Radical SAM Enzymes Involved in Tetrapyrrole Biosynthesis and Insertion

Gunhild Layer,* Martina Jahn, Jürgen Moser, and Dieter Jahn

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ABSTRACT: The anaerobic biosyntheses of heme, heme d_1, and bacteriochlorophyll all require the action of radical SAM enzymes. During heme biosynthesis in some bacteria, coproporphyrinogen III dehydrogenase (CgdH) catalyzes the decarboxylation of two propionate side chains of coproporphyrinogen III to the corresponding vinyl groups of protoporphyrinogen IX. Its solved crystal structure was the first published structure for a radical SAM enzyme. In bacteria, heme is inserted into enzymes by the cytoplasmic heme chaperone HemW, a radical SAM enzyme structurally highly related to CgdH. In an alternative heme biosynthesis route found in archaea and sulfate-reducing bacteria, the two radical SAM enzymes AhbC and AhbD catalyze the removal of two acetate groups (AhbC) or the decarboxylation of two propionate side chains (AhbD). NirJ, a close homologue of AhbC, is required for propionate side chain removal during the formation of heme d_1 in some denitrifying bacteria. Biosynthesis of the fifth ring (ring E) of all chlorophylls is based on an unusual six-electron oxidative cyclization step. The sophisticated conversion of Mg-protoporphyrin IX monomethylester to protochlorophyllide is facilitated by an oxygen-independent cyclase termed BchE, which is a cobalamin-dependent radical SAM enzyme. Most of the radical SAM enzymes involved in tetrapyrrole biosynthesis were recognized as such by Sofia et al. in 2001 (*Nucleic Acids Res.* **2001**, *29*, 1097−1106) and were biochemically characterized thereafter. Although much has been achieved, the challenging tetrapyrrole substrates represent a limiting factor for enzyme/substrate cocrystallization and the ultimate elucidation of the corresponding enzyme mechanisms.

KEYWORDS: *tetrapyrrole biosynthesis, heme, heme d_1, chlorophyll, radical SAM, CgdH, HemW, AhbD, AhbC, NirJ, BchE*
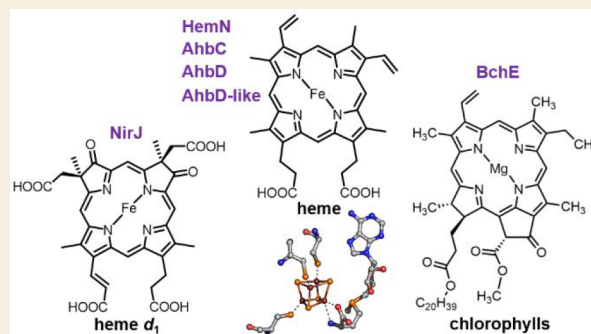
INTRODUCTION

Many members of the radical SAM enzyme family are involved in the biosynthesis of diverse natural products such as antibiotics, vitamins, and cofactors.[1,2] The biosynthesis of different tetrapyrrole cofactors such as heme, heme d_1, and bacteriochlorophyll represents a remarkable example for the multiple occurrence of radical SAM chemistry within a given pathway. Tetrapyrroles, often called the pigments of life, share a common structural core of four linked pyrrole rings forming either a closed, circular arrangement or a linear, open chain structure. Cyclic tetrapyrroles vary in the redox state of their ring system, the type of chelated metal ion, and the type of pyrrole ring substituents.[3] Prominent members of the cyclic tetrapyrroles are the magnesium-containing, green (bacterio)-chlorophylls that are essential for oxygenic and anoxygenic photosynthesis.[4] The yellow, nickel-containing coenzyme F_{430} plays an essential role in methanogenesis as part of the enzyme methyl-coenzyme M reductase.[5] Siroheme and heme d_1 are iron-containing isobacteriochlorins serving as prosthetic groups of assimilatory sulfite/nitrite reductases[6] and dissimilatory cytochrome cd_1 nitrite reductase,[7,8] respectively. The cobalt-containing corrins of the cobalamin or vitamin B_{12} class are part of methyltransferases, reductive dehalogenases, isomerases, and radical SAM enzymes.[9] However, the most ubiquitously found and versatile tetrapyrroles are hemes. These red, iron-containing porphyrins participate in various cellular processes including electron transfer, gas sensing and transport, signaling, catalysis, and transcriptional regulation.[10]

All members of the tetrapyrrole family are synthesized along a branched biosynthetic route starting from the first common precursor 5-aminolevulinic acid[3] (Figure 1).

Strikingly, there are multiple steps in the different branches of this pathway that are catalyzed via radical SAM chemistry. For instance, the synthesis of heme requires either one (CgdH) or two (AhbC, AhbD) radical SAM enzymes depending on the
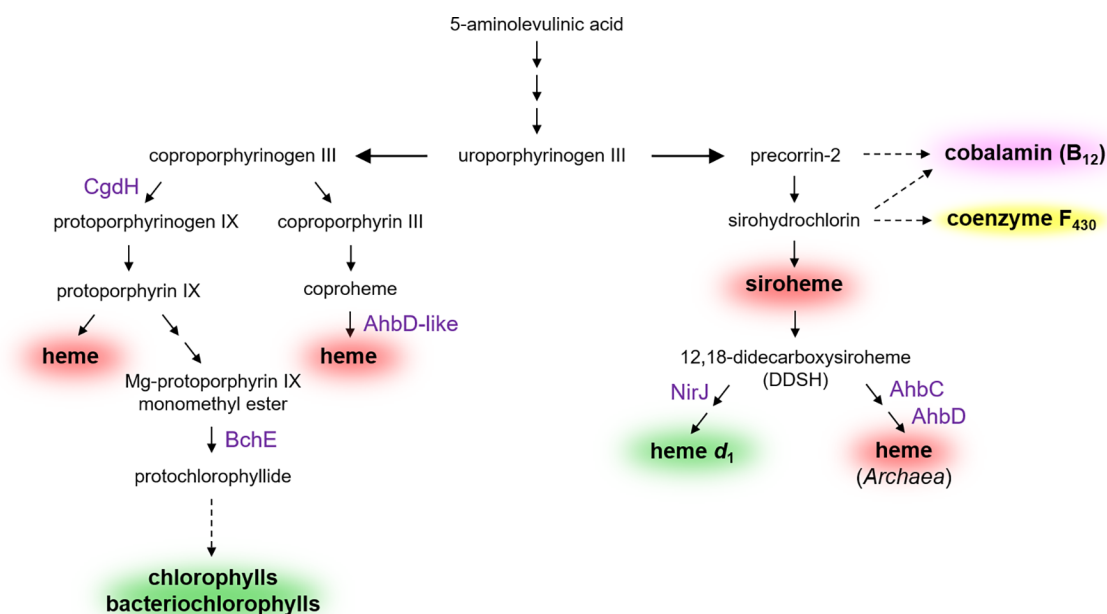
Figure 1. Overview of the branched tetrapyrrole biosynthesis pathway. Radical SAM reaction steps are highlighted by the respective enzyme names in violet. Note that the CgdH step is also carried out by CgdC in many organisms, and the AhbD-like step is also catalyzed by ChdC, both under aerobic conditions (see also main text). Arrows with solid lines represent one reaction step, and arrows with dashed lines indicate more than one reaction.
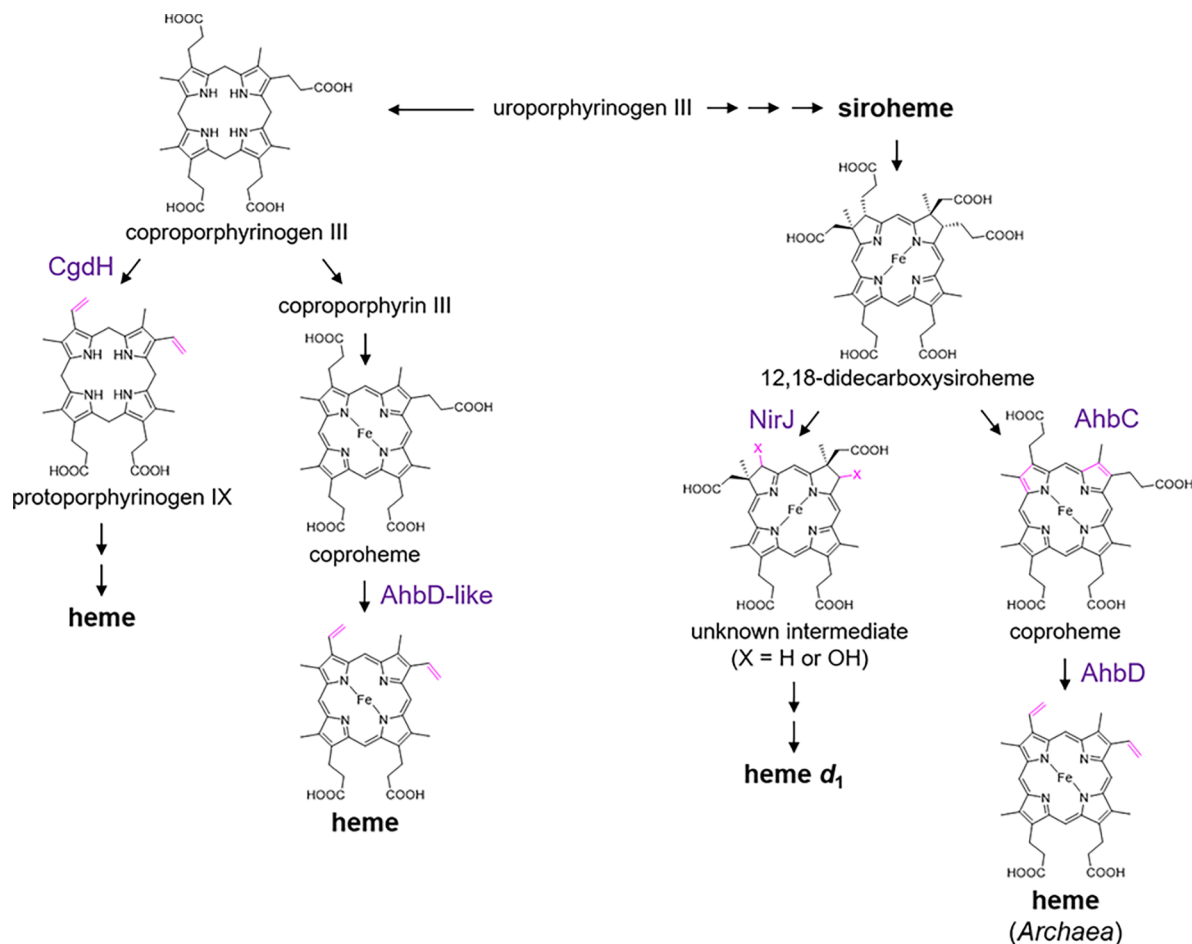

Figure 2. Currently known routes to heme and heme d_1. Radical SAM reactions are shown with chemical structures and are highlighted in pink. Note that the CgdH step is also carried out by CgdC in many organisms, and the AhbD-like step is also catalyzed by ChdC, both under aerobic conditions (see also main text).
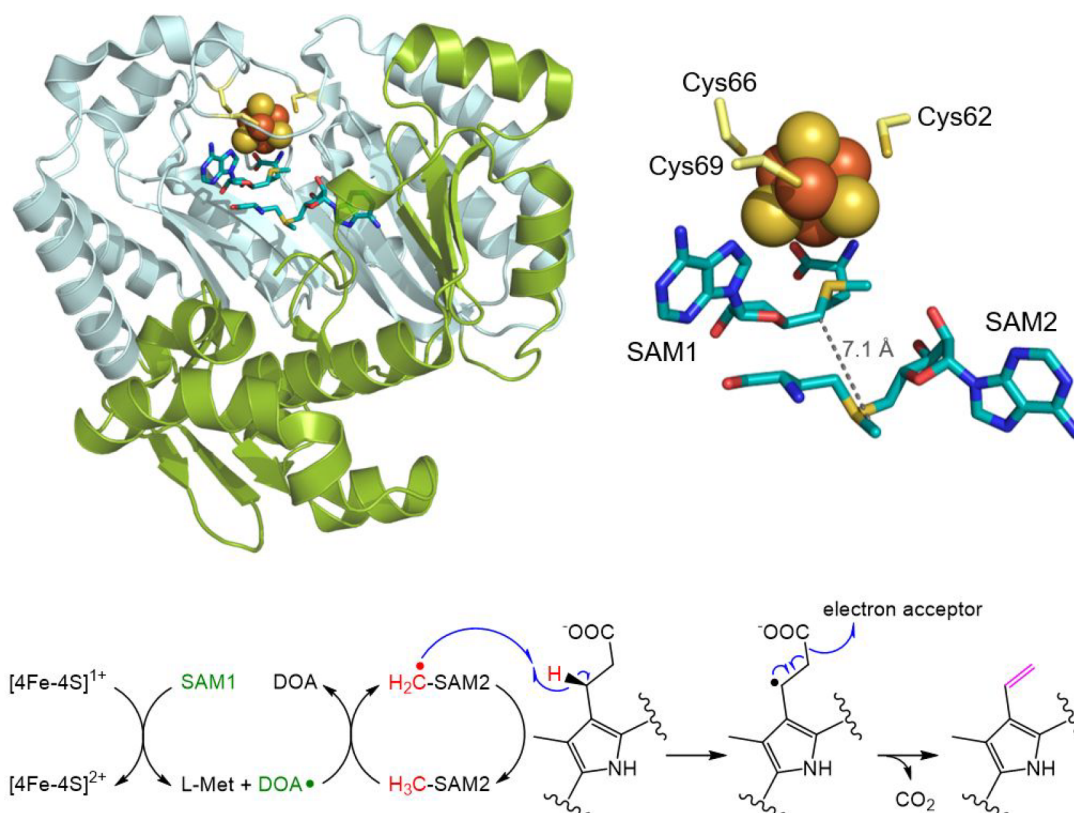
Figure 3. Crystal structure of CgdH from *E. coli* and catalytic mechanism. The partial TIM-barrel is shown in pale cyan, and the additional structural elements are depicted in green. On the right, the cofactor arrangement within the CgdH structure is shown in detail, with labels for both SAM molecules and the cysteine ligands coordinating the [4Fe−4S] cluster. The catalytic mechanism shown at the bottom is described in the main text.

organism. For the biosynthesis of heme d_1 or bacteriochlorophyll *a*, one radical SAM reaction was identified (NirJ or BchE), respectively. Moreover, HemW is a radical SAM heme chaperone involved in heme trafficking and insertion in some bacteria. Most of the radical SAM enzymes listed above were already bioinformatically predicted by Sofia et al. in 2001.[11] In this perspective, the research on the radical reaction steps of tetrapyrrole biosynthesis and the corresponding radical SAM enzymes will be reviewed briefly, also highlighting challenges and future directions.

◼ RADICAL SAM ENZYMES IN HEME BIOSYNTHESIS

Today, three different biosynthetic routes for heme formation are known.[12] The conversion of the general precursor 5-aminolevulinic acid to uroporphyrinogen III is common to all biosynthetic routes. Then the pathway branches to a "classical" route via protoporphyrin IX and two currently known "alternative" routes via either coproporphyrin III or siroheme[12] (Figure 2).

Both the protoporphyrin IX-dependent (PPD) and the coproporphyrin III-dependent (CPD) pathway require a total of four enzymatic steps to convert uroporphyrinogen III to heme *b*. The first step is shared by these routes and consists of the decarboxylation of uroporphyrinogen III to coproporphyrinogen III. Then the PPD route proceeds via the conversion of coproporphyrinogen III to protoporphyrinogen IX, followed by oxidation to protoporphyrin IX and finally iron insertion to yield heme *b*. Within this route, the radical SAM enzyme coproporphyrinogen III dehydrogenase (CgdH) catalyzes the oxygen-independent, oxidative decarboxylation of two propionate side chains of coproporphyrinogen III to the corresponding

vinyl groups of protoporphyrinogen IX. The oxygen-dependent counterpart of CgdH is the coproporphyrinogen III decarboxylase (CgdC) occurring in some bacteria and all eukaryotes. In the alternative CPD route, coproporphyrinogen III is first oxidized to coproporphyrin III followed by iron insertion. The resulting iron-coproporphyrin III (coproheme) is then converted into heme *b* by oxidative decarboxylation of two propionate side chains to the corresponding vinyl groups. Under aerobic conditions, this reaction is catalyzed by coproheme decarboxylase (ChdC), whereas a radical SAM enzyme similar to AhbD (see below) might be responsible for this reaction under anaerobic conditions. Finally, the siroheme-dependent (SHD) route comprises a total of six enzymatic steps. First, uroporphyrinogen III is methylated to precorrin-2, which is then oxidized to sirohydrochlorin, followed by iron insertion yielding siroheme. This key intermediate is then decarboxylated to 12,18-didecarboxysiroheme (DDSH). Finally, the two related radical SAM enzymes AhbC and AhbD catalyze the successive conversion of DDSH to coproheme and then to heme *b*, respectively.[12] Remarkably, the catalyzed conversion performed by either CgdH or AhbD is identical, although the respective substrates are different. Also, CgdH and AhbD do not share substantial amino acid sequence similarity besides the typical radical SAM features, and the mechanisms by which they catalyze the oxidative decarboxylation of propionate side chains to vinyl groups is different as described below.

◼ CgdH

The radical SAM enzyme coproporphyrinogen III dehydrogenase (CgdH, formerly called HemN) catalyzes the oxidative decarboxylation of the propionate side chains of coproporphyri-
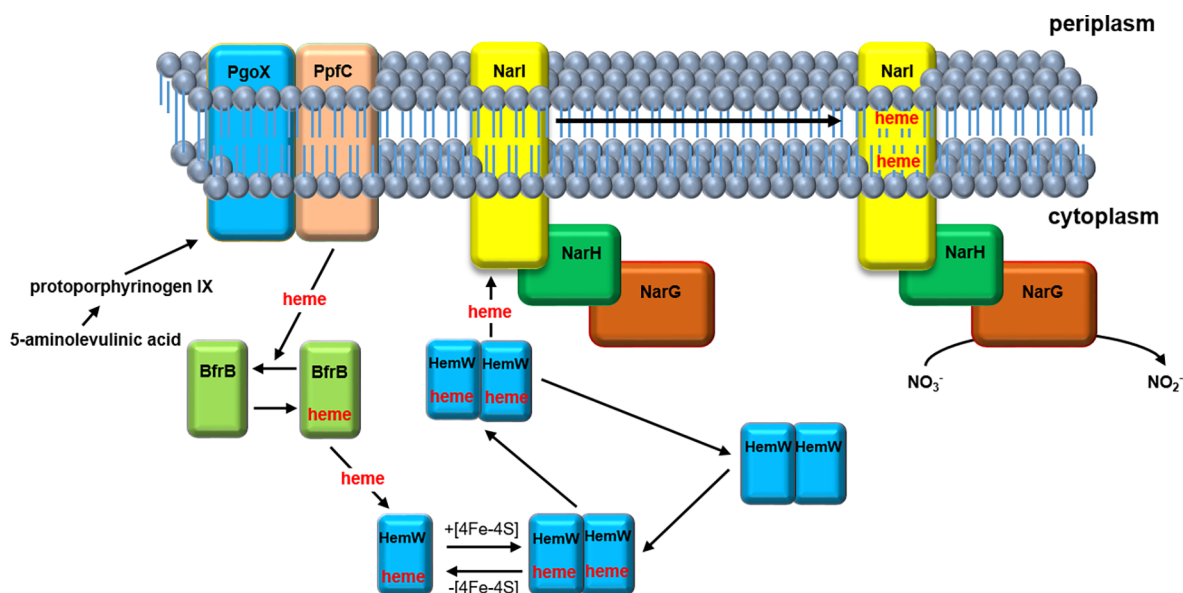
Figure 4. Model for the activity of the heme chaperone HemW. Heme *b* from heme biosynthesis (final enzyme protoporphyrin IX ferrochelatase, PpfC) is most likely transferred via bacterioferritin (BfrB, green) to the heme chaperone HemW (blue). Subsequently, the [4Fe−4S] cluster-containing HemW dimerizes and localizes to the membrane, where it interacts with its target protein NarI (yellow), the heme-containing subunit of the respiratory nitrate reductase NarGHI. After heme incorporation into apo-NarI, the holo-NarGHI is able to catalyze the reduction of nitrate to nitrite. PgoX = protoporphyrinogen IX oxidase.

nogen III rings A and B to the corresponding vinyl groups. The same reaction is catalyzed by the oxygen-dependent, cofactor-free CgdC under aerobic conditions in some bacteria and all eukaryotes.[12] While the aerobic reaction was discovered in the 1950s,[13] the anaerobic CgdH activity was first described by Tait at the end of the 1960s for *Rhodobacter sphaeroides*.[14−16] As observed for the oxygen-dependent reaction, an initial pro-*S*-hydrogen abstraction at the β-carbon of the propionate side chain of the substrate was subsequently described for CgdH.[17] Genetic approaches led to the isolation of the corresponding *cgdH* genes (named *hemN* at the time) in the 1990s.[18−20] CgdH was predicted to be a member of the radical SAM enzyme family by Sofia et al. in 2001.[11] However, due to the frequent misannotation of "HemNs" at the time, not all of the predicted sequences are true CgdHs involved in heme biosynthesis, which was clarified by Dailey et al. in 2015.[21] In 2002, the recombinant *Escherichia coli* CgdH was biochemically characterized as a radical SAM enzyme carrying a [4Fe−4S] cluster coordinated by three cysteine residues of the canonical CX_3CX_2C motif and conducting SAM cleavage during catalysis.[22,23] The crystal structure of CgdH, which was the first published structure of a radical SAM enzyme, revealed the presence of two SAM molecules in the active site[24] (Figure 3). While the cluster-coordinating SAM serves as the source for the initial 5′-deoxyadenosyl radical (DOA•), the second SAM was shown to act as a radical relay via a methylene radical intermediate.[25] The substrate radical of the CgdH reaction was characterized by electron paramagnetic resonance spectroscopy employing several deuterium-labeled substrates.[26] CgdH catalysis starts with the reduction of the [4Fe−4S] cluster and subsequent reductive cleavage of the SAM, resulting in the generation of methionine and DOA•. Then DOA• abstracts a hydrogen atom from the methyl group of the second SAM molecule bound in the active site, generating a SAM-based methylene radical.[25] Via subsequent pro-*S*-hydrogen abstraction at the β-carbon of the substrate propionate side chain, the substrate radical is formed.

Substrate radical fragmentation with final decarboxylation might be accompanied by the regeneration of the $[4Fe−4S]^{1+}$ by the released electron.[25] Several radical side reactions, most likely nonphysiological artifacts, were observed during CgdH in vitro assays.[25,27]

Among the radical SAM enzymes involved in tetrapyrrole biosynthesis, CgdH was the first to be characterized on the biochemical and structural level. Although the overall structure is known now for years and the catalytic mechanism is well-understood, several open questions remain. For example, the distance between the C5′ of SAM1 and the methyl group carbon of SAM2 in the crystal structure of CgdH is about 7 Å, which is too large for direct hydrogen abstraction of DOA• from the methyl group. However, in this context, it must be mentioned that the methionine moiety of SAM2 was rotationally disordered around the C5′−S bond in the crystal structure of CgdH,[24] which might be due to the fact that the substrate was not present. With the substrate coproporphyrinogen III bound in the active site, SAM2 might be less flexible and, moreover, might move closer to SAM1. Also, the understanding of the details of CgdH catalysis including the identification of important amino acid residues would greatly benefit from a structure with a bound substrate. Therefore, cocrystallization of CgdH with coproporphyrinogen III must be undertaken, although this is not trivial due to the extreme oxygen sensitivity of the substrate.

■ HemW

As a typical radical SAM enzyme, HemW contains three cysteines and one SAM, which coordinate a [4Fe−4S] cluster. Although the amino acid sequence of HemW exhibits a high degree of similarity to that of the coproporphyrinogen III dehydrogenase CgdH, its physiological function remained unknown for many years. A genetic investigation finally indicated the participation of HemW in the generation of cytochromes in *Lactobacillus lactis*. Next, stable heme binding by the recombinant HemW from *L. lactis* was shown.[28] The *E. coli*
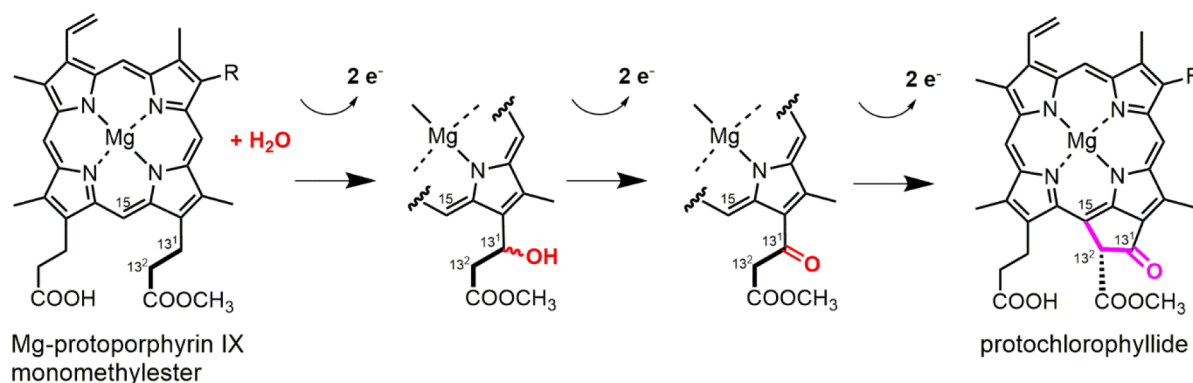
Figure 5. Proposed reaction intermediates of BchE catalysis. The synthesis of the fifth ring (ring E) of bacteriochlorophyll is formally described as oxidative six-electron (e^-) cyclization. R stands for ethyl or vinyl.

HemW was then demonstrated to be a heme chaperone catalyzing the insertion of heme b into the nitrate reductase NarGHI, which is part of an anaerobic respiratory electron transport chain of the bacterium.[29] Bacterioferritins and the heme-containing subunit NarI of NarGHI were shown to interact directly with HemW. Thus, bacterioferritins might serve as heme donors for HemW, whereas the cytochrome subunit NarI of nitrate reductase represents the target of HemW (Figure 4). For the heme transfer process, an intact [4Fe–4S] cluster is required. The human counterpart RSAD1 was shown to stably bind heme, indicating its heme chaperone function. The exact radical SAM-based mechanism of heme transfer remains to be determined.[28,29]

AhbC AND AhbD

Based on bioinformatic genome analysis, the involvement of AhbC and AhbD in an alternative heme biosynthesis pathway was proposed.[30,31] In 2011, the SHD route was elucidated, and the enzymatic activities of AhbC and AhbD were shown for the first time in cell-free extracts containing the recombinant enzymes from *Methanosarcina barkeri* (AhbC) and *Desulfovibrio desulfuricans* (AhbD). AhbC catalyzed the removal of the acetate side chains on pyrrole rings A and B of DDSH, resulting in coproheme formation.[32] In addition to this initial demonstration of AhbC activity, no further studies with purified AhbC have been published.

In the case of AhbD, the recombinant enzymes from *M. barkeri* (AhbD$_{Mb}$) and *D. vulgaris* (AhbD$_{Dv}$) were purified and characterized.[33−35] Purified AhbD catalyzes the oxidative decarboxylation of the two propionate side chains on pyrrole rings A and B of coproheme to the corresponding vinyl groups of heme b. The overall reaction proceeds in a stepwise manner with the occurrence of a monovinyl intermediate.[33] AhbD contains two [4Fe–4S] clusters.[33,35] The typical radical SAM cluster is coordinated by the three cysteine residues of the CX_3CX_2C motif and is essential for SAM cleavage. The second "auxiliary" cluster, proposed to be coordinated by four cysteine residues of the C-terminal motif $CX_2CX_5CX_2CX_{17}C$, is not required for SAM cleavage to occur. However, both clusters are essential for the overall decarboxylation reaction. For the auxiliary cluster, a role in electron transfer was proposed.[35] In the postulated reaction mechanism for AhbD, the initially formed DOA• directly abstracts a hydrogen atom from the substrate propionate side chain. The resulting substrate radical then undergoes fragmentation, yielding the vinyl side chain, potentially with release of CO_2. Similar to the CgdH-catalyzed

reaction, an electron acceptor is required for the uptake of the remaining electron. For AhbD, it was proposed that the central iron ion of the substrate and the auxiliary cluster might provide a pathway for electron transfer.[35] However, this hypothesis requires further investigation.

The overall reaction catalyzed by AhbD, that is, the conversion of propionate groups to vinyl groups, is identical to that catalyzed by CgdH. However, the substrates of AhbD and CgdH are different, and the two enzymes do not share substantial amino acid sequence identity. Although there is no experimental 3D structure available for AhbD, the enzyme is not predicted to bind two SAM molecules based on sequence analysis. For all of these reasons, the catalytic mechanisms of AhbD and CgdH are quite different. Much more research is required in the case of AhbD to understand the details of the reaction mechanism, including solving its structure and elucidating the electron transfer path after release of CO_2.

NirJ: A RADICAL SAM ENZYME IN HEME d_1 BIOSYNTHESIS

Heme d_1 is an iron-containing dioxoisobacteriochlorin that serves as essential cofactor in cytochrome cd_1 nitrite reductases.[36,7] The biosynthesis of heme d_1 is related to the SHD pathway to heme b.[32] The subsequent intermediate after siroheme is also DDSH, but then the heme d_1 route diverges. Instead of acetate side chain removal, the two propionate groups on pyrrole rings A and B are removed and replaced by keto functions. In the last step, the propionate side chain on pyrrole ring D is converted to an acrylate group. NirJ was proposed to be a member of the radical SAM enzyme family by Sofia et al. in 2001.[11] It shares some amino acid sequence homology with PqqE and MoaA, leading to frequent mis-annotation. Moreover, it shares substantial homology with AhbC, reflecting the use of the same substrate (DDSH) by both enzymes.[32] It was shown that purified NirJ catalyzes the reductive cleavage of SAM[37] and the removal of the two propionate side chains of DDSH.[38] However, due to oxidative side reactions during product extraction, the actual NirJ reaction product remains to be identified. NirJ most likely contains two iron−sulfur clusters with the "auxiliary" cluster coordinated by cysteine residues of a C-terminal $CX_2CX_5CX_{20−21}C$ motif.[38] Almost the same sequence motif is present in AhbC and AhbD (see above). The precise reaction mechanism of NirJ, including the true identity of the reaction product and the nature of the released side chain fragment, remains to be established.
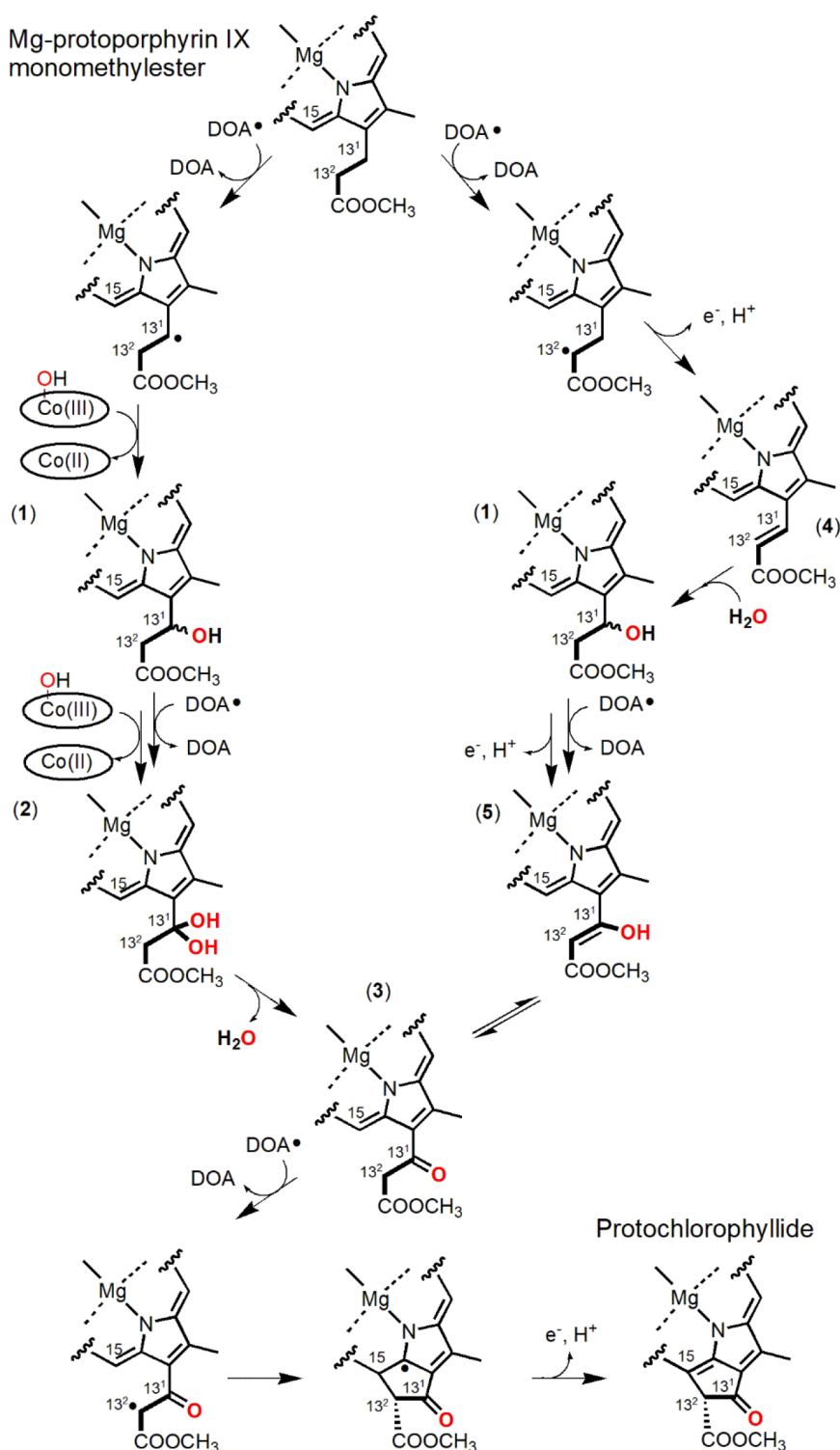
Figure 6. Two potential mechanisms for the cobalamin-dependent catalysis of BchE (left and right trace). The left-hand reaction sequence involves the attack of the hydroxyl group of hydroxocobalamin and proceeds via the geminal diol **2**. The right-hand mechanism has no direct cobalamin involvement and includes the formation of the enol **5**.

Most likely, NirJ and AhbC display highly similar 3D structures due to their high degree of amino acid sequence similarity. Nevertheless, there must exist characteristic differences between the two enzymes within their active sites, which give rise to the individual reactions catalyzed by each enzyme resulting in the formation of different products. Therefore, solving the crystal structures of NirJ and AhbC in complex with

their substrate DDSH will help to reveal details of their active site architectures and catalytic mechanisms.

■ **BchE INVOLVED IN OXYGEN-INDEPENDENT SYNTHESIS OF BACTERIOCHLOROPHYLL**

Chlorophyll (Chl) and bacteriochlorophyll (BChl) molecules belong to the most abundant pigments on the planet. These

molecules are synthesized from protoporphyrin IX, which also functions as a central intermediate of heme biosynthesis (Figure 1). The fifth ring (ring E) of Chls and BChls is of central importance for the harvesting of red light (>600 nm), and it is a distinguishing feature compared to other tetrapyrroles (Figure 5).[4] However, the biosynthesis of this part of the macrocycle is poorly understood.[39−41] Mg-protoporphyrin IX monomethylester (Mg-PME) acts as the substrate of two mechanistically unrelated Mg-PME cyclases, which carry out the synthesis of protochlorophyllide (Figure 5).

Substrate conversion can be formally described as oxygenation of the C-13^1 methylene to hydroxymethylene (two-electron oxidation), subsequent 13^1-oxo group formation (two-electron oxidation), and final C−C bond linkage between C-13^2 and C-15 (two-electron oxidation). The oxygen-dependent Mg-PME cyclase termed AcsF is conserved in higher plants, algae, cyanobacteria, and some purple proteobacteria.[42,43] AcsF was classified as a non-heme diiron monooxygenase, and ^{18}O isotopic labeling experiments demonstrated that the 13^1-oxo group arises from molecular oxygen.[44,45] The oxygen-independent Mg-PME cyclase is termed BchE and is found in photosynthetic purple bacteria. The enzyme shows mechanistic dichotomy as whole cell $H_2^{18}O$ labeling experiments revealed that the 13^1-oxo group derives from a water molecule.[46,47] The pioneering bioinformatics work of Sofia et al. identified BchE as a founding member of a group of radical SAM enzymes performing cobalamin-dependent catalysis.[11] The C-terminal domain of the protein carries the typical CX_3CX_2C sequence motif for the ligation of a [4Fe−4S] cluster. The presence of an N-terminal cobalamin binding domain was experimentally supported by BChl analyses of cobalamin-requiring *Rhodobacter capsulatus* mutant strains (*bluE* and *bluB*).[48] To date, the biochemical or spectroscopic verification of the cobalamin cofactor requirement is still pending, as all efforts for the purification of enzymatically active BchE were not successful. Only recently, a robust spectroscopic in vitro BchE activity assay was established, which makes use of a cellular extract from a *R. capsulatus* mutant strain with a blockage of an early step of the BChl biosynthesis pathway. External supply of the purified BchE substrate allowed for kinetic experiments and also for the $H_2^{18}O$-dependent isotope label transfer onto the carbonyl group (C-13^1-oxo) of protochlorophyllide. The assay system was stimulated in the presence of the cofactors NADPH and SAM and inhibited by the cofactor analogue S-adenosylhomocysteine as also described for other radical SAM enzymes. BchE was characterized as a membrane-associated enzyme, which is dependent on an additional cytosolic component with an estimated molecular mass between 3 and 10 kDa. Involvement of a BchE-specific electron donor protein of *R. capsulatus* was hypothesized because a soluble cytosolic *E. coli* extract was not functional as a substitute.[47] The solved three-dimensional structure of a distantly related cobalamin-dependent radical SAM enzyme (OxsB, involved in oxetanocin biosynthesis)[49] paved the way for the calculation of a three-dimensional BchE homology model as a basis for an extensive structure-guided mutagenesis approach. For that purpose, a BchE-deficient *R. capsulatus* strain was complemented in the presence of 24 variant *bchE* plasmids, and the overall synthesis of BChl was monitored. This in vivo complementation assay revealed the three ligands of the [4Fe−4S] cluster, an overall of three amino acid residues, which potentially interact with the SAM cofactor and five residues, which might function as ligands of the cobalamin cofactor.[47]

The enzymatic mechanism of cobalamin-dependent radical SAM enzymes has been well-characterized for methyl transferase reactions. In contrast, the H_2O-dependent oxygenation and cyclization of the BchE substrate is based on unprecedented radical SAM chemistry. Over the years, differing reaction sequences for the cobalamin-dependent catalysis of BchE have been proposed.[41,50,48] Two potential mechanisms are depicted in Figure 6 (left and right trace). The BchE reaction is always initiated by the reductive cleavage of SAM. The resulting DOA• abstracts the H-13^1 of the substrate, and the resulting radical attacks the hydroxyl group of a hydroxocobalamin (left trace). The resulting hydroxyl intermediate (**1**) undergoes the same hydroxylation reaction a second time to yield a geminal diol (**2**) that is equivalent to the keto intermediate (**3**). Alternatively, hydroxylation of C-13^1 might include sequential dehydrogenation and hydration of the substrate (right trace). Therefore, BchE abstracts atom H-13^2, and the resulting radical is then oxidized to the olefin (**4**) which is hydrated to form hydroxyl intermediate **1**. Subsequent dehydrogenation of this compound allows for the formation of the enol **5**, which subsequently tautomerizes to the keto intermediate **3**. For both mechanisms, the cyclization step might proceed through abstraction of the H-13^2 atom by DOA•. The resulting substrate radical then attacks C-15 of the macrocycle and after loss of an electron and a proton protochlorophyllide is formed.

For the final clarification of the reaction mechanism, important experimental hurdles still need to be overcome, for example, the recombinant production of enzymatically active BchE, the identification of the proposed electron donor, or the verification of the type of cobalamin cofactor (cyano-cobalamin, adenosyl-cobalamin, or hydroxo-cobalamin). For that purpose, an increasing repertoire of engineered *E. coli* strains is available which have been optimized for the production of (cobalamin-dependent) radical SAM enzymes.[51−53] Variant BchE proteins have the potential to accumulate the proposed reaction intermediates and organic synthesis might help to establish these molecules as artificial BchE substrates. Interdisciplinary work is required for the understanding of BchE catalysis which will broaden the repertoire of radical SAM chemistry by an unprecedented reaction.

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Notes

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■ ABBREVIATIONS

BChl, bacteriochlorophyll; Chl, chlorophyll; CPD, coproporphyrin III-dependent; DDSH, 12,18-didecarboxysiroheme; DOA, 5′-deoxyadenosine; Mg-PME, Mg-protoporphyrin IX monomethylester; PPD, protoporphyrin IX-dependent; SAM, S-adenosylmethionine; SHD, siroheme-dependent

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