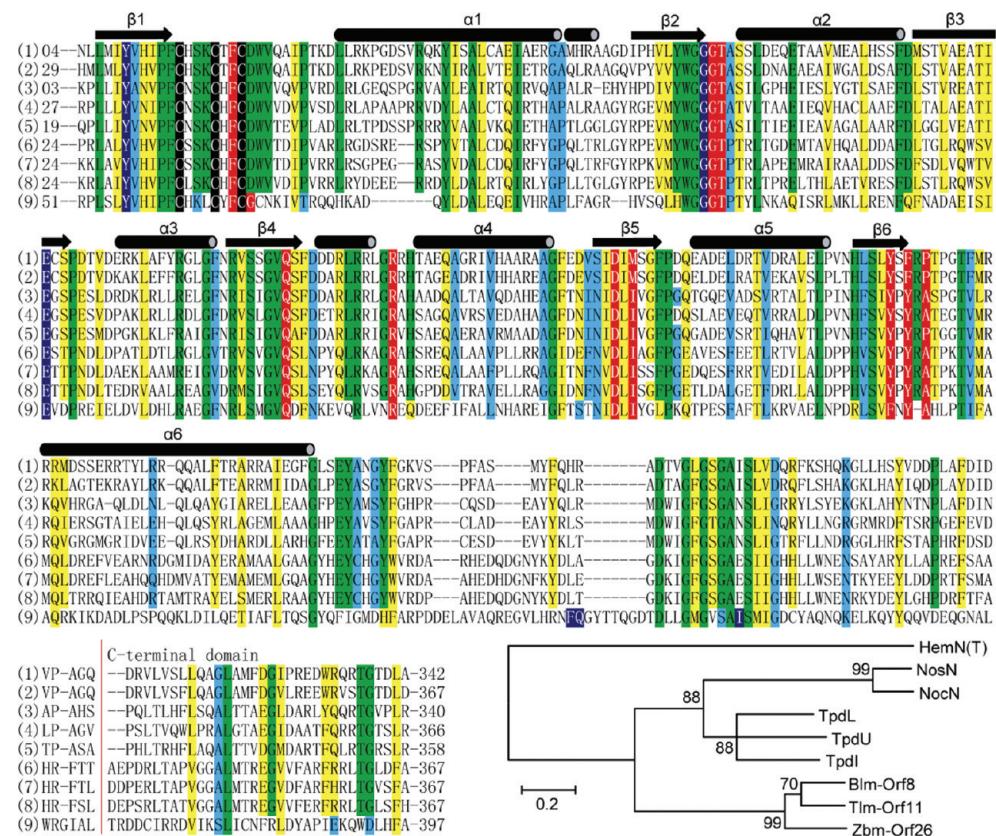
#### 4. Class C RSMTs: HemN-like Enzymes with Unknown Mechanisms

Class C RSMTs are HemN-like enzymes that contain a separate domain in the C-terminus in addition to the TIM-barrel radical SAM core (Figure 2). This class of enzymes includes NosN<sup>53</sup> and NocN<sup>54</sup> that catalyze the methylation of the 3-methyl-2-indolic acid (MIA) moiety<sup>51,55</sup> (Figure 7) in the biosyntheses of nosiheptide and nocathiacin (a nosiheptide structural analogue); TpdL, TpdL, and TpdU<sup>56</sup> involved in methylation of thiazole heterocycles in the biosyntheses of GE2270 and thiomuracin; and Blm-Orf8,<sup>57</sup> Tlm-Orf11,<sup>58</sup> and Zbm-Orf26<sup>59</sup> which may be responsible for methylation of the pyrimidine ring in the biosyntheses of the bleomycin family of antibiotics (Figure 1). Since class C RSMTs share high sequence homology with HemN, their function as methyltransferases might not be easily recognized, and many proteins identified in different genomes that are designated as oxygen-independent coproporphyrinogen III oxidases in the GenBank database may actually be class C RSMTs.

The methyltransferase activity of NosN involved in nosiheptide biosynthesis has been recently demonstrated.<sup>53</sup> Early labeling studies showed that the hydroxymethyl group at the C4 indolyl moiety of nosiheptide (Figure 1) was derived from the methyl group of SAM.<sup>60</sup> The hydroxymethyl group on the C4 indole ring of nosiheptide and nocathiacin

may be introduced by methylation by NosN<sup>53</sup> and NocN<sup>54</sup> at C4 of the MIA moiety, followed by hydroxylation on the resultant methyl group (Figure 7). Indeed, when *nosN* was deleted from the chromosome of the nosiheptide producer strain *Streptomyces actuosus*, the mutant produced an analogue of nosiheptide, which lacked the modification at C4 of the indole ring.<sup>53</sup> This result strongly supports the activity of the HemN-like protein NosN for methylation on C4 of the MIA moiety in nosiheptide biosynthesis.

The crystal structure of *E. coli* HemN showed that the enzyme consists of a  $(\beta\alpha)_6$  TIM barrel radical SAM core, a unique C-terminal domain and a so-called “trip-wire” domain at the beginning of the N-terminus.<sup>23</sup> All known class C RSMTs contain a C-terminal domain similar to that of HemN, which possibly participates in substrate binding. The  $(\beta\alpha)_6$  TIM barrel core domains of class C RSMTs share high sequence similarity with that of HemN, while the N-terminal “trip-wire” domain of HemN is absent from class C RSMTs (Figure 8). Interestingly, the crystal structure of HemN contains a second SAM molecule adjacent to the first SAM that binds to the [4Fe-4S] cluster. The exact role of the second SAM remains unknown, but its binding is clearly of physiological significance, as mutation of residues involved in the second SAM binding abolished the coproporphyrinogen III oxidase activity.<sup>61</sup> Most of the residues for SAM binding in



**FIGURE 8.** Multiple sequence alignment of class C RSMTs. Amino acid sequences were obtained from GenBank. (1) NosN from *Streptomyces actuosus*; (2) NocN from *Nocardia* sp. ATCC 202099; (3) TpdI from *Nomonuraea* sp. Bp3714-39; (4) TpdL and (5) TpdU from *Nomonuraea* sp. WU8817; (6) Blm-Orf8 from *Streptomyces verticillus*; (7) Tlm-Orf11 from *Streptoalloteichus hindustanus*; (8) Zbm-Orf26 from *Streptomyces flavoviridis*; and (9) HemN from *E. coli* that was used for prediction of the SAM-binding residues as well as the secondary structure elements in the core region of class C RSMTs. The three cysteine residues in the CxxxCxxC motif for binding of the [4Fe-4S] cluster are highlighted in black, while the red and dark blue highlighted residues are believed to be involved in binding of the first and second SAM molecule, respectively, as observed in the HemN crystal structure.<sup>23</sup> Completely conserved residues, highly conserved residues, and weakly conserved residues of class C RSMTs are denoted by green, yellow, and light blue highlighted font, respectively. The right lower inset is the maximum likelihood tree of the nine sequences in the multiple sequence alignment. Support for the clades is indicated by bootstrap values. HemN (T), the truncated version of *E. coli* HemN in which the N-terminal “trip-wire” domain<sup>23</sup> was deleted, serves as the outgroup for the phylogenetic tree. The data were analyzed using MEGA4 software.<sup>70</sup>

the ( $\beta\alpha$ )<sub>6</sub> TIM barrel core of HemN are conserved in class C RSMTs (Figure 8), indicating that these enzymes may also bind two SAM molecules. As mentioned previously, binding of a second SAM is consistent with the catalytic mechanism of all classes of RSMTs, as the proteins may use one SAM for generation of the dAdo radical and another for serving as the methyl donor. However, the separate binding sites for the two molecules of SAM differ from the apparent single binding site in the class A RSMT RlmN.<sup>27</sup> Despite methylating structurally similar aromatic heterocycles, the possibility that class C RSMTs employ a similar mechanism as that of class A is unlikely, as there are no conserved cysteines except the residues in the CxxxCxxC motif (Figure 8). In addition, NocN has only one cysteine residue beyond the three required for binding of the [4Fe-4S] cluster. This eliminates its ability to use the disulfide mechanism of class A RSMTs. Currently, no

other clues are available regarding the mechanism employed by class C RSMT. Therefore elucidation of their catalytic strategies awaits future research.

## 5. Conclusion and Outlook

The radical SAM superfamily is renowned for its remarkable catalytic diversity. As illustrated by the examples presented here, this diversity includes use of different mechanisms for the same type of reaction. RSMTs likely all catalyze hydrogen atom abstraction from a substrate, but how this hydrogen abstraction is utilized to achieve a methyl transfer reaction reflects the diverse chemistries utilized by the superfamily. Class A RSMTs use a premethylated protein and a self-hydrogen abstraction mechanism to initiate the methylation. Class B enzymes, on the other hand, appear to employ MeCbl, which can be viewed as a carrier of an inert methyl radical due

to the propensity of homolytic cleavage of the relatively weak carbon–cobalt bond. The mechanism of class C RSMT remains elusive at present. Future studies are expected to reveal the details of the catalytic mechanisms of these intriguing enzymes, which will likely entail interesting and surprising chemistries.

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## BIOGRAPHICAL INFORMATION

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## FOOTNOTES

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