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Novel chemistry and structural perspectives in vitamin B_{12}-dependent radical SAM enzymes

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Vitamin B_{12} is one of the most complex vitamins and an essential cofactor for humans, but also for bacteria, which have evolved different strategies for its biosynthesis and acquisition. For decades, vitamin B_{12} has been mainly assumed to be a source of nucleophilic methyl groups and radical initiators, in the forms of methyl-cobalamin and adenosyl-cobalamin. Recently, the importance and the chemical versatility of vitamin B_{12} have been reconsidered in light of the emergence of the superfamily of radical SAM enzymes. In this review, we provide an overview of vitamin B_{12}, its role, and its diversity within the human microbiota, and in the emerging family of B_{12}-dependent radical SAM enzymes. We focus on the diversity of B_{12}-dependent radical SAM enzymes from a mechanistic and structural perspective, along with their potential evolutionary links to other B_{12}-dependent enzymes.

1. Introduction

Vitamin B_{12} (also called cobalamin) is an essential vitamin for humans (Fig. 1). It is also a central metabolite in bacteria and its importance has been recently highlighted in the so-called superfamily of radical SAM enzymes.[1–4] This superfamily of enzymes catalyzes unrelated and often chemically challenging transformations, including post-translational modifications,[5–10] complex rearrangements,[11] and DNA modification,[12–14] along with cofactor[15,16] and metallic-center[17,18] biosynthesis. In the last decade, the importance of these enzymes has been demonstrated in bacterial and human metabolisms,[19,20] where they support key functions spanning from the biosynthesis of secondary metabolites to antiviral defense. Originally, the importance of radical SAM enzymes was recognized in a pioneering bioinformatics study, defining them as metalloenzymes containing one unusual [Fe_4S_4] cluster and using *S*-adenosyl-L-methionine (SAM) as an essential cofactor to initiate radical catalysis.[21] This study also predicted that several of these enzymes would utilize vitamin B_{12} as a cofactor. However, the *in vitro* demonstration that some radical SAM enzymes require cobalamin has been reported only relatively recently,[22,23] leading

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to the emergence of a novel enzyme family called the B_{12}-dependent radical SAM enzymes.

Being one of the largest classes of radical SAM enzymes,[23–33] these biocatalysts delineate a superfamily on their own, comprising more than 220 000 members. Early studies have shown that these enzymes are involved in the alkylation of unreactive carbon atoms,[23,25,30,31] *P*-methylation[22] and ring contraction.[29] More recently, these enzymes have been proposed to catalyze unrelated transformations, such as ring expansion[34] and thioether bond formation.[35] In all of these reactions, vitamin B_{12} plays a central but sometimes ill-understood role, underscoring the importance of this vitamin in bacterial metabolism and within the human microbiome.[1,36,37] Indeed, it is estimated that the vast majority of bacterial species that constitute this complex ecosystem[38,39] require vitamin B_{12} for their metabolism.[36,37] However, only a fraction of them have the biosynthetic machinery required to perform its *de novo* synthesis, and fierce competition exists to acquire this metabolite.[40]

This review focuses on the latest developments regarding vitamin B_{12} biosynthesis, and explores the structural and functional diversity of B_{12}-dependent radical SAM enzymes, and their catalytic and structural links with other B_{12}-dependent enzymes.

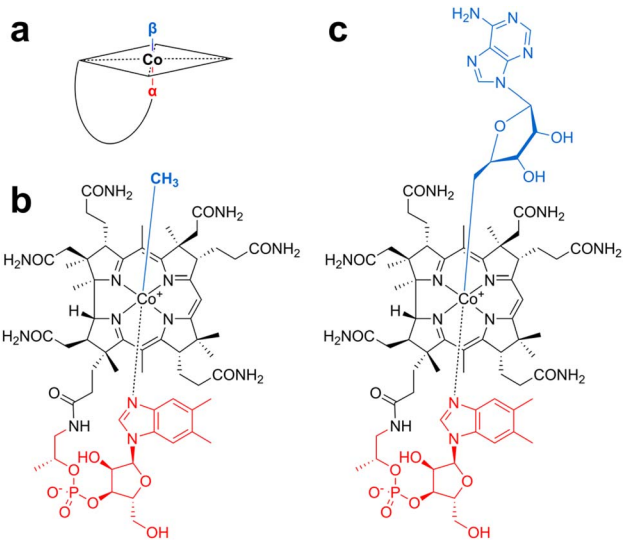

Fig. 1 Vitamin B_{12}. (a) A schematic representation of cobalamin, with α and β denoting the axial ligands positioned below and above the corrin ring, respectively. (b) The structure of methylcobalamin, featuring a methyl group coordinated at the β position. (c) The structure of adenosylcobalamin, with a 5′-deoxyadenosyl ligand at the β position.

2. Vitamin B_{12}

Vitamin B_{12} (cobalamin) is one of the eight known B vitamins and was identified in 1948 as a red pigment containing cobalt.[41,42] Its structure, determined a few years later,[43] revealed that it is built based on a substituted tetrapyrrole (corrin) ring containing a central cobalt atom. This ring is generally connected by a three-carbon moiety and a 3′-phosphoribose linked to an unusual nucleotide, 5,6-dimethylbenzimidazole (DMB),

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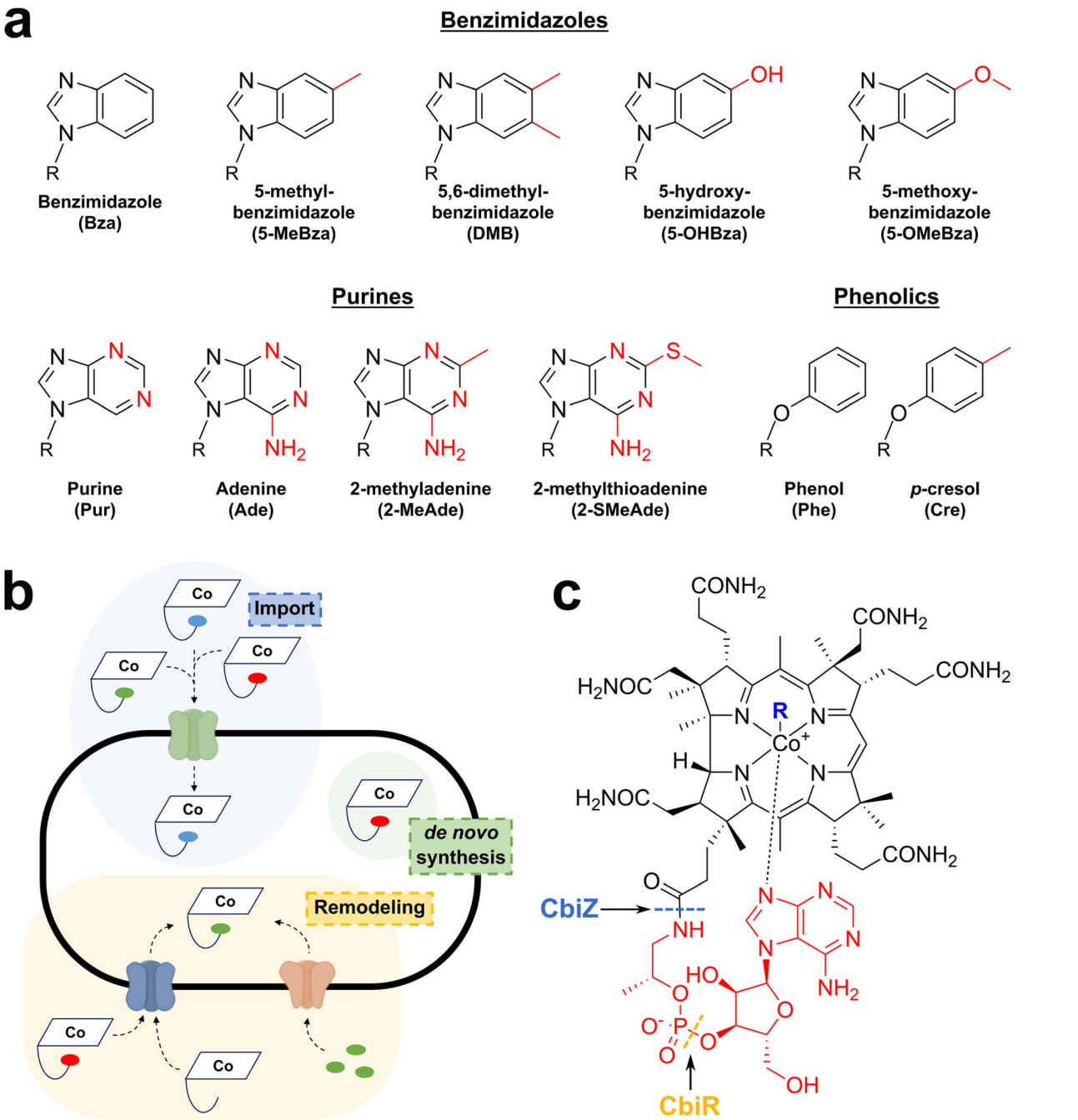

Fig. 2 Vitamin B_{12} and the microbiome. (a) The structures of the lower ligands found in cobamides. (b) Possible pathways for cobamide acquisition in the human microbiome. (c) The structure of pseudocobalamin with the cleavage sites of remodeling enzymes (CbiZ and CbiR) indicated with dashed lines.

which coordinates the cobalt atom in the lower axial position (α face) (Fig. 1). All of these structural features give vitamin B_{12} unique reactivity and functions. To add to this complexity, vitamin B_{12} usually coordinates at its upper axial position (β face) a methyl or an adenosyl group in methyl- and 5′-deoxyadenosylcobalamin (MeCbl and AdoCbl), respectively. In humans, these two forms are used by two distinct enzymes: methionine synthase, which catalyzes the conversion of homocysteine to methionine, and the mitochondrial enzyme methylmalonyl CoA mutase, which converts methylmalonyl CoA to succinyl CoA. A deficiency in vitamin B_{12} leads to severe pathologies, which explains why a sophisticated trafficking pathway has been evolved in humans.[44] Interestingly, not only do these two enzymes use two distinct forms of vitamin B_{12}, but they also exploit its two reactivities.[45] Indeed, MeCbl is used in S_N2-type reactions to transfer a methyl group as a carbocation to a nucleophilic acceptor, while AdoCbl is used exclusively in radical reactions by generating the 5′-deoxyadenosyl radical, following Co–carbon bond homolysis. In bacteria, recent

studies exploring the human microbiota have drawn a more complex picture of the role and requirement of vitamin B_{12}.

2.1. Vitamin B_{12} and cobamides in the microbiome

Although vitamin B_{12} is the most studied, it is part of a larger family of cofactors called cobamides. Indeed, the investigation of several bacterial systems has revealed that, besides the upper axial ligand, the nature of the lower axial ligand is also variable, leading to a wide diversity of cobamide cofactors (Fig. 2a). Surprisingly, in several environments, including the human gut, vitamin B_{12} has been reported to be a minority among the cobamides produced,[36,37,46] in spite of its critical requirement for the host and the microbiome. Indeed, it has been recently recognized that vitamin B_{12} profoundly impacts the gut microbiome by promoting a more diverse ecosystem with potential health benefits to the host.[47,48] A recent systematic review of the literature supports that vitamin B_{12} is associated with changes in gut microbiota by increasing alpha-diversity and shifting the microbiome composition.[48] However, depending on the cobalamin form and uptake, the effects of vitamin B_{12} appear to differ and to be indirectly mediated by propionate production, which has a positive role in gut integrity and host health.

Why do bacteria produce such a diversity of cobamides? One hypothesis is that this structural diversity is connected with vitamin B_{12} acquisition. Indeed, most bacterial species cannot *de novo* synthesize cobamides, but instead possess importing systems (Fig. 2b). Altering the cobamide structure might thus provide a fitness advantage by preventing their import by competing species. In support of this evolutionary hypothesis, it has been shown that while *Bacteroides thetaiotaomicron* can use only benzimidazolyl and purinyl-cobamides,[36] other bacteria, such as *Sporomusa ovata*, favor phenolyl-cobamides.[49] Remarkably, to circumvent this issue, many bacteria, including *Dehalococcoides mccartyi*,[50] *Rhodobacter sphaeroides*,[51] *Vibrio cholerae*,[52] and the gut bacteria *Akkermansia muciniphila*[53] are capable of "cobamide remodeling". This remodeling is performed by unrelated biosynthetic pathways such as CobS in *V. cholerae*, the amidohydrolase CbiZ in *R. sphaeroides*, and the phosphodiesterase CbiR in *A. muciniphila*[53] (Fig. 2c). After the removal of the lower ligand, the imported cobamides can be further processed by the salvaging pathways in order to produce relevant cobamides that support bacterial growth. Although these pathways are likely to provide fitness advantages by repurposing cobamides, the structural and functional diversity of these remodeling enzymes appears to be the result of convergent evolution, underscoring the physiological relevance and importance of vitamin B_{12}.

Another hypothesis regarding the diversity of cobamides is connected to the multiplicity of B_{12}-dependent enzymes and the reactions they catalyze. It is plausible that some cobamides are specifically tailored to fulfill dedicated catalytic functions. To date, this has not been experimentally validated, and further research will be necessary to gain a comprehensive understanding of the structural and functional diversity of cobamides.[37,54]

2.2. Novel insights into vitamin B_{12} biosynthesis

Among its unique features, cobalamin is the only vitamin synthesized exclusively by bacteria.[55,56] It is produced *via* two related though genetically distinct routes called the aerobic and anaerobic pathways.[57] The distribution of these pathways is uneven, with anaerobic pathways being the most widespread. Although both pathways require the bismethylation of uroporphyrinogen III to precorrin-2 as the first step, they rapidly diverge. The biosynthesis of vitamin B_{12} has been described in excellent reviews,[44,56,57] and is almost fully elucidated. However, the final steps of its biosynthesis, which involve the synthesis and attachment of the lower nucleotide loop, have not been entirely clarified.[58,59]

In the aerobic pathway, the key role of the oxygen-dependent enzyme BluB, which converts FMN to DMB, has been demonstrated[60–62] (Fig. 3). For the anaerobic biosynthesis of DMB, five genes (BzaABCDE) are required, and 5-aminoimidazole ribotide (AIR), an intermediate in the purine biosynthesis pathway, is the central precursor.[59] Another difference is that DMB is not synthesized as a free group but likely as a ribotide derivative in the anaerobic pathway. Indeed, it was recently shown that BzaC, the first methyl transferase involved in the conversion of AIR to DMB (Fig. 3), prefers 5-OHBza-ribotide over 5-OHBza as a substrate. Interestingly, the activity of BzaC on this asymmetric precursor provides a structural rationale for the observed regioselectivity of this conversion.[59]

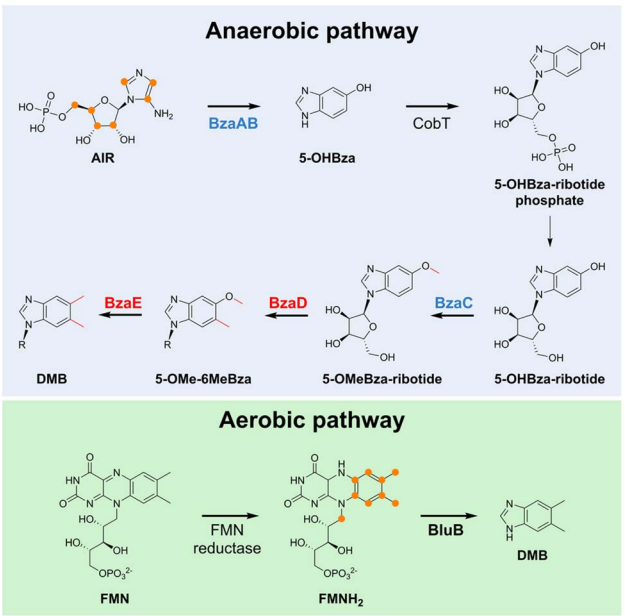

Fig. 3 Anaerobic and aerobic biosynthetic pathways for the DMB moiety of vitamin B_{12}. In the aerobic pathway, FMN is reduced and then its oxidative fragmentation by BluB leads to the formation of DMB. In the anaerobic pathway, starting from AIR (5-aminoimidazole ribotide), the BzaBCDE enzymes produce 5-OHBza-ribotide, which is further converted into cobalamin containing a DMB moiety. Conserved carbon atoms in the final product (DMB) are highlighted by orange circles.

The conversion of 5-OMeBza into DMB remains an open question. The final steps are predicted to involve two B_{12}-dependent radical SAM enzymes: BzaD and BzaE. BzaD likely catalyzes the methylation of a Csp^2-atom to form 5-OMe-6-MeBza, reminiscent of the reaction catalyzed by TsrM[23,25] (see below). The BzaE-catalyzed conversion of 5-OMe-6-MeBza to DMB is enigmatic and will require further studies to be clarified. The anaerobic biosynthesis of DMB involves not only unique chemistry but also cobalamin-dependent enzymes, which is a fascinating conundrum.

3. Catalytic functions of vitamin B_{12}

Until recently, vitamin B_{12}-dependent enzymes were classified into three broad classes: the isomerases/mutases, the methyltransferases, and the more recently characterized reductive dehalogenases,[63,64] as described below.

3.1. Radical B_{12}-dependent enzymes: isomerases, mutases, and reductases

Isomerases/mutases were considered until recently as the largest class of vitamin B_{12}-dependent enzymes.[63] They include diol dehydratases,[65] ethanolamine ammonia-lyase, lysine 5,6-aminomutase,[66] methylmalonyl-CoA mutase, methyleneglutarate mutases,[67] glutamate mutase[68–71] and ribonucleoside-triphosphate reductase.[72] These enzymes use AdoCbl to catalyze radical reactions, and their mechanism can be summarized as follows: upon substrate binding, AdoCbl is homolytically cleaved, generating the 5′-deoxyadenosyl (5′-dA•) radical and cob(II)alamin. The highly reactive 5′-dA radical abstracts a substrate H-atom and generates a substrate radical that undergoes either a 1,2 rearrangement (isomerases) or carbon skeleton moiety intramolecular migration (mutases). Re-abstraction of the product radical H-atom leads to reaction termination and AdoCbl recombination (Fig. 4 and 5a). Among these enzymes, class II ribonucleotide reductases are outliers, as the 5′-dA radical abstracts a hydrogen atom from a cysteine residue, generating a thiyl radical within the enzyme's active site. This radical species, in turn, abstracts a substrate H-atom and serves as a radical reservoir.[12,73]

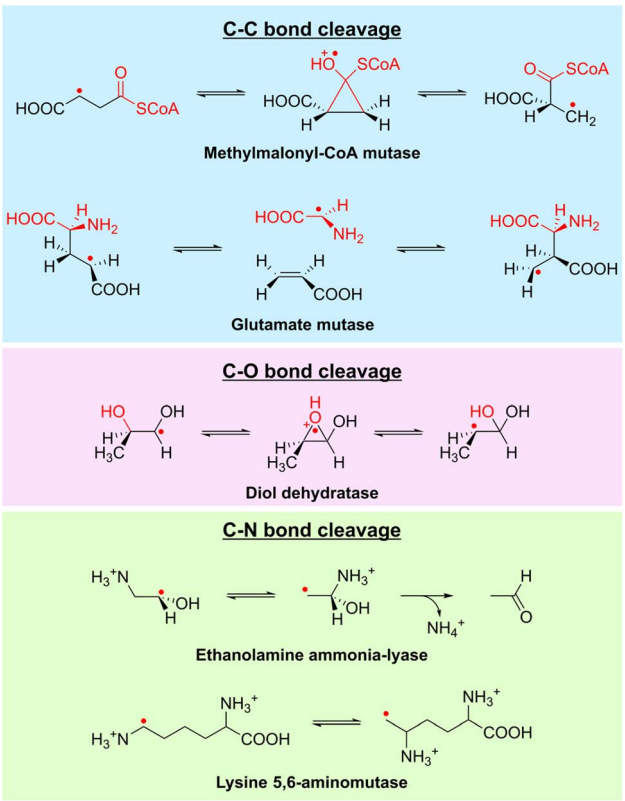

Fig. 4 Representative reactions of the isomerase/mutase family. Isomerases and mutases have been shown to be involved in C–C, C–O and C–N bond cleavage and formation.

3.2. Methyltransferases

B_{12}-dependent methyltransferases were identified at the same period as isomerases, more than six decades ago.[74] The most widely investigated systems are certainly methionine synthase, which catalyzes the conversion of homocysteine into methionine[63,64,67,75–80] and the corrinoid iron-sulfur protein (CFeSP), involved in methyl transfer in the Wood-Ljungdahl carbon fixation pathway.[63,81–84] In this pathway, the methyl group is transferred to different metallic sites, with one of two Ni ions[85] present in the acetyl-CoA synthase active site serving as its penultimate acceptor, before formation of the final product (Fig. 5b).

Both enzymes use the same general mechanism, with methyl being transferred from MeCbl(III) to an acceptor and the formation of the super-nucleophile Cob(I), which is methylated back by methylenetetrahydrofolate. Interestingly, extensive characterization by the Banerjee and Ragsdale laboratories has demonstrated that both enzymes can be inactivated in a Cob(II) state, which needs further reduction and a SAM molecule, as a more efficient methyl donor (Fig. 5c)[63,64,76,77,81,86] for enzyme reactivation and subsequent turnovers. This reactivation cycle appears as an impressive trick to prevent the irreversible inactivation of the enzyme and the loss not only of the protein but also of vitamin B_{12}, which is a metabolically costly cofactor. Indeed, it was estimated that oxidative inactivation occurs once every 2000 turnovers, which is significant.

3.3 Reductive dehalogenases

The third group of vitamin B_{12}-dependent enzymes is the reductive dehalogenases.[87,88] This group has been identified more recently and proved to usually contain, in addition to a variety of corrinoid cofactors (*e.g.*, norpseudo-B_{12}), iron-sulfur clusters like in the CFeSP protein.[89–91] These enzymes play a crucial role in organohalide respiration and have the ability to reductively cleave carbon-halogen bonds found in a variety of environmentally hazardous chemicals. They are, therefore, fascinating biocatalysts for bioremediation. The role of the corrinoid cofactor has been elucidated only recently, thanks to advanced EPR and structural analyses.[87,88,92] As a consensus, in these enzymes, the active form of the corrinoid cofactor is Cob(I).

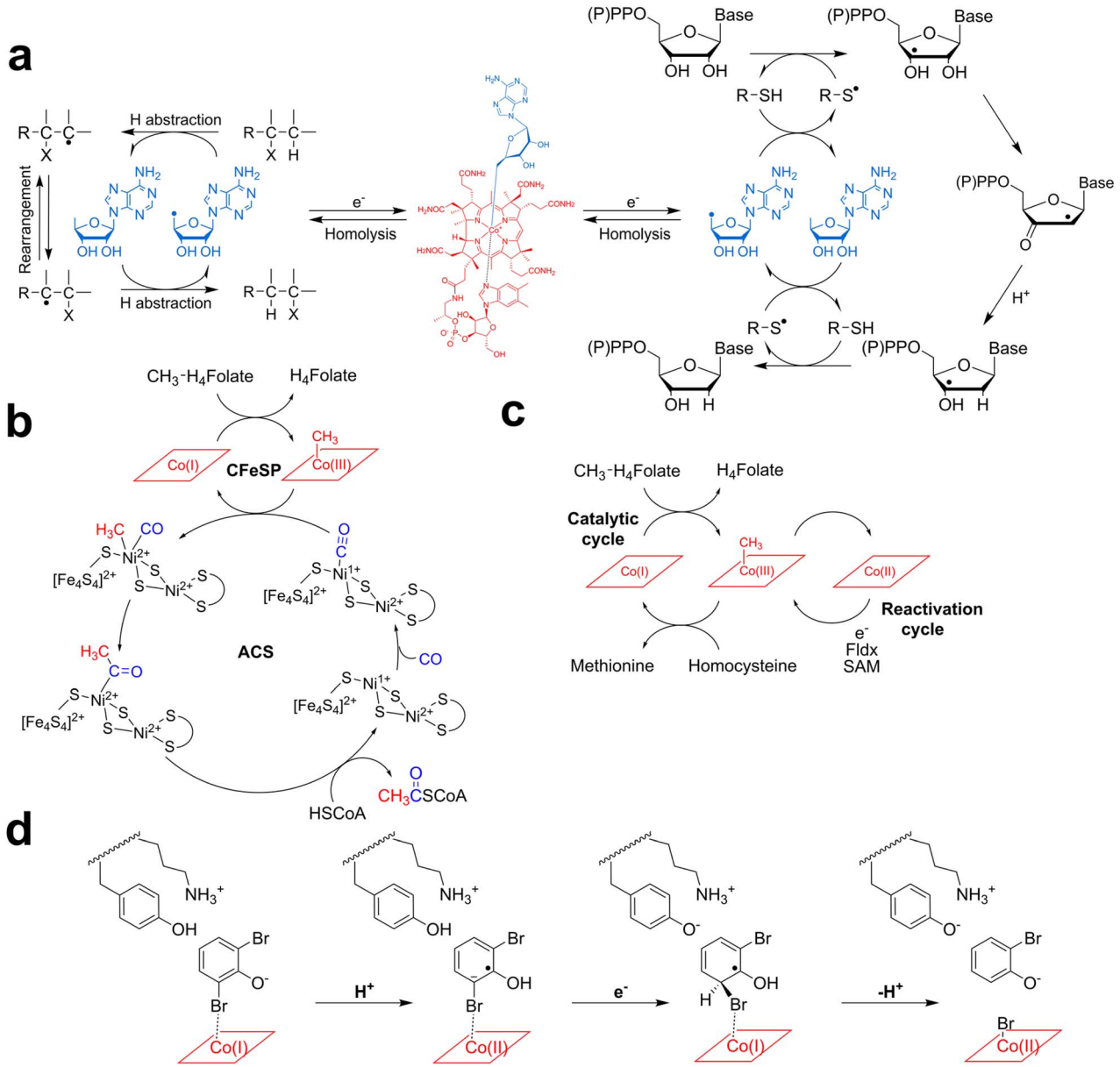

Fig. 5 General reaction mechanisms of B_{12}-dependent radical enzymes. (a) The general mechanism of isomerases/mutases compared to the one of ribonucleoside-triphosphate reductase. (b and c) Methyltransferases: the corrinoid iron-sulfur protein (CFeSP) with the A-cluster of acetyl-CoA synthase shown. This bimetallic Ni–Ni center is bridged to an $[Fe_4S_4]$ cluster. Ni_p (proximal nickel) serves mainly in electron transfer and the stabilization of intermediates, while Ni_d (distal nickel) binds the methyl group from CFeSP; (c) methionine synthase; and (d) dehalogenases.

For the reductive dehalogenase from *Nitratireductor pacificus* (NpRdhA), a direct interaction was observed between the cobalt atom of cobalamin and the substrate (Fig. 5d). A mechanism has been proposed with cobalamin directly catalyzing halogen abstraction through the formation of a halogen–cobalt bond, which could be followed either by heterolytic or homolytic bond cleavage, with the transient formation of a halogen-Cob(III)alamin or an aryl radical intermediate. In contrast, a direct interaction with the substrate was not evidenced for the tetrachloroethene reductive dehalogenase from *Sulfurospirillum multivorans* (PceA).[88] Thus, long-range electron transfer in the enzyme's active site may also play a crucial role in catalysis. For this enzyme, a homolytic cleavage of the C–Cl bond was favored because of the strong stability of the C–Cl bond (~80–100 kcal mol^{-1}) and the detection of Co(II) and radical intermediates in related systems.[93,94] Further studies will be required to determine whether there is a common mechanism for dehalogenases or if, as is commonly observed for B_{12}-dependent enzymes, they have evolved distinct reactivity. Of note, the function of the iron-sulfur clusters and the process leading to the reduction of Cob(II) into the catalytically competent Cob(I) remain to be fully clarified.

As illustrated by these three classes of enzymes, vitamin B_{12} is a powerful cofactor that can support different types of

chemistry (*i.e.*, nucleophilic *vs.* radical) and distinct reactions, from alkylation reactions and isomerization to the cleavage of halogen–carbon bonds. It is, therefore, not surprising that novel roles have emerged for this unique cofactor in the largest and most versatile superfamily of enzymes, the radical SAM enzymes.[22,23]

4. B_{12}-dependent radical SAM enzymes

The current release of the radical SAM enzyme superfamily includes more than 720 000 proteins.[1–3,95–97] These enzymes, recognized 20 years ago as an emerging superfamily,[21] form the most diverse and largest superfamily of enzymes. For instance, they catalyze an impressive diversity of radical-based reactions in a myriad of metabolic pathways,[3,98,99] including DNA synthesis[73] and repair,[13,14,100,101] and protein and nucleic acid modifications[7,8,102–109] as well as cofactor and vitamin biosynthesis.[15,16,110–115] These enzymes also play a central role in the growing family of ribosomally-synthesized and post-translationally modified peptides (RiPPs), which are major metabolites from the human microbiota and represent attractive alternatives to traditional antibiotics.[23–25,30,95,116–120] However, as more than half of the different classes have no experimentally validated functions, radical SAM enzymes are still a frontier in biochemistry.

As a general principle, these metalloenzymes are characterized by a $[Fe_4S_4]^{2+/1+}$ cluster coordinating the SAM cofactor and employ a radical-based mechanism, with some notable exceptions such as TsrM.[23,25,27,121,122] To initiate their reaction, one electron is transferred to the catalytic $[Fe_4S_4]^{2+/1+}$ cluster, inducing, like in B_{12}-dependent isomerases and mutases, the homolytic cleavage of a SAM molecule and the generation of the transient 5′-dA radical.[123–125] This highly reactive species usually activates a C–H bond by abstracting an H-atom from the substrate, resulting in the formation of a carbon-centered radical intermediate.[3,126,127] However, depending on the nature of the substrate, some variations have been evidenced, including the radical addition of 5′-dA to the substrate,[128,129] and recently, the generation of alternative radical species.[130] How these reactions are controlled by the protein matrix and how they proceed are still matters of debate; however, substrate reactivity likely plays a major role.[1,127]

Despite more than 30 years of investigation and the early prediction that vitamin B_{12} could be an essential cofactor for several radical SAM enzymes, this hypothesis has only recently been experimentally validated. The difficulties in expressing and investigating B_{12}-dependent radical SAM enzymes that utilize an $[Fe_4S_4]$ cluster and cobalamin, which are oxygen- and light-sensitive cofactors, combined with the fact that some of these enzymes are only able to accommodate the B_{12} cofactor during their translation,[25,26,32,33,131] likely explain why our understanding of this superfamily of enzymes remains limited.

Over the last decade, B_{12}-dependent radical SAM enzymes have been shown to catalyze a diverse range of reactions. Notably, this family includes a large group of methyltransferases that are unique biocatalysts capable of forming a carbon–carbon bond between a methyl group and an unactivated carbon atom[23,25,28,31,33,131–136] (Fig. 6). By far, these C-methyltransferases are the most studied and represented group within this superfamily, alkylating usually Csp^3-atoms on a broad diversity of molecules from carbohydrates and antibiotics (Fosfomycin,[134,137] Gentamicin[133]) to various metabolites and proteins (Fig. 6). Intriguingly, the first two enzymes investigated in this group were PhpK[22] and TsrM,[23,25,32] which are a P- and a Csp^2-methyltransferase, respectively. In sharp contrast to the methylation of inert Csp^3-atoms, the reaction catalyzed by these two enzymes can proceed either by radical or nucleophilic chemistry (Fig. 6 and 7).

Alongside these methyltransferases, B_{12}-dependent radical SAM enzymes that catalyze unrelated transformations, such as ring formation, ring contraction, and thioether bond formation, have been sporadically identified in the bacteriochlorophyll,[138] oxetanocin A[29] and carbapenem[35] biosynthetic pathways, respectively.

In these distinct reactions, the role and nature of the cobalamin cofactor are unclear. For instance, in OxsB, it has been proposed that cobalamin could serve as an electron sink, while in BchE, since two H-atom abstractions are required, AdoCbl might be involved in catalysis.

4.1. Mechanisms of B_{12}-dependent radical SAM enzymes

Known mechanisms of the B_{12}-dependent radical SAM enzymes include both radical and nucleophilic chemistry (Fig. 6). For B_{12}-dependent radical SAM enzymes catalyzing the methylation of a Csp^3-atom,[23,25,28,31,33,131–136] the consensus mechanism

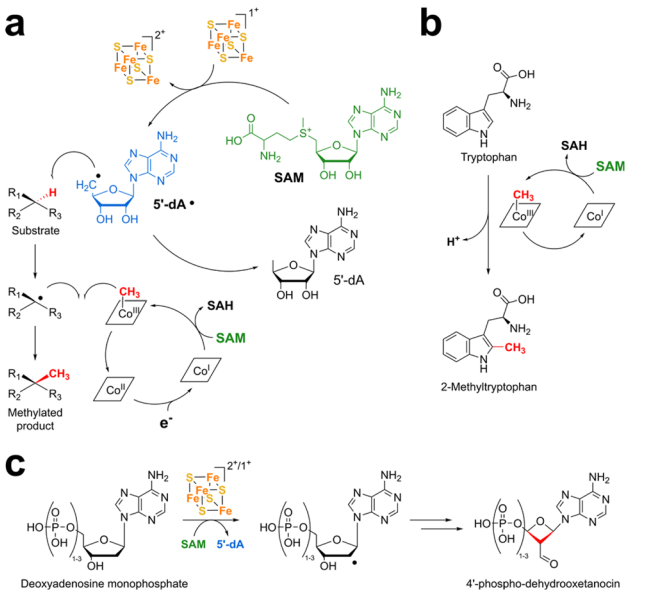

Fig. 6 The proposed mechanism for representative B_{12}-dependent radical SAM enzymes catalyzing radical and nucleophilic chemistry. (a) The general mechanism of Csp^3-B_{12}-dependent methyl transferases. (b) The mechanism of TsrM, a Csp^2-B_{12}-dependent methyl transferase. (c) The mechanism of OxsB, a Csp^2-B_{12}-dependent radical SAM enzyme catalyzing oxidative ring contraction.

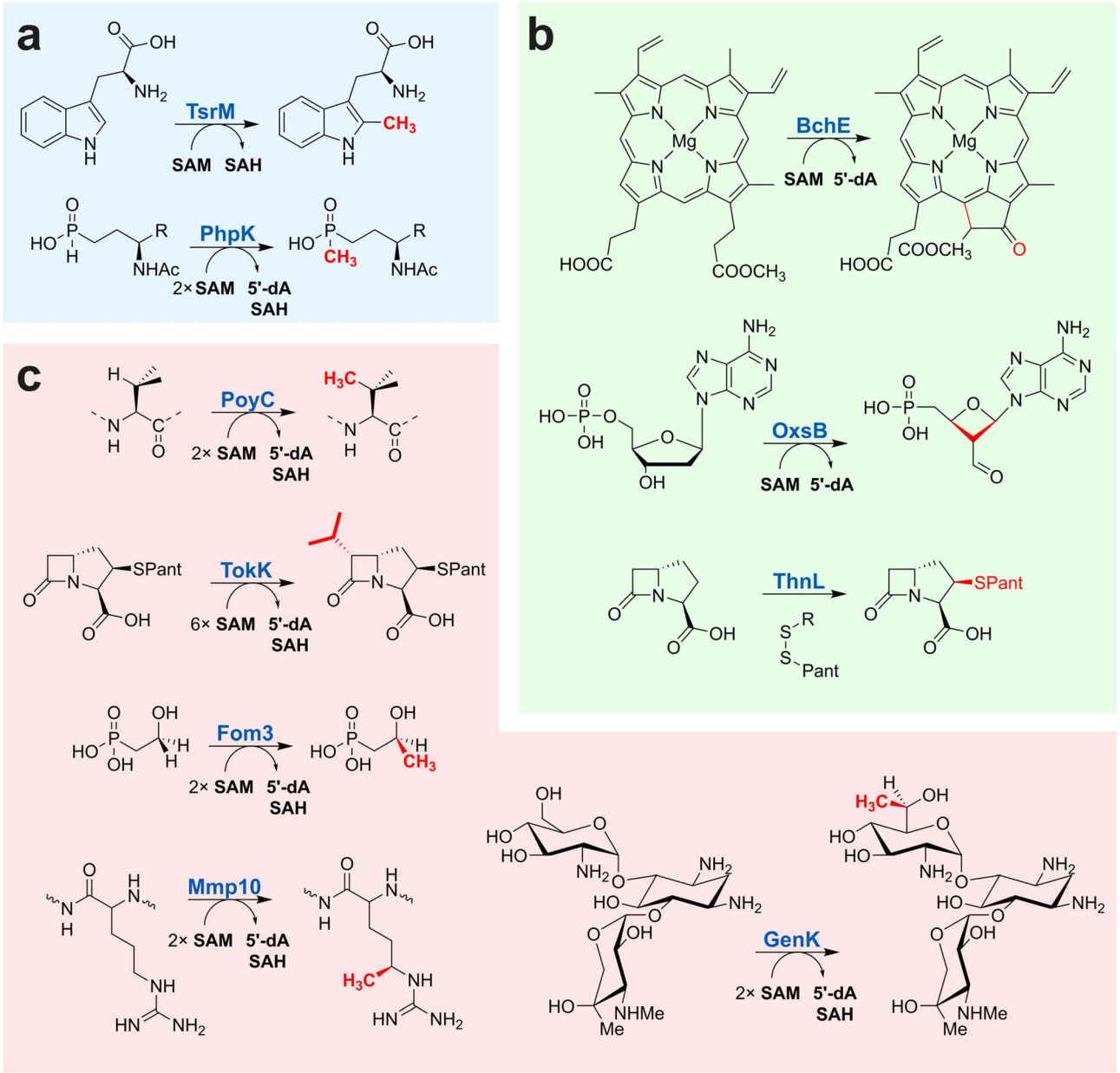

Fig. 7 Reactions catalyzed by B_{12}-dependent radical SAM enzymes. B_{12}-dependent radical SAM enzymes are involved in (a) the methylation of Csp^2-hybridized and P-atoms, (b) various reactions such as ring contraction and thioether bond formation and (c) the methylation of Csp^3-hybridized atoms.

involves a dual use of the SAM cofactor as both a methyl donor and a radical initiator, with the concomitant formation of SAH (*S*-adenosyl-homocysteine) and 5′-dA, respectively (Fig. 6a). In most cases, methylation occurs with inversion of the stereochemistry of the methylated carbon atom (*i.e.*, a double double-inversion process);[134,137,139–144] however, some exceptions where the stereochemistry of the alkylated carbon atom is retained are known.[139]

Intriguingly, for the Csp^2-methyltransferase TsrM, the currently accepted mechanism implies a nucleophilic displacement without a clear role for the conserved $[Fe_4S_4]$ cluster, although the latter is functionally critical[23,25,121,122] (Fig. 6b). Finally, the other enzyme whose mechanism has been investigated in detail is OxsB, catalyzing oxidative ring contraction. While it has been demonstrated that OxsB radically cleaves SAM and abstracts a substrate H-atom, the function of its cobalamin cofactor is unclear; however, it has been exploited to alter the fate of the reaction, leading to the formation of methylated products.[29,145]

4.2. A base-off vitamin B_{12} in the active site of radical SAM enzymes

Thanks to the spectroscopic and structural investigations of several unrelated enzymatic systems in the last decade,

significant advances have been made in our understanding of the role of cobalamin in B_{12}-dependent radical SAM enzymes. The first spectroscopic study was published in 2016 and focused on the characterization of TsrM.[27] This study not only showed that the [Fe_4S_4] cluster does not directly interact with SAM, but also that the cobalamin cofactor was likely in a base-off configuration, two conclusions supported by a subsequent structural analysis.[121] Thus, while in solution vitamin B_{12} essentially exists in a hexacoordinated base-on form with the DMB moiety binding the α-face of the corrin-bound cobalt atom (Fig. 2), it is decoordinated in TsrM. Intriguingly, the structural analysis of OxsB[146] and the recent structures of three methyltransferases, TokK,[147] Mmp10,[33] and QCMT,[148] revealed a similar base-off configuration of the cobalamin cofactor supporting a general trend within this superfamily (see Section 4.3).

The displacement of the DMB moiety has a profound impact on modulating not only the strength, but also the reactivity of the Co–carbon bond localized *in trans*. It also increases the molecular surface by about 300 $Å^{-2}$. This binding mode is far from being exclusive to B_{12}-dependent radical SAM enzymes. Indeed, from methionine synthase[80] to human and bacterial mutases,[149–151] many B_{12}-dependent enzymes have been shown to have a base-off cofactor.[152] In these enzymes, the cobalt-atom is coordinated by the protein itself, generally through a histidine residue in the consensus sequence DxHxxG, resulting in a "base-off/His-on" cobalamin. With this conformation, the hexacoordination of the cobalt atom is preserved. Other enzymes, such as the pyridoxal-phosphate (PLP)-dependent enzymes lysine-5,6-amino-mutase[153] and ornithine-4,5-amino-mutase,[154] also share this binding mode. The CFeSP[82,83] and the reductive dehalogenases,[87,88,92] despite having a base-off cobalamin, do not have a protein residue in direct interaction with the α-face of the corrin-bound Co-atom. Thus, the Co-atom exhibits a penta-coordination with a methyl group or the substrate present on the β-face, a configuration similar to the one encountered in B_{12}-dependent radical SAM enzymes. In contrast, many B_{12}-dependent enzymes, such as diol dehydratases,[155,156] ethanolamine ammonia lyase,[157] and B_{12}-dependent ribonucleotide reductase,[158] possess a base-on cobalamin in their active site.

4.3. Structural diversity of B_{12}-dependent radical SAM enzymes

Unraveling the structure of B_{12}-dependent enzymes has proven challenging, with the first structural information published in 2014,[159] and the first holo-structure released in 2017.[29,160] Recently, the structural characterization of seven B_{12}-dependent radical SAM enzymes, including TsrM,[121] TokK,[147] Mmp10,[33] QCMT,[148] and two proteins of unknown function,[161] has laid the foundation for comprehending the structural and mechanistic diversity of these enzymes (Fig. 8). Structurally, these enzymes are characterized by a typical radical SAM domain organized in a fold related to the TIM barrel. This domain houses a catalytic [Fe_4S_4] cluster coordinated by the canonical radical SAM motif CXXXCXXC. In addition to this TIM barrel domain, these

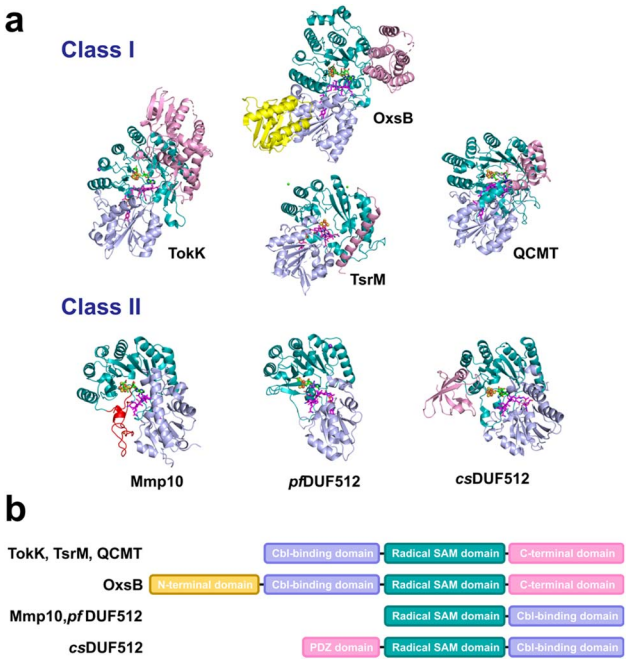

Fig. 8 B_{12}-dependent radical SAM enzyme structures. (a) Structures of class I (TokK, OxsB, TsrM, QCMT) and II (Mmp10, *cs*DUF512, *pf*DUF512) B_{12}-dependent radical SAM enzymes shown as cartoons and colored by domain. The cobalamin-binding domain is colored in light blue, the radical SAM domain in teal and the N-terminal domain of unknown function and the helix bundle domain of OxsB are depicted in yellow and pink, respectively. The iron loop in Mmp10 is colored in red. The C-terminal domains in TokK and TsrM and the PDZ domain in *cs*DUF512 are shown in pink. B_{12}, Cob and AdoCbl are shown as magenta sticks. The SAM (SAM cleavage products in TokK, aza-SAM in both TsrM and *cs*DUF512 and SAH in *pf*DUF512) is depicted using green sticks, and the radical SAM [Fe_4S_4] cluster is shown with orange and yellow spheres. (TokK: PDB 7KDY; OxsB: PDB 5UL4; TsrM: PDB 6WTF; QCMT: PDB 9CCB; Mmp10: PDB 7QBS; *cs*DUF512: PDB 9CG1; *pf*DUF512: PDB 9CG2). (b) Domain organization in B_{12}-dependent radical SAM enzymes.

enzymes possess a cobalamin-binding domain, which anchors the cobalamin cofactor. This second domain adopts a general Rossmann fold configuration, with some variations regarding the number of beta (β) strands and alpha (α) helices, and their relative orientation to the radical SAM domain, defining two distinct classes of B_{12}-dependent radical SAM enzymes (Fig. 8).

The first class encompasses OxsB, TsrM, TokK, and QCMT. Despite catalyzing three distinct reactions (*i.e.*, ring contraction, nucleophilic and radical methylation, respectively), these enzymes share similar structural features with a Rossmann fold constituted of five β-strands and five connecting α helices. In addition, the respective orientation of the cobalamin cofactor and the [Fe_4S_4] cluster are similar, with a distance of approximately 11 Å between both metallic centers, ideally suited to sandwich the substrate. The only notable difference in these enzymes' active-sites is the tetra-coordination of the [Fe_4S_4] cluster in TsrM by a glutamate residue, presumed to preclude radical chemistry.

The second class of B_{12}-dependent radical SAM enzymes was initially revealed by the investigation of Mmp10,[33] which

catalyzes the methylation of Arg285 in the active site of methyl coenzyme M reductase (MCR), the central enzyme in methanogenesis. For this enzyme, the B_{12}-domain is located in the C-terminal position, and the radical SAM domain is in the N-terminal position, in contrast to the majority of B_{12}-dependent radical SAM enzymes (Fig. 8). Moreover, despite catalyzing a radical reaction, Mmp10 possesses a tetra-coordinated cluster like TsrM. In this enzyme, the Rossmann fold comprises 4 β-strands and 4 α-helices. Interestingly, a recent study has shown that unrelated enzymes possessing a DUF512 domain[161] share the same overall structure with a notably similar cobalamin binding domain. In these enzymes, the average relative distance between both metallic centers is 12 Å and, in the absence of the substrate, the radical SAM $[Fe_4S_4]$ cluster is fully coordinated. In the case of Mmp10, it has been shown that upon substrate binding, a unique active-site conformational change allows SAM to bind to the $[Fe_4S_4]$ cluster, supporting the initiation of the radical reaction. It remains to be shown if DUF512 proteins exhibit a similar behavior.

Although there is no direct interaction between the protein and the α-face of the cobalamin cofactor, B_{12}-dependent radical SAM enzymes typically feature a residue in the lower axial position of cobalamin that plays a role in enzyme catalysis. Proteins that catalyze radical-dependent methylations provide a hydrophobic environment to the lower face using distinct residues (*i.e.*, Trp76, Leu322, Leu366, and Leu286 in TokK, Mmp10, *cs*DUF512, and *pf*DUF512, respectively) (Fig. 9), while enzymes that do not catalyze radical methylation have a more hydrophilic environment (*i.e.*, Arg69 and a water molecule & Asn186 in TsrM and OxsB, respectively). These residues likely prevent the hexacoordination of the cobalamin cofactor, weakening the Co–C bond and increasing its reactivity.

Another intriguing feature is the presence, between the cobalamin cofactor and the $[Fe_4S_4]$ cluster, of a generally hydrophobic residue (*i.e.* Trp215, Tyr23, F95 and Y47 in TokK, Mmp10, *cs*DUF512 and *pf*DUF512 respectively), with the exception of TsrM which possesses a polar residue (Glu236) (Fig. 9). While the function of this residue is unclear, it likely plays an important role in tuning the cobalamin redox potential. Mutation of this or the lower axial residue has been shown to strongly impair enzyme activity,[121,147] emphasizing the importance of the cobalamin's surrounding environment. Of note, OxsB, which does not use cobalamin for methyl transfer but most likely as an electron sink, does not possess this stacked residue, and its lower face is solvent accessible. Further studies will be required to ascertain the exact role of these residues, but they likely contribute to finely tuning cobalamin reactivity.

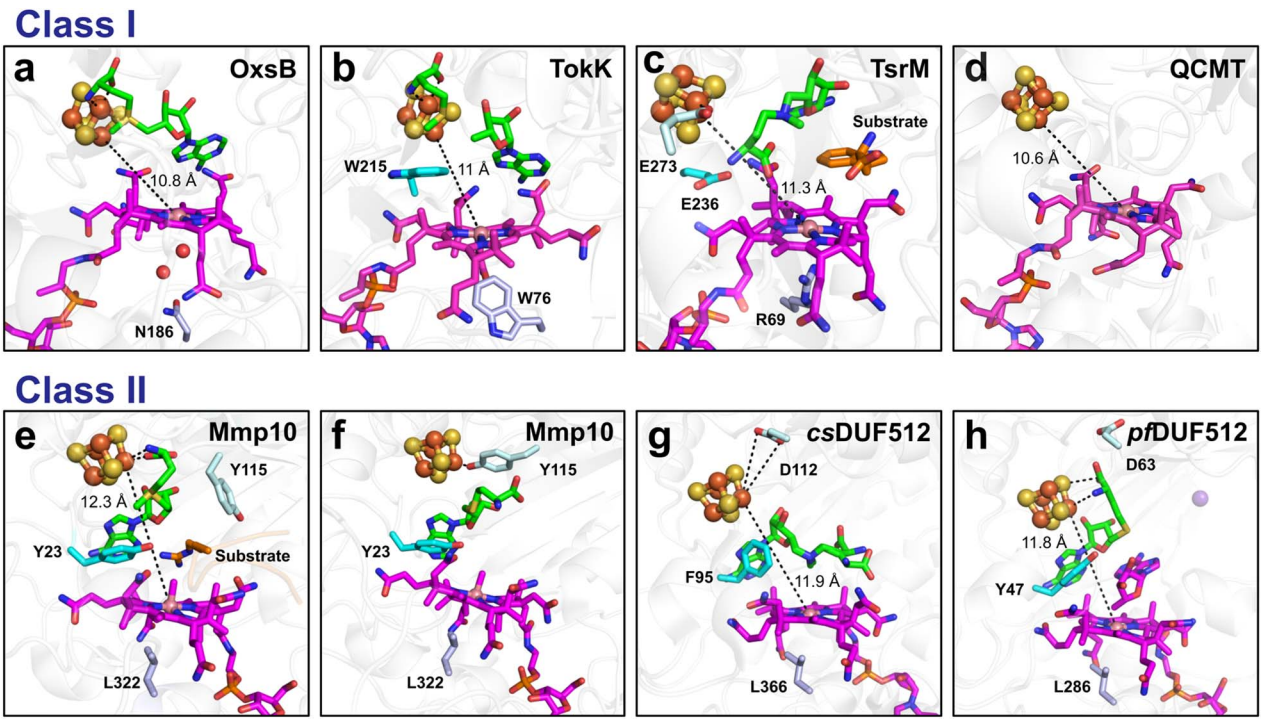

Fig. 9 Structures of the active sites of B_{12}-dependent radical SAM enzymes showing the positions of key residues. Close-ups of the active sites of (a) OxsB, (b) TokK, (c) TsrM, (d) QCMT, (e) Mmp10 with and (f) without substrate, (g) *cs*DUF512 and (h) *pf*DUF512. Residues involved in the tetra coordination of the cluster (E273/TsrM, Y115/Mmp10, D112/*cs*DUF512 and D63/*pf*DUF512) are shown in pale cyan. Amino acids in the lower axial position of cobalamin (N186-water107/OxsB, W76/TokK, R69/TsrM, L322/Mmp10, L366/*cs*DUF512, L286/*pf*DUF512) are shown in light blue. Residues between the cobalamin cofactor and the $[Fe_4S_4]$ cluster (W215/TokK, E236/TsrM, Y23/Mmp10, F95/*cs*DUF512, Y47/*pf*DUF512) are shown in cyan. The cobalamin cofactor is shown in magenta, the $[Fe_4S_4]$ cluster in orange and yellow spheres, and the SAM (SAM cleavage products in TokK, aza-SAM in both TsrM and *cs*DUF512 and SAH in *pf*DUF512) in green. Substrates are shown in orange. The distance between the cobalt atom of cobalamin and the proximal iron from the $[Fe_4S_4]$ cluster is indicated with a black dashed line. (TokK: PDB 7KDY; OxsB: PDB 5UL4; TsrM: PDB 6WTF; QCMT: PDB 9CCB; Mmp10: PDB 7QBS; *cs*DUF512: PDB 9CG1; *pf*DUF512: PDB 9CG2).

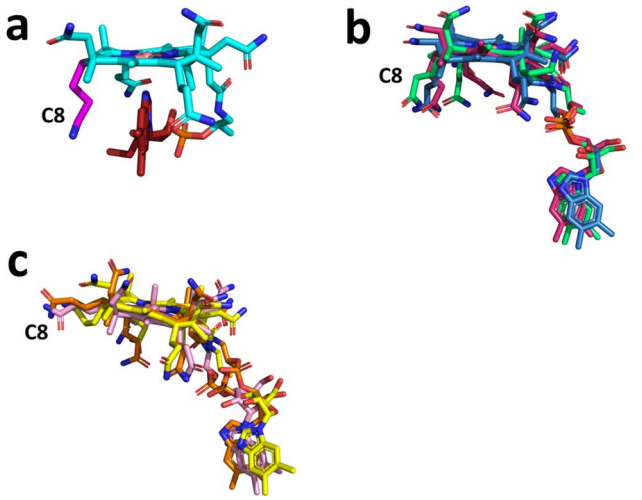

Fig. 10 Conformation of the cobalamin cofactor in B_{12}-dependent SAM enzymes. (a) Base-on cobalamin, (b) cobalamin cofactor in OxsB (green), TsrM (blue) and QCMT (pink), and (c) cobalamin cofactor in Mmp10 (light pink), TokK (orange) and *pf*DUF512 (yellow).

In all the structures solved with a bound cobalamin, the cofactor is in the base-off configuration, with the DMB moiety displaced from the Co-atom (Fig. 10). Intriguingly, among these seven structures, we can define two groups that differ by the orientation of the C8 side chain. While QCMT, TsrM, and OxsB have a configuration similar to base-on cobalamin (Fig. 10a and b), Mmp10, TokK, and DUF512 proteins adopt a more planar configuration of the tetrapyrrole ring (Fig. 10c). Although this conformational constraint is likely to have a substantial effect on cobalt reactivity, it is not generic to enzymes catalyzing radical methyl transfer reactions (*e.g.* Mmp10 & TokK) since in QCMT, the geometry of the tetrapyrrole ring is similar to the one found in base-on cobalamin.

5. Structural relationships with other iron-sulfur and B_{12}-dependent enzymes

As mentioned above, the association of a $[Fe_4S_4]$ cluster and a cobalamin cofactor is not unique to B_{12}-dependent radical SAM enzymes and has been previously shown in other enzyme families, including the reductive dehalogenases and the CFeSP. The structures of two distinct types of reductive dehalogenases, NpRdhA[87] and PceA,[88] have revealed striking structural similarities within this enzyme family, with notably a $[Fe_4S_4]$ cluster and a cobalamin binding domain located in the C- and N-terminal regions, respectively, along with additional domains. However, in contrast to B_{12}-dependent radical SAM enzymes, the cobalamin cofactor resides in the innermost region of the enzyme's core, whereas two $[Fe_4S_4]$ clusters are located near the protein surface. Remarkably, one of these clusters is positioned within the van der Waals distance to the tetrapyrrole ring while the other one is located beneath cobalamin, consistent with their function as an electron shuttle (Fig. 11).

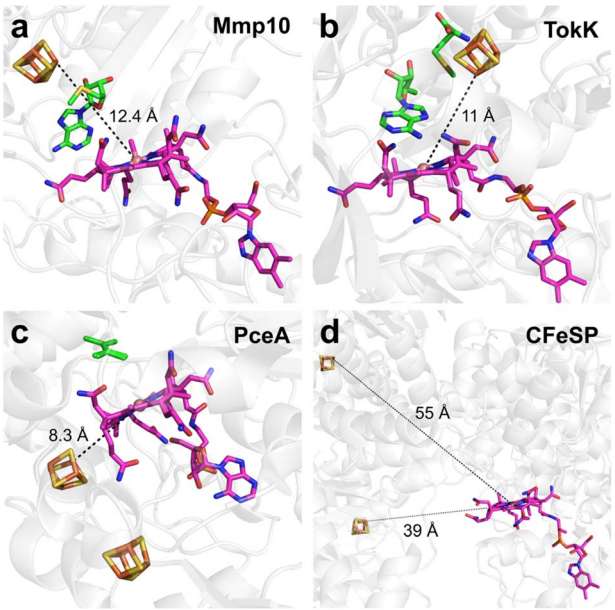

Fig. 11 $[Fe_4S_4]$ clusters and cobalamin organization in (a and b) B_{12}-dependent radical SAM methyltransferases (Mmp10 & TokK), (c) reductive dehalogenases and (d) CFeSP. The cobalamin cofactor is in magenta and the $[Fe_4S_4]$ clusters are shown using orange and yellow sticks. When present, cofactors and substrates are shown in green. (Mmp10: PDB 7QBT; TokK: PDB 7KDX; PceA: PDB 4URO and CFeSP: PDB 4DJD). The distance between the cobalt atom of cobalamin and the proximal iron from the $[Fe_4S_4]$ cluster is indicated with a black dashed line.

In CFeSP, the relative organization of the $[Fe_4S_4]$ cluster and the cobalamin cofactors also differs. Indeed, while both cofactors are located within the same subunit, the distance between the cobalt atom and the nearest iron of the $[Fe_4S_4]$ cluster is ~39 Å compared to 8.3 Å in reductive dehalogenases and ~11–12 Å in B_{12}-dependent radical SAM methyltransferases. Here again, the function of the $[Fe_4S_4]$ cluster is to shuttle electrons. However, in CFeSP, which catalyzes nucleophilic chemistry, the purpose of the $[Fe_4S_4]$ cluster is to reactivate the enzyme by reducing the cobalt cofactor from Cob(II) to Cob(I).

Hence, while B_{12}-dependent radical SAM methyltransferases, CFeSP, and reductive dehalogenases use cobalamin and iron-sulfur clusters for catalysis, they use both cofactors for distinct functions and chemistry.

6. Functional perspective on B_{12}-dependent SAM enzymes

B_{12}-dependent radical SAM enzymes have been shown to be involved in diverse transformations with novel functions likely to emerge within this superfamily. Using sequence similarity networks,[162] we can have an estimate of their structural and functional diversity (Fig. 12). By analyzing 83 589 accession IDs sorted into 37 236 "meta-nodes", it is possible to delineate more than 100 groups. The largest group contains about one-third of the nodes, and the majority of the groups have less than 100 unique protein sequences. To date, structural information has

been obtained only for discrete groups (red labels in Fig. 12), with the largest ones still unexplored. All groups, with at least one functionally characterized enzyme, include C-methyltransferases, which appear to be scattered throughout the B_{12}-dependent radical SAM network.

With the exception of OxsB, enzymes involved in non-methylation reactions belong to the largest group of B_{12}-dependent radical SAM enzymes, and show no clear structural features that would allow them to be distinguished from alkylating enzymes. Similarly, some B_{12}-dependent radical SAM enzymes have been shown or predicted to catalyze non-radical methylations, such as TsrM,[23,25,121,181] or ClоN6.[163] However, they belong to distinct groups and appear to share more functional than structural similarities. Further characterization of the structural features and cofactor content of these different groups is necessary before a comprehensive understanding of this emerging enzyme family can be obtained. For instance, Mmp10, with only a radical SAM and a B_{12}-binding domain, might represent one of the simplest scaffolds,[33] but its structural analysis revealed unanticipated complexity, with four distinct metallic centers, including the first mononuclear iron identified within this superfamily. Finally, although sequence similarity networks are useful for encapsulating radical SAM enzyme diversity, they may also include unrelated enzyme architectures. For instance, several radical SAM enzymes,[184] which do not bind cobalamin (*i.e.*, AprD4 and homologs of the 3-amino-3-carboxypropyl transferase, ArsL[130]) but have domains remotely related to the Rossmann fold, are present within this SSN (Fig. 12). However, unexpected discoveries are likely to be made along the way while exploring this constellation of biocatalysts.[139,186,187]

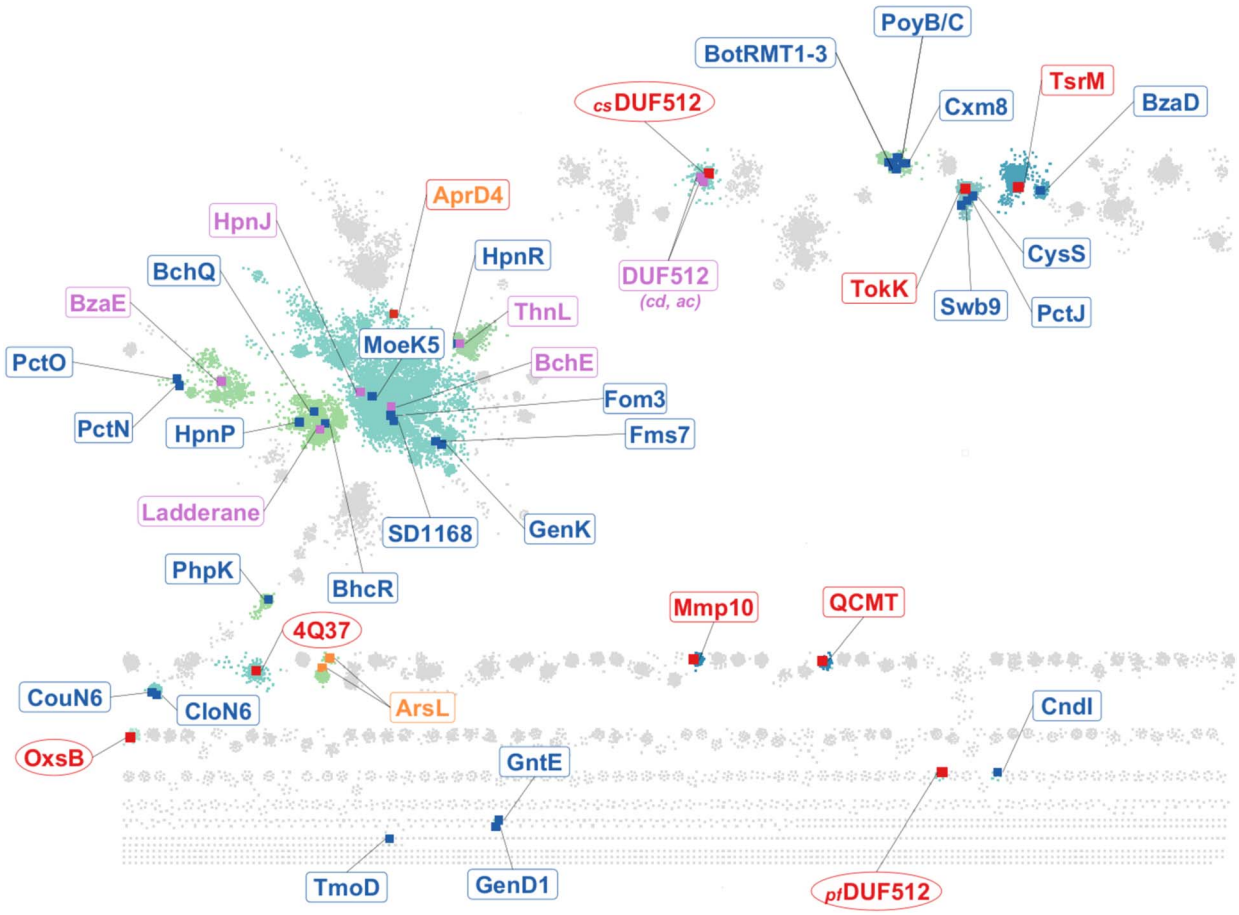

Fig. 12 The sequence similarity network of B_{12}-dependent radical SAM enzymes. In blue, non-structurally characterized methyltransferases: Csp^2-methyltransferases: CouN6, CloN6,[163] BzaD;[59] P-methyltransferase: PhpK;[22] iterative methyltransferases: PoyB,[164] CysS,[135] Swb9,[165] BchQ,[166] TmoD;[167] Csp^3-methyltransferases: PoyC,[131] PctJ,[168] BotRMT 1–3,[169] HpnR,[170] Fms7,[171] GenK,[133,172] Fom3,[31,137] MoeK5,[173] SD1168,[174] BchR,[166] HpnP,[170] PctO,[175] PctN,[175] CndI,[176] GntE/GenD1,[177] Cmx8. In purple: enzymes catalyzing other or unknown transformations: BchE,[178] Ladderane,[179] ThnL,[35] BzaE,[59] HpnJ,[180] *cd*DUF512,[161] *ac*DUF512.[161] In red, structurally characterized enzymes with known functions (rounded rectangles): TsrM,[23,25,121,181] Mmp10,[33,182] QCMT,[148,183] TokK,[147] and other or unknown transformations (ellipses): OxsB,[29] 4Q37,[159] *cs*DUF512,[161] *pf*DUF512.[161] In orange, unrelated enzymes such as[184] AprD4 and ArsL.[130] The network was generated from the list of Uniprot IDs of all current B_{12}-dependent radical SAM enzymes (clusters 2.1, 1.4 and 1.8 on *radicalsam.org*[2]) using the Enzyme Function Initiative (EFI) enzyme similarity tool (EFI-EST) with an alignment score of 60. To improve the visualization of the network with cytoscape,[185] we chose the representative node ("rep node") representation where sequences sharing > 50% identity are grouped into the same meta-node.

Conclusions and future outlook

B_{12}-dependent radical SAM enzymes, despite their broad distribution and key roles in major biosynthetic pathways from vitamin to antibiotic biosynthesis, have been investigated only recently. While initially mainly regarded as methyltransferases, in the last decade, these enzymes have been shown to catalyze novel reactions that remain to be fully deciphered.[139,186] Notably, in non-methyl transfer reactions, the role and exact nature of the cobalamin cofactor remain to be clarified.

Structural analysis has provided unexpected insights into the mechanisms of these unique biocatalysts. First, it has recently been discovered that B_{12}-dependent radical SAM enzymes can adopt two distinct architectures, with the B_{12}-binding domain at the N- or C-terminus, delineating two classes of enzymes (Fig. 8).[29,33,147,161] More intriguingly, several of these enzymes exhibit tetra-coordination of the radical SAM [Fe_4S_4] cluster using, in addition to the three canonical cysteine ligands, another protein residue. This conformation, never observed since the inception of this enzyme family 25 years ago,[21] is likely important for these enzymes to switch from nucleophilic to radical chemistry. However, due to the limited number of structures solved, notably in the presence of genuine cofactors and substrates, it remains unclear whether these are isolated cases or a general trend within this emerging family of enzymes.

The cobalamin cofactor has been shown to adopt a base-off coordination, as observed in other B_{12}-dependent enzymes. However, in some B_{12}-dependent radical SAM enzymes, the tetrapyrrole ring has a planar conformation with an axial C8 side chain. The purpose of this distinct geometry is unclear, but, together with key residues that interact with both faces of the cobalamin cofactor, it likely tunes the reactivity and redox potential of the Co-center.

Despite the outstanding progress made in the last decade, much remains to be explored within this superfamily. As detailed here, we still lack structurally characterized representatives for the main groups of B_{12}-dependent radical SAM enzymes. Some key aspects of their catalysis, such as the factors that control their stereochemistry or the ability of some of them to catalyze iterative methyl–transfer reactions, have only been marginally investigated. A deeper understanding of these biocatalysts will be necessary to harness their powerful biosynthetic potential, particularly the formation of C–C bonds between unreactive carbon atoms, chemical prowess unique to radical SAM enzymes.[95,139] B_{12}-dependent radical SAM enzymes likely hold surprises in store, notably regarding their chemical versatility and their ability to catalyze unprecedented reactions.

Author contributions

O. B., M.T. N & A. B: original draft. All authors contributed to the figures, review, and editing of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data that support some findings of this study, notably the SSN, are available from the corresponding author, O. Berteau, upon reasonable request.

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