Diversity of the reaction mechanisms of SAM-dependent enzymes

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Abstract  S-adenosylmethionine (SAM) is ubiquitous in living organisms and is of great significance in metabolism as a cofactor of various enzymes. Methyltransferases (MTases), a major group of SAM-dependent enzymes, catalyze methyl transfer from SAM to C, O, N, and S atoms in small-molecule secondary metabolites and macromolecules, including proteins and nucleic acids. MTases have long been a hot topic in biomedical research because of their crucial role in epigenetic regulation of macromolecules and biosynthesis of natural products with prolific pharmacological moieties. However, another group of SAM-dependent enzymes, sharing similar core domains with MTases, can catalyze nonmethylation reactions and have multiple functions. Herein, we mainly describe the nonmethylation reactions of SAM-dependent enzymes in biosynthesis. First, we compare the structural and mechanistic similarities and distinctions between SAM-dependent MTases and the non-methylating SAM-dependent enzymes. Second, we summarize the reactions catalyzed by these enzymes and explore the mechanisms. Finally, we discuss the structural conservation and catalytical diversity of class I-like non-methylating SAM-dependent enzymes and propose a possibility in enzymes evolution, suggesting future perspectives for enzyme-mediated chemistry and biotechnology, which will help the development of new methods for drug synthesis.

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1. Introduction

S-adenosylmethionine (SAM) is a dominant metabolic intermediate and ubiquitous cofactor in all living organisms. The majority of SAM-dependent enzymes are responsible for methylation and are generally called methyltransferases (MTases). SAM-dependent MTases are involved in many biopathways, including pathways involved in the modification of biopolymers (such as lipids, proteins and nucleic acids) and biosynthesis of small-molecule metabolites.

SAM-dependent MTases have long been a hot topic in biomedical research. Methylation of proteins and nucleic acids is of great significance in epigenetic regulation. To date, considerable efforts have been made to develop therapeutic agents targeting DNA and protein MTases whose abnormal activity is thought to underlie the pathology of various diseases, such as cancer, diabetes, and Alzheimer’s disease. The significance of MTases is also underscored by their crucial roles in the biosynthesis and biocatalytic modification of natural products (NPs). NPs and their derivatives provide a large scope for fragment-based drug design and for skeleton modification of prolific pharmacological scaffolds, such as phenazine, benzoquinone, pyrimidine, porphyrin, flavonoid, and benzenediolk lactone scaffolds. These compounds exhibit great potential in the treatment of cancer, inflammation, Alzheimer’s disease and many other human diseases.

Methylation is an indispensable intermediate step for diversification of the NP skeleton in biosynthetic pathways, exhibiting a preponderance in chemo-, regio- and stereo-specific synthesis and ecosfriendly characteristics. Thus, a very large number of MTases have potential applications in the industrial synthesis of bioactive compounds. For example, vanillin and isovanillin regioisomers can be generated by engineered catechol 0-methyltransferases (COMTs) and the catechol 4-O-methyltransferase SfAC. One of the flavonoid derivatives, 7-O-methyl aromadendrin, can be obtained from p-coumaric acid by engineering SuOMT-2 in Escherichia coli. A significant family of alkaloid benzylisoquinolines can be obtained by using 6-OMT, cochlaurine NMT and 4′-OMT in cascade synthesis.

Intriguingly, there is a group of SAM-dependent enzymes that share highly similar core domains with MTases but catalyze diverse types of reactions such as decarboxylation, oxidation, cyclization and hydroxylation. In addition, radical SAM-dependent (RS) enzymes, which have been widely studied in radical chemistry, also exhibit the ability to catalyze multiple reactions. In this review, we will discuss the structure, reaction and catalytic mechanism of multifunctional SAM-dependent enzymes and compare them with typical MTases in terms of structure and mechanism to provide detailed insight into the applications of these enzymes. This review covers the novel SAM enzymes with class I core domains and several typical radical SAM enzymes, which catalyze non-methylation reactions.

2. SAM-dependent enzymes

2.1. Classification and structure

The methyltransferase family is one of the superfamilies of SAM-dependent enzymes. Since the first structure of C5-cytosine-DNA-methyltransferases was obtained, MTases have been continuously studied. In general, MTases can be divided into five classes based on their structural topologies. Class I MTases are characterized as having a full Rossmann fold with a seven-stranded β-sheet and flanking α-helices that form a doubly wound open αβα-sandwich, showing a central topological switchpoint and a β-hairpin at the carboxyl end of the sheet. The glycine-rich (GxGxG or GxG) motif in the first β-sheet and a strongly conserved acidic residue at the end of β2-sheet are hallmarks of SAM binding. The auxiliary N-terminus is flexible for substrate recognition and polymerization in some cases. Class MTases constitute one of the main groups of MTases involved in NP biosynthesis, and some SAM-dependent enzymes catalyzing the nonmethylation reactions of NPs (enzymes independent on the [4Fe-4S] cluster) also share similar core topologies with class I MTases, which will be discussed below. Very similarly, class IV MTases show half of the Rossman fold but differ in the unique C-terminus that tucks back and forms a “knot” (Fig. 1D). Class II, III and V MTases exhibit considerably different topologies compared to class I MTases. The class II MTases core domain is dominated by a long antiparallel β-sheet at the center of the enzyme flanked by groups of helices. SAM is bound to the RxxxGY motif, positioned in a shallow groove at the edge of β-sheet (Fig. 1B). The active site of class III MTases is anchored into a large cleft by two αβα-domains containing five β-strands and four helices in each domain, indicating a large pocket between the N- and C-terminal domains for substrate binding. SAM is tightly folded in the active site but does not bind to the GxGxG motif, which is also conserved in class III MTases (Fig. 1C). Class V MTases are composed of a series of β-strands and a knot-like C-terminus tucked under the surface loop. SAM is bound in the shallow cleft proximal to the C-terminus (Fig. 1E). Although these five classes of MTases comprise complete, partial or small Rossmann folds, they vary markedly in overall topologies, SAM conformation and binding manner.

Radical SAM (RS) enzymes belong to another superfamily responsible for methylation. They generate high-energy radical molecules that can methylate unreactive non-nucleophilic centers of substrates and catalyze a wide range of reactions in addition to methylation. This superfamily shares a common partial (αβ)n TIM barrel fold or full TIM barrel in some cases, in which a β-sheet is anchored inside by peripheral α-helices, sharing a lateral opening manner at the active site. This open β-sheet is covered by a C-terminal domain and part of the N-terminal domain (Fig. 2A and C). The [4Fe-4S] cluster is bound at the top of the partial TIM barrel core domain, stabilized by SAM and a highly conserved cysteine-rich motif in the loop following the β1-strand. A neighboring SAM molecule coordinates one Fe ion of the cluster through its amide nitrogen and carboxylate oxygen in a bidentate fashion, and the other three Fe ions coordinate with the cysteine residues in the cysteine-rich motif in the loop following the β1-strand. A neighboring SAM molecule coordinates one Fe ion of the cluster through its amide nitrogen and carboxylate oxygen in a bidentate fashion, and the other three Fe ions coordinate with the cysteine residues in the cysteine-rich motif in the loop following the β1-strand. A neighboring SAM molecule coordinates one Fe ion of the cluster through its amide nitrogen and carboxylate oxygen in a bidentate fashion, and the other three Fe ions coordinate with the cysteine residues in the cysteine-rich motif in the loop following the β1-strand. A neighboring SAM molecule coordinates one Fe ion of the cluster through its amide nitrogen and carboxylate oxygen in a bidentate fashion, and the other three Fe ions coordinate with the cysteine residues in the cysteine-rich motif in the loop following the β1-strand.
| Reaction type                        | Enzyme                  | Source            | Catalytic domain                                                                 | Key ligand                        | PDB code   | Ref.     |
|-------------------------------------|-------------------------|-------------------|----------------------------------------------------------------------------------|-----------------------------------|------------|----------|
| Decarboxylation                     | HemN                    | *Escherichia coli*| Partial TIM barrel in N-terminus                                                 | [4Fe-4S] cluster, two SAMs        | 1OLT       | 20       |
| Decarboxylation and hydroxylation   | RdmB                    | *Streptomyces*    | Class I MTases core domain (a Rossmann-like fold) in C-terminus                 | SAM                               | 1XDS       | 21       |
| Ring-opening reaction               | ChuW                    | *Escherichia coli*| Partial TIM barrel in N-terminus                                                 | [4Fe-4S] cluster, two SAMs        | –          | 22       |
| Cyclization                         | YtkT                    | *Streptomyces*    | Partial TIM barrel in N-terminus                                                 | [4Fe-4S] cluster, two SAMs        | –          | 23       |
| Cyclization                         | C10P                    | *Streptomyces*    | Partial TIM barrel in N-terminus                                                 | [4Fe-4S] cluster, two SAMs        | –          | 24       |
| Cyclization                         | CMASs (PcaA, CmaA1, CmaA2) | *Mycobacterium* | Class I MTases core domain (a Rossmann-like fold) in C-terminus                 | SAM, a bicarbonate ion            | 1LI1, 1KPG, 1KPI | 25       |
| Cyclization                         | CFASs                   | *Lactobacillus*   | Class I MTases core domain (a Rossmann-like fold) in C-terminus                 | SAM, a bicarbonate ion            | 5Z9O, 6BQC | 26, 27   |
| SAM cyclization                     | VioH                    | *Cysotobacter*    | Class I MTases core domain (a Rossmann-like fold) in C-terminus                 | SAM                               | –          | 28       |
| Formation of double bond and bis(spiroacetal) | SlnM                    | *Streptomyces*    | Class I MTases core domain (a Rossmann-like fold) in C-terminus                 | SAM                               | –          | 29       |
| Carbocation-mediated cyclization    | TleD                    | *Streptomyces*    | Class I MTases core domain (a Rossmann-like fold) in C-terminus                 | SAM                               | 5GM2       | 30       |
| [4+2] cycloaddition                | SpnF                    | *Saccharopolyspora* | Class I MTases core domain (a Rossmann-like fold) in C-terminus                 | SAM                               | 4PNE       | 31       |
| Pericyclization                     | Lepl                    | *Aspergillus*     | Class III MTases core domain in C-terminus and dehydrogenase-ferrochelatase module in N-terminus | SAM                               | 6IX5       | 32       |
| Methylation, dehydrogenation and chelation | CysG                   | *Salmonella*      | Class III MTases core domain in C-terminus and dehydrogenase-ferrochelatase module in N-terminus | SAM, NAD+                          | 1PJS       | 33       |
| Methylation, epoxidation and isomerization | PsoF                   | *Aspergillus*     | SAM-containing MTase domain and FAD-containing monoxygenase domain              | SAM, FAD                          | –          | 34       |

Not applicable.
phospholipid synthase (CFAS)\(^\text{26}\) shares a C-terminal \((\alpha/\beta)7\)-fold, and the phospholipids are located in a tunnel, extending from the surface between the N- and C-termini to the central cleft. Moreover, TleD\(^\text{30}\), SpnF\(^\text{31}\) and LepI\(^\text{32}\), which catalyze cyclization, all share a typical Rossmann fold in the C-terminus with an extensive N-terminus for dimerization and a tightly covered active site. Nevertheless, these enzymes show some differences in the conformation and length of the N-terminal domain (Fig. 5). The N-terminal domain in SpnF does not insert itself into another subunit but simply anchors its own active site\(^\text{31}\), while LepI has a relatively large N-terminus for dimerization in a swapped manner and a completely compacts the substrate cavity via a leucine-rich coiled coil\(^\text{32}\).

2.2. General catalytic mechanisms

2.2.1. \(S_{\text{N}}2\) or \(S_{\text{N}}2\)-like methylation

In the methylation of natural products, \(S_{\text{N}}2\) nucleophilic replacement is one of the most universal synthetic mechanisms (Fig. 3A). The proper distance and orientation of the nucleophilic center in the vicinity of the electron-deficient methyl moiety are prerequisites for \(S_{\text{N}}2\)-like methylation\(^\text{13}\). The methyl donor atom in SAM and acceptor atom in the substrate are located in a linear arrangement as required by the methyl transfer reaction, which is achieved by reorientation of adjacent residues or flexible loops of the enzyme upon binding. The reaction is followed by a nucleophilic attack on the active methyl group in SAM, inducing the cleavage of C–S bonds. \(S_{\text{N}}2\) or \(S_{\text{N}}2\)-like methylation in natural product biosynthesis relies on three different mechanisms\(^\text{13}\). The first mechanism, which is associated with proximity and desolvation, was originally discovered in salicylic acid carboxyl methyltransferase\(^\text{44}\). For this mechanism, the catalytic reaction does not require residues or components in the solvent, depending instead on the proper environment being formed by the architecture of the active site. In the active site of DnrK, the possible general base Tyr142 is observed to be in proximity to the substrate, but mutagenesis of Tyr142 does not have a substantial effect on the catalytic reaction\(^\text{45}\), indicating that DnrK uses the proximity- and desolvation-based mechanism to perform methylation (Fig. 3B). The second mechanism, called general acid/base-mediated catalysis, uses residues in optimal orientation (histidine, arginine or other basic residues, as usually observed) to subtract hydrogen atoms from substrates, for instance, Arg201 and Tyr78 in Coq\(^\text{46}\), His120 in CouO\(^\text{47}\), Tyr226 in CbiL\(^\text{48}\) and Arg111 in NirE\(^\text{49}\). In NirE, Arg111 acts as an essential base to deprotonate C-20 of substrate and promotes electron transfer, facilitating the subsequent methylation of C-2 of substrate. Glu114 is also essential for correction of the orientation of Arg111 (Fig. 3C). While, instead of using residues in enzymes as general bases, cyclopropane fatty acyl phospholipid synthases mostly adopt a novel mechanism that depends on bicarbonate as a base\(^\text{26}\).

The third mechanism is referred to as the metal-dependent mechanism (Fig. 3D). Metal ions in the active site serve as general bases for deprotonation of the substrate through coordination with the substrate or alter the pK\(_a\) of the phenolic hydroxyl group and promote deprotonation. Caffeoyl coenzyme A 3-O-
methyltransferases (CCoAOMT) follows this mechanism, in which a Ca\(^{2+}\) ion alters the \(pK_a\) of the hydroxyl group and assists in the formation of an oxyanion adjacent to the electron-deficient methyl group on SAM, thereby promoting subsequent methylation\(^50\).

2.2.2. Radical-based reactions

Although many biological methylations can be achieved via \(S_N2\) or \(S_N2\)-like replacement, an increasing number of enzymatic methylations have been reported to proceed via radical-based mechanisms\(^43,51\). At the start of the enzymatic reactions, one co-ordinated SAM molecule is cleaved to yield 5′-deoxyadenosyl radical (5′-dAdo\(^\cdot\)) as a potent oxidant. 5′-dAdo\(^\cdot\) initiates various reactions through two pathways: 5′-dAdo\(^\cdot\) directly activates the substrate and drives the subsequent reactions or abstracts the hydrogen from the methyl group of the second SAM molecule in the enzyme to form radical SAM, yielding 5′-deoxyadenosine (5′-dAdoH) at the same time. The radical SAM then acts on the substrate and drives various reactions (Scheme 1). Highly active radical intermediates dramatically enrich the reactions catalyzed by radical SAM enzymes, but great effort is needed to reveal the underlying mechanism to increase efficiency and decrease the formation of byproducts by radical intermediates.

3. Nonmethylation reactions catalyzed by SAM-dependent enzymes

3.1. Decarboxylation

In 2003, Layer et al.\(^20\) determined the crystal structure of HemN, which functions as an oxygen-independent coproporphyrinogen III oxidase during heme biosynthesis. HemN combines two SAM cofactors and an iron-sulfur cluster. The structure of the HemN complex shares the three-quarter-barrel catalytic domain, harboring two SAM cofactors and a [4Fe-4S] cluster in a flattened loop. The active site inside the TIM barrel with a conserved cysteine motif shields the aggressive radical intermediates from the surrounding medium. Variation in the basic architecture may disrupt the selective binding of the substrate and cofactor, leading to correct binding of both large and small substrates\(^20\). Initially, it

Figure 2 Overall structure, close-up view and topology diagram of a representative radical SAM enzyme (HemN, PDB: 1OLT) are shown in a cartoon and a topology diagram. The core TIM barrel is defined as the region from the N-terminus of the strand (\(\beta1\)) leading to the cluster-binding loop to the C-terminus of the sixth strand (\(\beta6\)). In the representative enzyme HemN\(^3\), the core TIM barrel containing residues from Leu53 to Asn241 is highlighted\(^1\). (A) Overview of HemN. The partial (\(a/\beta\))\(_{6}\)TIM barrel is conserved in the majority of radical SAM enzymes. The special \(\alpha4\) protrudes from the partial TIM barrel, and its C-terminus is oriented toward a cluster-binding loop (between \(\beta1\) and \(\alpha1\)). (B) Close-up view of the [4Fe-4S] cluster and SAM binding mode. Three cysteines (Cys62, Cys66 and Cys69 in HemN) on the loop following the first main strand in the TIM barrel are conserved for cluster binding and coordinating with three Fe ions (colored in magenta), which is referred to as the cysteine-rich motif. The amide nitrogen and carboxylate oxygen of SAM coordinate to the fourth Fe ion. The SAM adjacent to the cluster binds at the top of the TIM barrel, and the glycine-rich motif (Gly112, Gly113 and Thr114 in HemN) is defined in many radical SAM enzymes, but other SAM-binding motifs are difficult to identify on account of the large overlap of the SAM-binding regions in different enzymes. Here, we labeled both hydrophilic and hydrophobic residues of one SAM-binding region in HemN. The second SAM molecule is present in some cases, such as HemN, BioB and LipA\(^7\), but the binding mode is not discussed here. (C) Topology diagram of HemN. In both the cartoon and topology diagram, \(\alpha\)-helices and \(\beta\)-strands in the TIM barrel are colored orange and blue, variant domains are colored grayish-white, SAM is colored pink, Fe ions in the cluster are colored magenta, S atoms are colored yellow, residues are colored cyan, and hydrogen bonds are labeled with red dashed lines.
was believed that two bound SAMs produce two radical intermediates and are involved in two decarboxylation reactions, but this proposed mechanism does not perfectly explain the indispensable role of the second SAM molecule. A newly reported mechanism clearly explains the role of the two SAM molecules in oxidative decarboxylation (Scheme 2A). One SAM molecule forms a $\text{S}^\prime\text{-dAdo} \cdot$ radical and abstracts a hydrogen atom from the methyl group of another SAM. The resulting radical SAM then abstracts the $\beta$-hydrogen atom of the carboxylate and enhances the release of carbon dioxide to form a terminal olefin. Another enzyme, HemW of *Lactococcus lactis*, shares 28% identity with HemN of *E. coli*, but unlike HemN, this enzyme cannot perform heme biosynthesis and shows no activity for oxidative decarboxylation. In 2018, Haskamp et al. reported that HemW harbors two SAM molecules and a [4Fe-4S] cluster. They show sequence similarity and are responsible for the biosynthesis of heme $d_1$, heme and protoheme.

Anthracyclines are a series of natural compounds with aromatic polyketides produced by *Streptomyces* bacteria and are usually used as anticancer drugs. The biosynthesis of anthracyclines involves various reactions, including glycosylation, hydroxylation, decarboxylation and methylation, catalyzed by numerous enzymes. DnrK and RdmB are two of these enzymes and are responsible for methylation and hydroxylation, respectively. RdmB, an aclacinomycin-10-hydroxylase, is a methyltransferase homolog that simultaneously catalyzes decarboxylation and hydroxylation with dependence on SAM, and this reaction is a key step in the biosynthesis of the polyketide antibiotic $\beta$-rhodomycin. The overall structure of RdmB shows a C-terminus containing a Rossmann-like fold formed by a five-stranded $\beta$-sheet and seven $\alpha$-helices and an auxiliary N-terminus for dimerization. SAM is bound by a glycine-rich motif next to the substrate but is oriented in an unsuitable position for methyl transfer, demonstrating the impossibility of transmethylation. Arginine-assisted decarboxylation is the initial reaction in RdmB. Then, the substrate with large aromatic rings is further stabilized.
by several hydrophobic residues targeting the vicinity of C-10 of the substrate, forming a cage for accommodating lone-pair electrons of oxygen and then generating a hydroperoxide. After the release of the substrate, a reductant from the enzyme reduces the hydroperoxide to a hydroxy group (Scheme 2B). RdmB shares 52% sequence identity and a similar three-dimensional structure with DnrK (an $O$-MTase involved in the biosynthesis of the anthracycline daunorubicin), with differences in three variable regions around the active sites (R1, R2 and R3 shown in Fig. 4A).

Grocholski et al. used chimeragenesis to explore why DnrK and RdmB present almost the same structure but totally different functions. They inserted an additional serine (Ser297) in the active site R1 of DnrK (Fig. 4A and B), and the variant DnrK-Ser (DnrK-S) exhibited complete reversal of the DnrK activity from methylase to monooxygenase. The structure of the variant DnrK-S shows a conserved phenylalanine with a similar orientation in both DnrK-S (Phe296) and RdmB (Phe300), but there is a distinct glutamine (Gln295) in DnrK at the same position (Fig. 4C). It is proposed that the phenylalanine serves as a gate that closes the channel and prevents solvent entry into the active cavity, and under this condition, water-assisted neutralization of the carbanion formed after decarboxylation would not occur in DnrK-S and RdmB (Scheme 2B). The negative charge of the carbanion is then stabilized by the polyphenolic moiety and the adjacent positive charge of SAM or its analog sinefungin. This delocalization of electrons helps in substrate-assisted activation of oxygen to overcome the similar spin barrier, similar to the manner in which other cofactor-independent monoxygenases work. Upon completion of oxidation, the peroxo-substrate is released and reduced by the reagent outside the active site cavity. Notably, C-10 decarboxylation may occur, leading to the formation of the carbanion in both native DnrK and RdmB. This process is initiated by Arg302 in DnrK and Arg307 in RdmB, but the phenylalanines (Phe298 in DnrK-S and Phe300 in RdmB) and glutamine (Gln295 in DnrK) are crucial modulators that determine whether water-assisted neutralization of the carbanion occurs and further determine whether the enzyme functions as a methyltransferase or monoxygenase (Scheme 2B). This reversal of activity caused by chimeragenesis greatly broadens the scope of application and will inspire further research for protein engineering and antibiotic reform.

3.2. Ring-opening reaction

In heme metabolism, the degradation of heme is also of great significance for iron homeostasis and cell signals in diverse physiological and pathological processes. Modification of heme scaffolds may be a potential strategy for modification of drugs with similar skeletons. The radical-based enzymes ChuW in E. coli O157:H7 and HutW in Vibrio cholerae are involved in this pathway. ChuW catalyzes the degradation of heme in an oxygen-independent manner in vitro, resulting in the ring-opening of tetrapyrrole and the liberation of iron aided by two SAM molecules. The catalytic mechanism of degradation is proposed to be consistent with the HemN-like radical process, in which the first SAM molecule is activated through homolytic cleavage and the subsequent 5′-dAdo abstracts a hydrogen atom from another SAM molecule (SAM2) to yield 5′-dAdoH. Then, the radical SAM attacks the substrate and initiates methyl transfer and rearrangement of the porphyrin ring, leading to iron release and ring opening (Scheme 3). ChuW utilizes a radical SAM system to provide the oxidant in an anaerobic environment instead of oxygen in aerobic spaces. Initially, ChuW was annotated as HemN, but this annotation was refuted based on evidence that ChuW could not rescue the function of HemN in HemN knockout Salmonella enterica. Interestingly, both of these proteins are
crucial in the metabolism of heme and share similar functional domains, but HemN is responsible for the formation of heme, whereas ChuW catalyzes the degradation of heme; further structural exploration is needed to determine the key residues for catalysis.

3.3. Cyclization

Members of the spirocyclopropane family, including yatakemycin (YTM), CC-1065 gilvusmycin, duocarmycin A and duocarmycin SA76−80, share a highly active cyclopropane moiety, which indicates their exceptionally potent cytotoxicity81,82. The coplanarity of the three carbon atoms, short and π-like C—C bonds and strong C—H bonds of the cyclopropane moiety make it a good choice for improvement of metabolic stability and brain permeability and prompt alteration of pKₐ during drug discovery9. Many agents containing cyclopropane have entered the clinical phase in targeted tumor therapy and infectious disease research83−85. However, difficulties in regioselectivity are a constant concern in chemical synthesis, and multisubstituted cyclopropanes are no exception86. Based on the critical role and challenging synthesis of these compounds, biosynthesis of cyclopropane continues to attract attention. In the biosynthesis of YTM, YtkT catalyzes the formation of a spirocyclopropane ring through radical cyclopropanation at the sp²-carbon of the aromatic ring87 (Scheme 4A). YtkT is a radical SAM enzyme that catalyzes cyclopropanation of YTM-T to yield YTM and shares sequence identity with HemN23. It has a conserved cystine-rich motif, which is a classic motif for [4Fe-4S] cluster binding. In addition, the HemN-like radical enzyme C10P98 and methyltransferase C10Q88 constitute a cyclopropanase system for cyclopropanation of CC-1065. C10P is the first to participate in the reaction. One SAM molecule in proximity to the metal cluster forms 5′-dAdo• and abstracts a proton from the methyl group of another SAM molecule. Then, the radical SAM adds to C-11 in substrate 1 and generates radical intermediate 2, which abstracts a proton from the solvent and generates 3 (Scheme 4B). Next, assisted by His138 in C10Q, the hydroxy group at the 6-position of 3 is deprotonated, which leads to electron rearrangement, followed by the formation of cyclopropane via an intramolecular S₈₂cyclization mechanism.

SAM-dependent enzymes are also used in the conversion of lipid double bonds to cyclopropanes, such as the cyclopropanase Jaw589,90, cyclopropane mycolic acid synthases (CMASs)25,91 and cyclopropane fatty acid synthases (CFASs)92−94. Three CMASs (PcaA, CmaA1 and CmaA2)25 and CFASs26,27 show similar class I MTase core domains in the C-terminus for substrate binding and catalysis but differ in the N-terminal domain for lipid binding. The
linkage between the two domains is indicative of a hinge that hides the substrate in the active site and is responsible for the closing or opening of the active site for catalysis and substrate release. A bicarbonate ion is present in the active site of some CMASs and CFASs, acting as a general base to deprotonate the hydrogen of the carbocation intermediate, and is a key component in CMAS- and CFAS-mediated cyclopropanation. Vioprolides A–D make up a class of antifungals with potent immunomodulatory effects produced by the myxobacterium Cystobacter violaceus Ch vi35. Vioprolides A and C possess a
4-methylazetidinocarboxylic acid (MAZ) key moiety. However, the MAZ moiety is rarely found in natural products, except in bonnevillamide A, which is isolated from *Streptomyces* sp. GSL-6B. The class I MTase-like enzyme VioH catalyzes the $S_N2$ reaction in SAM to form azetidinecarboxylic acid (AZE), followed by a methylation reaction catalyzed by the radical SAM enzyme VioG to yield the MAZ moiety (Scheme 4D). It is proposed that VioH deprotonates the amino group of SAM and stimulates the intermolecular cyclization of SAM molecules, but the structural details need to be further clarified.

Salinomyzin is a polyether antibiotic containing a bis(spiroacetal) core structure. The unique structure endows it with the ability to chelate metal ions and outstanding potency in killing cancer stem cells, indicating the significance of understanding its biosynthetic mechanism. The formation of its $\Delta^{18,19}$ double bond and bis(spiroacetal) is catalyzed in an unprecedented manner by the SAM-dependent enzyme SlnM. SlnM is moderately homologous to TcmP, an $O$-MTase and contains a glycine-rich motif for SAM binding. Further biochemical studies have elucidated the indispensability of SAM and acidic residues for completion of the reaction. Initially, the movement of electrons from the oxygen atom on C-17 to C-19 enhances the acid-assisted dehydration process and facilitates the attack on C-17 by the hydroxy group on C-13, converting the corresponding cyclohexanone to the desired products (Scheme 5A). The electronegative sulfur atom of SAM increases the acidity of amino

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**Scheme 3** Ring-opening reaction catalyzed by ChuW.

**Scheme 4** Cyclization catalyzed by SAM-dependent enzymes. (A) Methylation and cyclopropanation catalyzed by YtkT. (B) Proposed mechanism of radical-mediated cyclopropanation catalyzed by C10P and C10Q. (C) Proposed mechanism of bicarbonate-mediated cyclopropanation catalyzed by CMASs and CFASs. (D) Cyclization and methylation catalyzed by VioH and VioG.
acids around the active site, presumably by loss of the activation of the neutrally charged SAH and the remaining function of the positively charged sinefungin (analog of SAM).

Teleocidin B possesses an indolactam-based alkaloid and is a potent protein kinase C activator\textsuperscript{104}. Terpene cyclization usually follows a cationic mechanism as an initial step in organisms. The carbocation is usually produced by the release of a pyrophosphate, protonation of an epoxide ring or carbon–carbon formation\textsuperscript{104–106}. In the biosynthesis of teleocidin B from teleocidin A1 (TelA1), TleD catalyzes the formation of carbonium ions by methyl transfer from SAM and then initiates terpene cyclization\textsuperscript{107} (Scheme 5B). TleD forms a hexamer domain-swapped pattern and shares an additional N-terminal $\alpha$-helix inserted into the typical class I MTase fold domain of its adjacent subunit\textsuperscript{30}.

**Scheme 5** Multiple reactions catalyzed by the indicated enzymes. (A) Proposed mechanism for the formation of the spiro-ring catalyzed by SlnM. (B) Proposed mechanism of carbocation-mediated cyclization catalyzed by TleD. (C) $[4+2]$ and $[6+4]$ cycloaddition and Cope rearrangement catalyzed by SpnF. (D) Dehydration, branch pericyclization and rearrangement catalyzed by Lepl. SAM and its analogs are shown in the box. Ovals in gray represent residues or corresponding enzymes. Proposed electron transfers are indicated by curved arrows. MTA, 5'-deoxy-5'-(methylthio) adenosine; DA, Diels–Alder reaction; IMDA, intramolecular Diels–Alder reaction; HAD, hetero-Diels–Alder reaction.
The additional N-terminal α-helix anchors the active site to form a compact pocket that can accommodate only TelA1, while water molecules cannot enter. Glu153 and Glu181 form hydrogen bonds with the substrate, promoting its adoption of the proper conformation. Tyr21 serves to maintain the correct position of the additional N-terminal α-helix, which may facilitate the generation of the enclosed active site. Notably, the geranyl group of TelA1 shows alternative rotation, which might lead to two conformations (Scheme 5B), namely, a Re-face stereocenter and a Si-face stereocenter at C-25\(^+\),C-10\(^+\). Considering that methyl transfer is a driving step in the cascade reactions and that methyl acceptors and donors must adopt a proper distance, the Re-face stereocenter was discerned as the dominant stereocenter. The proposed mechanism of action of TelA1 is that methyl transfer occurs first, and then, the carboxylation is formed to initiate the subsequent cyclization (Scheme 5B). The hydrophobic pocket strictly ensures the occurrence of the correct methyl transformation and substrate binding. Additionally, TelE shows very low or even no sequence identity with class I MTases but is closely homologous to SpnF, a SAM-MTase-like enzyme that catalyzes [4+2] cycloaddition that potentially proceeds through the Diels–Alder mechanism\(^31,108,109\) (Scheme 5C). Structural comparison showed that the additional N-terminal α-helix is a common feature of MTase-like cyclases in TelE and SpnF, tightly anchoring the hydrophobic active site for catalysis (Fig. 5A and B).

The reaction of multiple regioselective and stereoselective carbon centers and are widely applied in the total synthesis of bioactive complexes\(^10\). Due to the lack of biosynthetic pericyclic reactions in natural product synthesis, the natural synthetic pathway of leporin has attracted much attention\(^111\). In 2017, the SAM-dependent dehydratase and multifunctional pericyclicase Lepl was identified\(^114\); this enzyme is responsible for the formation of the dihydropropyn core of leporin via a bifurcated mechanism. Lepl shares structural homology with several class I O-MTases, such as OxaC\(^115\), mitomycin-7-O-methyltransferase\(^116\), and PhzM\(^117\). Lepl catalyzed pericyclization converges bifurcated reactions, including an E2-like anti-elimination dehydration, an intramolecular Diels–Alder (IMDA) reaction and hetero-Diels–Alder (HDA) reaction occurring via an ambimodal transition state, and a retro-Claisen rearrangement, to form one desired product (Scheme 5D).

Neutrally charged SAH is found to inhibit the reaction, while positively charged SAM and sinfungin activate the enzyme\(^32,118\). S\(^5\)-Deoxy-S\(^5\)-(methylthio) adenosine (MTA) also shows inhibitory effects and was confirmed as occupying the substrate-binding site by structural analysis\(^118\). To gain further insight into the structural mechanism and into the regulatory effects of SAH and sinfungin, the structures of Lepl and its complexes were determined successively\(^32,118\). Lepl forms a homodimer in an asymmetric form mediated by a domain-swapped N-terminal domain, and one of the helices is involved in hiding the surface of the active site in another subunit (Fig. 3C). Several bulky and hydrophobic residues have been observed to eliminate water and create a large hydrophobic cavity for substrate accommodation. Adjacent to SAM is a wide substrate entry tunnel, and many water-filled tunnels are observed on the backside of the cavity at the domain interface. Lepl catalyzes dehydration through an anti-elimination mechanism, in which His133 acts as a general base to deprotonate 4-OH and Arg295 in the trans conformation to assist the release of the anti-periplanar OH group (Scheme 5D). Second, in the subsequent cycloaddition, a bond rotation of the diene occurs to improve shape complementarity in the active site. Endo TS-1 is subsequently generated with a low-energy conformation derived from the reactive geometry and by aligning hydrophilic residues, His133 and Arg295 in particular. This product then branches into two pathways, namely, direct HDA reaction and indirect IMDA reaction, to form the final product. Through the IMDA pathway, an ene adduct is generated, and the ene product is converted to the final product leporin C through a highly polarized TS-2 followed by retro-Claisen rearrangement, which is electrostatically established by the positively charged imidazolium of His133. LepE eliminates water molecules and stabilizes boat-like TS-2 by reducing the energy of rearrangement and changing the electronic properties of the substrate utilizing the cationic residues His133, Arg197 and Arg295 in the final step\(^119\). Notably, although the adjacent cofactor SAM does not directly interact with the substrate or contribute any allosteric effects, it defines the active site binding environment via the electrostatic effect of sulfonium or the ammonium analog and stabilizes the highly polarized TS-2\(^32\). This novel role of SAM is called “electric field catalysis”\(^121\). A parallel pattern is also observed in Slm\(^122\) and RdmB\(^21\). Another SAM-dependent pericyclase, SpnF, catalyzes the [4+2] cycloaddition\(^11\), in which Tyr23 and Glu152 serve as a lid to anchor the active site. Despite several studies about SpnF\(^108,126,127\), the role of SAM and further details are still unknown. Further understanding of the molecular mechanism of these enzymes might prompt the design of versatile and efficient pericyclics.

3.4. Methylation, dehydrogenation and chelation

CysG is a multifunctional enzyme from S. entericus that catalyzes three steps in the biosynthesis of siroheme and seems to be the prototypical siroheme synthase since it is the only enzyme involved in transforming uro'gen III to siroheme. CysG forms a homodimer, catalyzing three reactions: SAM-dependent tetrapyrrole C-2 and C-7 methylations from uroporphyrinogen III to precorrin-2, NAD\(^+\)-dependent tetrapyrrole dehydrogenation and metal chelation\(^28,129\) (Scheme 6A). In Bacillus megaterium, CysG is separated into three enzymes: the SAM-dependent uro'gen III methyltransferase (SUMT) SirA, precorrin-2 dehydrogenase SirC and sirohydrochlorin ferrochelatase SirB\(^30\). In Saccharomyces cerevisiae, there are only two proteins, namely, Met1p (functioning as MTase) and Met8p (functioning as both dehydrogenase and ferro-chelatase)\(^131\). Additionally, as a member of the extended class III MTase family, CysG\(^6\) showed 25% and 45% sequence identity with CbiE\(^132\) (Fig. 6A and B). CysG and its homologs control the branchpoint in the biosynthesis of siroheme or cobalamin (Fig. 6A). CysG forms a homodimer, and two functional domains are linked by a flexible loop\(^128\) (Fig. 6C), named CysG\(^a\) (residues 214–457, containing domains I\(^a\) and II\(^a\) and cofactor SAM; homologous to SirA and Met1p\(^1\)) and CysG\(^b\) (residues 1–213, containing domains I\(^b\), II\(^b\) and III\(^b\) and cofactor NAD\(^+\); homologous to Met8p and SirC\(^3\)). SUMT shows similarity with the class III MTases-like CysG\(^b\) domain, except in some extended loops in SUMT\(^133\). In the opposite position of the polypeptide chain of the CysG dimer, the cofactor NAD\(^+\) is located at the edge of domain I\(^b\), and its nicotinamide group inserts itself into the cleft formed by I\(^b\) and III\(^b\). Another critical finding for the enzymes is the novel posttranslational modification of phosphor-Ser128 projecting into the active site of CysG\(^b\), the S128D variant of which destroys the dehydrogenation of
ferrochelation function but without dysfunction of dimethylation activity. This enzyme is similar to isocitrate dehydrogenase, which is also regulated by phosphorylation at serine\(^{134,135}\). This might be interpreted as the addition of negatively charged phosphate conflicting with precorrin-2, which is full of negatively charged carboxyl groups, leading to low affinity of the substrate. This finding of selective elimination in the appointed function can further be used to engineer proteins for biocatalysis. The homolog of CysG has also been studied\(^{136-138}\). Comparison of SirC, Met8p and CysG showed that the NAD-bound active site adopts various conformations. Arg159 is positioned in the active-site cleft in SirC, but the relevant arginine is located away from the active sites of CysG and Met8p\(^ {137}\). It is also confusing that SirC, a dehydrogenase without chelatase activity, is found to bind Co(II) and Cu(II) in the same manner as Met8p, and this observation needs to be further explored to determine the requirements for chelatase activity\(^ {137}\).

3.5. Methylation, epoxidation and isomerization

Pseudostrin A is a member of the pseudostrin family, which exhibits a wide variety of bioactivities, such as inhibition of immunoglobulin E production\(^ {139}\) and induction of cell differentiation in PC12 cells\(^ {140}\). The great significance of pseudostrin A comes from the skeleton containing the spiro-ring core structure and the closely related compounds azaspirene and synerazol. In the main step of the pseudostrin biosynthetic pathway, the conversion from the foremost precursor azaspirene to pseudostrin A is realized by PsoF\(^ {34}\). PsoF was initially found to be a bifunctional fusion protein that catalyzes C-methylation and epoxidation by the methytransferase (MT) domain and FAD-containing monoxygenase (FMO) domain, respectively\(^ {137}\). The enzyme CTB3 was also found in Cercospora nicotianae\(^ {141}\). To study PsoF-MT, the analog N-acetylcysteamine (NAC) thioester was used to mimic the natural substrate. Methylated polyketide was observed in trans-

![Scheme 6](image-url)
orientation. The FMO domain is responsible for stereospecific epoxidation of C-10,11. Then, C-11 and C-13 are further non-enzymatically attacked by water molecules through the SN2 or SN2ʹ mechanism to obtain the diol isomer (Scheme 6B). Another study also revealed that PsoF played a role in the trans-to-cis isomerization of C12–C13 in the PsoE-associated pathway intermediate presynerazol, making it a trifunctional enzyme. As proposed, the PsoF-FMO domain oxidizes the sulfur atom of followed by syn-elimination, which releases glutathione (GSH) to yield stereoisomer. The cis-containing intermediate remains in the active site and undergoes subsequent epoxidation. Unlike other multifunctional enzymes that catalyze consecutive reactions, it is unusual that PsoF does not adopt a consecutive mechanism of action and instead takes up two unrelated steps in the cascade. This may be explained by the selectivity of the FMO domain, which favors an extensive polyketide chain with phenylalanine and possibly a spiro-ring, and such compounds are downstream of the pathway and fit in the substrate cavity of PsoF-FMO.

4. Conservation and diversity of SAM-dependent enzymes

Rossmann fold is the key feature of class I MTases, which occurs repeatedly in a large number of SAM-binding enzymes including the non-methyltransferases. Therefore, the structural conservation and catalytical diversity of class I-like non-methylating SAM-dependent enzymes are further explored in our review, while radical SAM enzymes have been comprehensively discussed and are not included here.

The relevance of the class I-like non-methylating SAM-dependent enzymes has been analyzed from the sequences, structures and catalytic traits. The structure-based sequence alignment analysis shows that the regions containing Rossmann fold are generally well aligned, and most of the critical residues interacting with SAM or stabilizing the Rossmann fold structure in characterized motifs are identified (Fig. 7). Similar to the MTases, the acidic residues (Asp/Glu colored in green) and glycine-rich fragments (colored in blue) in motif I are highly conserved, which polarize water molecules and interact with carboxypropyl moieties of SAMs, respectively. The Asp/Glu acidic residues in motif IV, hydrophobic residues at the end of motif V and glycine in motif VI are partially conserved, with one or two exceptions. However, the acidic residues in motif II and motif III are absent in non-methyltransferases, while they are conserved in the typical Rossmann-fold MTases which interact with adenosyl base of SAM molecule. The absence of the acidic residues in motif II and III may represent the evolutionary diversity of the non-methyltransferases in the binding modes of SAM and ultimately end up with distinct catalytic reactions and functional promiscuities. Because the methylating and non-methylating reactions share distinct mechanisms to initialize the catalysis and the different chemical relativities of the substrates also require diverse circumstances of the binding pockets, it is difficult to draw any further marker in the sequence divergence to label the capability of catalysis and substrate specificity among the SAM-dependent enzymes.

The structural similarity of structurally-reported non-methyltransferases has been analyzed using Dali server. As expected, they share highly conserved Rossmann fold in C-terminus and a distinct N-terminus. The variable N-terminus plays diverse roles in dimerization, substrate specificity, or other functions, which results in the wide range of RMSD values ranging from 3.1 to 6.2 when taking RdMB as the reference structure.

Through the simple meta-analysis of the class I-like non-methylating SAM-dependent enzymes, some of them show...
consistency in function and evolutionary relationship. Except for the acidic residues in motif II and III, most of them exhibit the invariant key residues in Rossmann fold for SAM binding. However, the role of other conserved or variant residues is still less understood. The SAM-dependent enzymes mentioned are "tailored to function". Galperin and Koonin\(^{146}\) used the term "entatic state", that is "a catalytically poised state", to discuss the evolutionary relationship and the interplay of common and unique features among enzymes, which was originally proposed in 1968\(^{147}\). The class I MTases and class I-like non-methylating SAM-dependent enzymes in this review commonly share similar poised states preserved by the conservation of specific residues, while other variant residues are responsible for catalytic specificity of the enzymes, leading to distinct transition states for different reactions. That is to say, during evolution, the random mutations of amino acids in canonical Rossmann folds contribute to the diversity of enzymes, but not all the new entatic state of the protein is stable, and only the minority of neo-folded protein are viable which may reserve the main feature of Rossmann fold and mechanism\(^{13}\). Consequently, it is necessary to attach importance on the experimental characterization to correctly annotate the function of enzymes with the canonical domain.

5. Conclusions and outlook

The abundant evidence for SAM-dependent enzymes reviewed here apparently reveals the tailored functions and prolific substrate acceptance of these conserved class I MTase structures, which is referred to as catalytic promiscuity. Nonmethylation can be directly or indirectly dependent on SAM. In reactions that directly depend on SAM, such as cyclopropanation, SAM provides the methylene group through the SN2-like or RS mechanism. On the other hand, in RdmB\(^{21}\), SlnM\(^{29}\) and LepI\(^{32,118-125}\), SAM does not sacrifice itself but acts as an electron stabilizer through its positively charged sulfur center, which is reminiscent of "electric field catalysis"\(^{121-125}\). Some SAM-dependent enzymes catalyze methylation and other reactions by two discrete domains with different cofactors. For example, CysG catalyzes methylation, dehydrogenation and metal chelation though two domains in the opposite direction\(^{128}\); PsoF catalyzes methylation, isomerization and epoxidation through the MTase domain and FAD-containing monoxygenase (FMO) domain\(^{34}\). These enzymes may have a "switch" to lock or release functions. For example, epigenetic modification of serine in CysG can lock the activity of the FAD-containing domain.

These facts indicate the great potential of engineering SAM enzymes to generate new enzymes with pluripotency and conversion functions. The conversion of MTase to monoxygenase is a good example\(^{66}\). By comparing the key residues in DnrK and its homolog RdmB, a crucial phenylalanine in RdmB could serve as a gate to prevent water from entering the active site (Fig. 4). Thus, an amino acid was inserted into DnrK to reorient the phenylalanine in wild-type DnrK. The small alteration dramatically eliminated the MTase activity of DnrK and converted the enzyme to a monoxygenase (Scheme 2B).

Compared to chemical synthesis, enzymatic synthesis is a versatile method to obtain products with high chemo-, regio- and stereo-specificity. Enzymes accelerate reactions through stabilization of intermediates and products, resulting in preferred dynamics and thermodynamics. Due to the predominant roles of natural products in biomedical research, these groups of SAM-dependent enzymes might be good candidates for biocatalysis and cascade reactions in drug synthesis. Further investigation on the structure, molecular basis and biological functions of these enzymes is likely to spur significant advances in enzyme engineering and future application in a wide range of biosynthesis.

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Diversity of the reaction mechanisms of SAM-dependent enzymes

Author contributions

Qiu Sun and Mengyuan Huang generated the manuscript draft. Yuquan Wei edited and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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