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REVIEW

Recent advances in the biosynthesis of modified tetrapyrroles: the discovery of an alternative pathway for the formation of heme and heme d_1

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Abstract Hemes (*a*, *b*, *c*, and *o*) and heme d_1 belong to the group of modified tetrapyrroles, which also includes chlorophylls, cobalamins, coenzyme F_{430}, and siroheme. These compounds are found throughout all domains of life and are involved in a variety of essential biological processes ranging from photosynthesis to methanogenesis. The biosynthesis of heme *b* has been well studied in many organisms, but in sulfate-reducing bacteria and archaea, the pathway has remained a mystery, as many of the enzymes involved in these characterized steps are absent. The heme pathway in most organisms proceeds from the cyclic precursor of all modified tetrapyrroles uroporphyrinogen III, to coproporphyrinogen III, which is followed by oxidation of the ring and finally iron insertion. Sulfate-reducing bacteria and some archaea lack the genetic information necessary to convert uroporphyrinogen III to heme along the "classical" route and instead use an "alternative" pathway. Biosynthesis of the isobacteriochlorin heme d_1, a cofactor of the dissimilatory nitrite reductase cytochrome cd_1, has also been a subject of much research, although the biosynthetic pathway and its intermediates have evaded discovery for quite some time. This review focuses on the recent advances in the understanding of these two pathways and their surprisingly close relationship via the unlikely intermediate siroheme, which is also a cofactor of sulfite and nitrite reductases in many organisms. The evolutionary questions raised by this discovery will also be discussed along with the potential regulation required by organisms with overlapping tetrapyrrole biosynthesis pathways.

Keywords Heme · Heme d_1 · Tetrapyrrole biosynthesis · Siroheme · Modified tetrapyrrole · Alternative heme biosynthesis

Tetrapyrroles and the pigments of life

The hemes belong to a family of metallo-prosthetic groups that are all based on the same structural framework assembled from four polymerized pyrrole rings (labeled A–D) that are arranged into a large macrocycle. A pyrrole is a five-membered ring containing one atom of nitrogen. Modified tetrapyrroles participate in a diverse range of fundamental biological processes and are indispensable components of the metabolism of practically all organisms on earth; they include molecules such as the chlorophylls, cobalamins, siroheme, heme d_1, and coenzyme F_{430}. They play a central role in electron transfer-dependent energy-generating processes from photosynthesis to methanogenesis. The secret to the successful integration of tetrapyrrole-derivatives into so many biological systems reflects the photodynamic properties of the macrocycle and its ability to bind a range of metals. The structures of all modified tetrapyrroles are based on the molecular blueprint of uroporphyrinogen III (uro'gen III, Fig. 1), the first macrocyclic intermediate of the tetrapyrrole pathway, where four pyrrole rings are connected by four bridging carbon atoms to generate the overall structure. Variation in the oxidation state of the macrocycle, prototropic

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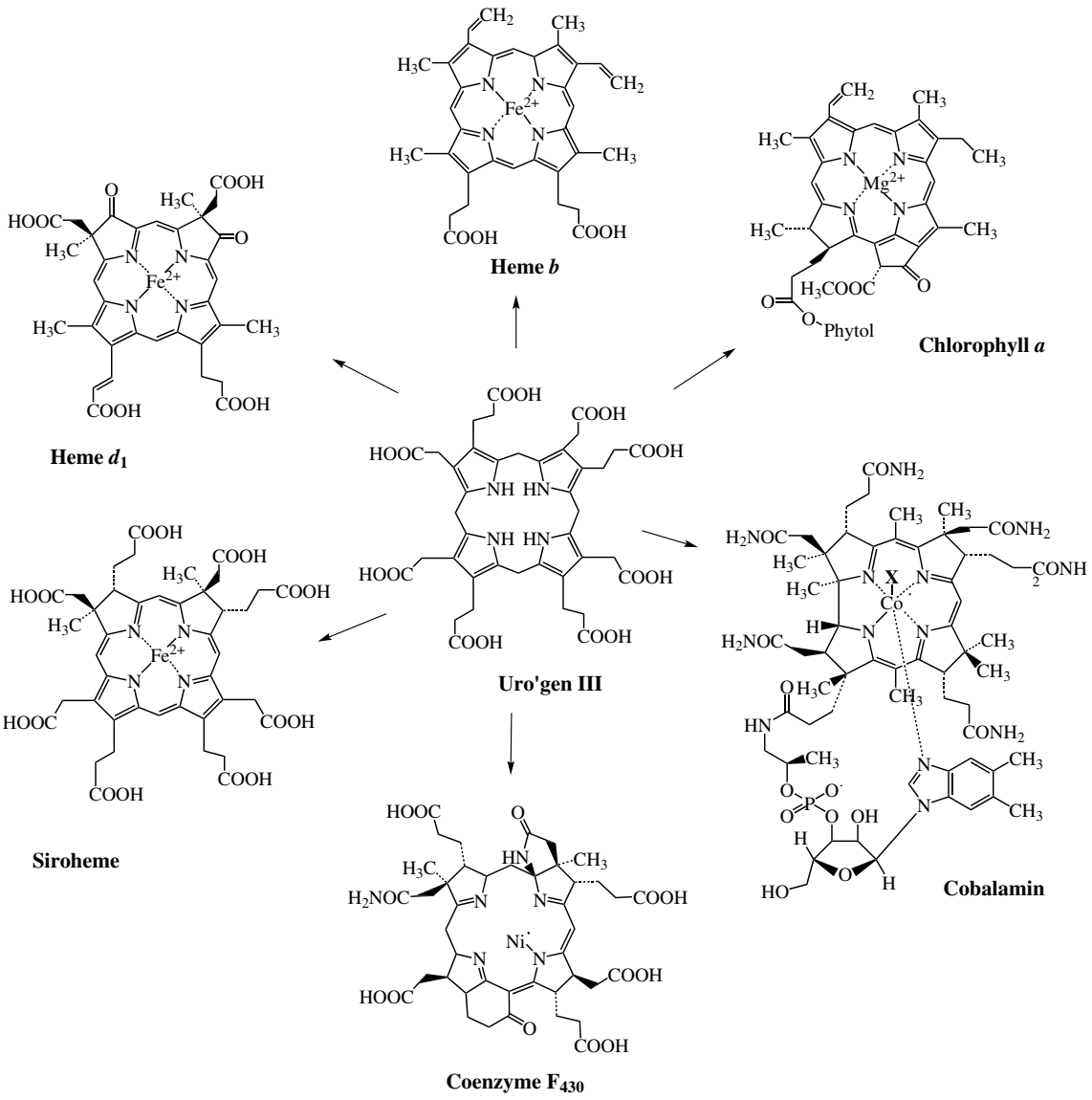

Fig. 1 The structures of important representatives of naturally occurring tetrapyrroles and their common precursor uroporphyrinogen III

rearrangements assisted by regiospecific methylations and insertion of different metal ions accounts for the variation in the chemical properties and color of this family of compounds.

Heme *b* (also called protoheme or Fe-protoporphyrin-IX), for example, represents a fully oxidized derivative of uro'gen III, is an iron-containing porphyrin and, when bound to globin, is responsible for the red color of blood. The chlorophyll macrocycle contains one less double bond and is a magnesium-containing chlorin that is responsible for the green color of leaves. Because of their color and the essential role these molecules play in so many biological processes, they are often referred to as the "pigments of life" [1]. The essential functions of these molecules are briefly described below:

The most commonly found tetrapyrrole in nature is chlorophyll. The biosynthesis of this magnesium-containing pigment is the most notable process on earth, with an estimated 10^9 tons being produced (and broken down) each year with the change in the seasons [2]. Chlorophyll is responsible for harvesting and trapping light during photosynthesis and converting it into the chemical energy of the cell. This process produces oxygen as a byproduct, therefore giving rise to life on earth as it is known today [3]. The bacterial equivalent to chlorophyll is bacteriochlorophyll. It is solely employed in anoxic photosynthesis and probably displays the evolutionary ancestor of chlorophyll [4, 5]. The role of the Mg^{2+} has received relatively little attention, but a key point is that this cation is unable to undergo oxidation and reduction under any feasible physiological

conditions and provides a rigidity to the macrocyle alongside conferring the ability to bind to axial ligands. There is evidence that Zn^{2+} can substitute for Mg^{2+} and it is possible that the greater abundancy of Mg is the reason for its 'selection' over Zn as the cation in chlorophyll.

Members of the cobalt-containing corrinoids such as cobalamin (vitamin B_{12}) are characterized by a missing methine bridge in the tetrapyrrole structure, which means that rings C and D are linked directly [6]. Different forms of cobalamin facilitate different types of enzymatic transformations including rearrangement, methylation, and reductive dehalogenation processes. Coenzyme F_{430} incorporates nickel and serves as prosthetic group of methyl-coenzyme M reductase, an enzyme involved in archaeal methanogenesis [7, 8]. In fact, cobalamin and coenzyme F_{430} join forces to help mediate the final two stages in methanogenesis and are responsible for the production of over a billion tons of methane gas every year [8].

Heme *b* is the prosthetic group of hemoglobin and myoglobin that is involved in the transport of oxygen [9]. Different forms of heme are integral components of cytochromes and are involved in electron transport chains associated with energy recovery. These are found in almost all forms of life [10]. Heme *b* is also a cofactor of many enzymes including catalases, peroxidases, and cytochrome P_{450}, which are involved in detoxification processes [11, 12]. The molecule is also part of gas sensing systems, can serve as a source of iron, act as transcriptional regulator or be involved in the control of pathogenicity [13].

The lesser-known modified tetrapyrroles include heme d_1 and siroheme. The heme-containing cytochrome cd_1 is the only known protein harboring the cofactor heme d_1 and one of the two enzymes responsible for the reduction of nitrite to nitric oxide in denitrifying bacteria [14]. Siroheme, an iron-containing isobacteriochlorin, is the cofactor of assimilatory sulfite and nitrite reductases for which the reaction products are sulfide and ammonium respectively [15].

Structure of heme

Hemes and heme d_1 are very different molecules. Hemes (which include heme *a*, *b*, *c*, and *o*) are iron-porphyrins, whereas heme d_1 is not actually a heme at all, but rather is an iron-containing dioxo-isobacteriochlorin. True hemes are derivatives of iron-protoporphyrin IX. The iron coordinated in the heme macrocycle can occur in two different oxidation states, FeII or FeIII, and fine tuning to the redox potential of the cofactor is achieved by changes to its environment, both axial ligands provided by the protein, and the general chemical properties of the heme pocket in the protein. This contributes greatly towards the functional diversity of the heme cofactor [16].

The heme macrocycle displays a system of fully conjugated double bonds causing heme to be photochemically active giving rise to characteristic absorption patterns in the visible spectrum and its strong coloration. The properties of light absorption are altered by relocation of the double bonds, which is achieved through substitutions on the ring and are also dependent on the protein the cofactor is embedded in [16, 17]. Further variations in functionality arise from the type of substituents on the pyrrole rings and interaction of the cofactor as heme can be covalently attached to the protein such is the case for cytochrome *c*. Hemes are classified according to their modifications relative to heme *b*. An overview of the substituents of hemes *a*, *b*, *c*, and *o* and their positions of modification on the macrocycle is shown in Fig. 2a; [17, 18]. Heme *c* differs from heme *b* through its covalent attachment to a protein through thioether bonds that are formed between the cysteines of the protein and the two vinyl groups of the heme [18]. The

Heme	R_1	R_2	R_3
a	$-C_{17}H_{29}O$	$-CH=CH_2$	$-CH=O$
b	$-CH=CH_2$	$-CH=CH_2$	$-CH_3$
c	$-C(H)(CH_3)$-S-protein	$-C(H)(CH_3)$-S-protein	$-CH_3$
o	$-C_{17}H_{29}O$	$-CH=CH_2$	$-CH_3$

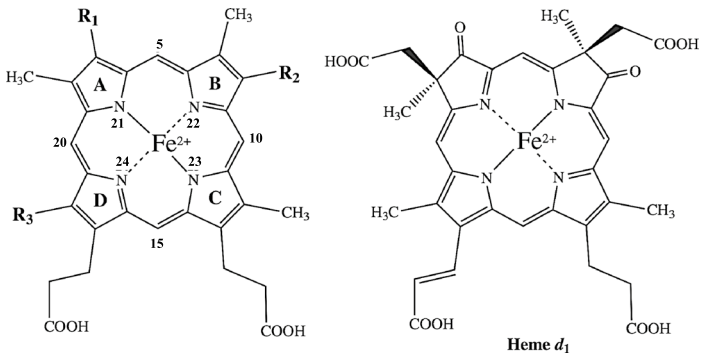

Fig. 2 Structures of biological important variants of heme [17] and the dioxo-isobacteriochlorin heme d_1. **a** Ligands at different residues on the heme macrocycle are donated R_1, R_2, and R_3 and listed on the *left*. In archaea, the side chains at R_1 of heme *a* and *o* can have longer groups [171]. The pyrrole rings are labeled *A–D* in a clockwise direction, the bridging carbon atoms are numbered 5, 10, 15 and 20, and the nitrogens 21–24 according to IUPAC nomenclature. **b** In comparison, the dioxo-isobacteriochlorin heme d_1

cysteine residues involved in heme binding follow a conserved CXXCH binding motif. Hemes *a* and *o* differ in that they both have farnesyl additions to ring A and heme *a* additionally a formyl group at ring D [19].

Heme not only plays an important role as a prosthetic group to many proteins processes but it also represents an important iron repository. Pathogens that do not synthesize this cofactor themselves have evolved intricate systems for heme scavenging from their host as this is essential for their survival [13, 20] and some of these processes represent important drug targets. The photodynamic properties of tetrapyrroles, especially the porphyrins, have been utilized in medicine through the use of photodynamic therapy. This application is based on the production of reactive oxygen species (ROS) from molecular oxygen after exposure of the porphyrin to light [21]. ROS are short-lived species that have a destructive effect on their close environment and for that reason the process has been used to treat certain cancers [22] and microbial infections [23], and has also been adapted for herbicidal and insecticidal activities [21].

Structure and function of heme d_1

The structure of heme d_1 was solved in 1985 by NMR and UV–Vis spectroscopy [24] and further confirmed through a total synthesis [24–26]. From the experimental evidence it became clear that heme d_1 is a dioxo-isobacteriochlorin, with a number of structural features distinguishing it from other modified tetrapyrroles (Fig. 2b). Compared to heme *b*, the vinyl groups at positions C3 and C8 are replaced by electron withdrawing carbonyl groups and positions C2 and C7 carry acetate side chains as well as methyl groups leading to the saturation of pyrrole rings A and B. The final difference is the presence of an acrylate side chain at position C17 at ring D replacing the propionic acid side chain at this position in heme *b*.

Heme d_1 plays a key role in the function of cytochrome cd_1 nitrite reductase and thereby contributes to the global nitrogen cycle. Four sequential reactions are responsible for the stepwise reduction of nitrate (NO_3^-) to dinitrogen gas (N_2). Denitrification, i.e., complete reduction of nitrate to nitrogen via the gaseous intermediates nitric oxide and nitrous oxide, is a respiratory process used by many bacteria under the condition of low oxygen or complete anaerobiosis [27, 28]. The enzymes involved in the denitrification process are coupled to respiratory electron transport chains and the intermediate nitrogen-oxide(s) serve as the terminal electron acceptors. This is the only biological process by which the soil-fixed nitrogen is returned as gas to the atmosphere. Thus, denitrification is commonly used for removing excess nitrate/nitrite from sewage, groundwater, and wastewater [29].

During the denitrification process, the first committed step involves the conversion of nitrite to nitric oxide and water that is catalyzed by dissimilatory nitrite reductases. There are two main dissimilatory nitrite reductases, a heme-containing cytochrome cd_1 nitrite reductase (NirS) and a copper-containing nitrite reductase (NirK). Their presence in different bacterial species is mutually exclusive [30]. In fact, the biological function of heme d_1 is exclusive to cytochrome cd_1 nitrite reductase, NirS. Well-studied denitrifiers include *Pseudomonas aeruginosa*, *Paracoccus denitrificans*, *Paracoccus pantotrophus*, *Roseobactor denitrificans*, and *Pseudomonas stutzeri*, all of which contain NirS.

The structure of the periplasmic cytochrome cd_1 nitrite reductase from *P. pantotrophus* was first published in 1995 [31], revealing that each monomer consists of two domains. The smaller, N-terminal domain, binds a *c*-type heme and serves as the center for accepting electrons from donor protein(s) [32]. The larger C-terminal domain carries the non-covalently attached heme d_1 that acts as the site of nitrite reduction. Both the bacterial source and the oxidized/reduced state of NirS, together with axial heme ligands in cytochrome cd_1 nitrite reductase, affect its biochemical and spectroscopic properties [33].

Due to the presence of the carbonyl side chains, the ferric/ferrous redox couple potential of heme d_1 should be more positive than that of other iron-porphyrins, iron-chlorins, and isobacteriochlorins. A value of +175 mV has been estimated in a variant form of cytochrome cd_1 in which the heme *c* potential was perturbed by mutation to be well removed from the value for the heme d_1 and any cooperativity between the potentials had been removed [34]. This value is higher than that normally found for a heme *b* in proteins, but there are examples of similar or even greater positive values found for heme *b* in proteins. Thus, although the redox potential of the heme d_1 means that it is suitable to receive electrons that pass through the bc_1 complex and is also tuned to permit the reduction of nitrite to nitric oxide but not to ammonium, the redox potential alone cannot be the explanation for the occurrence of the structural features of heme d_1 in this enzyme. A variety of investigations over recent years have shown that the reduced form of the heme d_1 has both a higher affinity for the anionic substrate nitrite and a lower affinity for the product nitric oxide than would have been expected by extrapolation from the properties of ferric *b*-type heme which is the form, relative to the ferrous species, with a higher affinity for nitrite and lower affinity for nitric oxide. What this means for the cytochrome cd_1 mechanism is that the nitrite probably binds to the fully reduced form whereupon reduction of nitrite to give nitric oxide bound to ferric heme d_1 is followed by electron transfer from the *c*-type heme. Thus, the ferric heme d_1 NO adduct is reduced to

the ferrous state and then NO is released. This description explains why the enzyme needs to be able to hold two electrons on each polypeptide chain for a one-electron reaction, but does not itself explain exactly why the unusual substituents are present on the heme d_1. Some insight into this has been provided by an advanced biophysical study [35] but the description is not complete.

Overview of heme biosyntheses

The biosynthesis of heme can be divided into three major sections: (1) The formation of the tetrapyrrole building block 5-aminolevulinic acid (ALA), (2) the synthesis of the first macrocyclic intermediate uro'gen III, and (3) the decoration of the ring. In most organisms, heme is synthesized via a highly conserved pathway that is initiated with the synthesis of the first common metabolite ALA. This small, five-carbon aminoketone is the precursor to all tetrapyrroles. There are two distinct and unrelated routes leading to ALA formation. The first pathway to be described is termed C-4 or 'Shemin pathway', which starts from glycine and succinyl coenzyme A and is found in animals, fungi, and the α-group of proteobacteria [36, 37]. The second route for ALA synthesis is referred to as the C-5 pathway, and is unique in that it utilizes glutamyl tRNA as a substrate for something other than protein synthesis. The C5 pathway is present in plants, most bacteria, and archaea [38, 39]. Organisms that make tetrapyrroles operate one or other of these routes although it has been shown in the photosynthetic organism *Euglena gracilis* that both pathways are functional [40, 41].

The middle section of heme synthesis involves the construction of the tetrapyrrole framework upon which all modified tetrapyrroles are derived. These three steps are found in all organisms that make tetrapyrrole-derivatives and involve the incorporation of eight molecules of ALA into the first macrocyclic intermediate, uro'gen III. In brief, this is achieved by the condensation of two molecules of ALA into the pyrrole porphobilinogen (PBG), four of which are oligomerized to the linear pre-uroporphyrinogen (also known as hydroxymethylbilane). Ring closure and inversion of the terminal D ring of the bilane results in the formation of the type III isomer of uro'gen III (Fig. 3). This is the only unsymmetrical isomer of uro'gen, which may have been selected as the template for tetrapyrrole synthesis since it provides a molecular spatial handle for further specific transformation of the molecule.

The final stage of heme synthesis involves modifications to some of the side chains of the macrocycle, followed by aromatization and iron insertion to give protoheme or heme *b*. The net effect is to make the macrocycle more hydrophobic, presumably allowing the molecule to integrate more easily into proteinaceous environments. The initial steps of the macrocycle modification result in the decarboxylation of the acetate side chains (giving coproporphyrinogen III), which is followed by the oxidative decarboxylation of the 'northern' propionate side chains (on rings A and B) to give protoporphyrinogen IX. The removal of six electrons and six protons from the macrocycle introduces aromaticity generates planarity and color, and is a prerequisite to the chelation of iron that produces protoheme. It had been thought that although this part of the pathway proceeded via the same intermediates, there was some variation in the nature of the enzymes that catalyzed the various transformations. The reason for the variation in the nature of the enzymes reflects the fact that some organisms utilize specific aerobic or anaerobic pathways for heme synthesis.

Amongst Prokaryotes, there is a higher degree of variation along the later steps of the pathway [42] that imply an altogether different pathway for heme synthesis. For instance, in sulfate-reducing bacteria, as well as some archaea, orthologues to any of the enzymes responsible for the conversion of uro'gen III to heme are completely absent. Indeed, recently it has been shown that the pathway proceeds via the intermediate precorrin-2, a bis-methylated derivative of uro'gen III, which is also a precursor to cobalamin, cofactor F_{430}, siroheme, and heme d_1. It has therefore been referred to as the **a**lternative **h**eme **b**iosynthesis pathway and the enzymes involved have been given the abbreviation Ahb [43, 44]. In light of the discovery of an alternative heme biosynthetic pathway, which will be discussed later, the two routes for heme synthesis will be referred to as the 'classic' and 'alternative' pathways. Along the classical pathway, ALA represents the exclusive source of all the carbon and nitrogen atoms that are integrated into heme. An overview is given in Fig. 3.

In Eukaryotes, the enzymes involved in heme biosynthesis are highly conserved members of the classic pathway and are well characterized. In humans, enzyme dysfunction between ALA and heme gives rise to a series of metabolic disorders called the porphyrias. This is an umbrella name that covers one of seven separate disorders that have a range of symptoms that are dependent upon which biosynthetic step is affected. The symptoms include neurological problems, possibly due to the accumulation of ALA, and photosensitivity due to the photodynamic properties of accumulated porphyrins.

ALA syntheses

C-4 or Shemin pathway

In the C-4 pathway, the synthesis of ALA is achieved in a single step through the condensation of glycine and

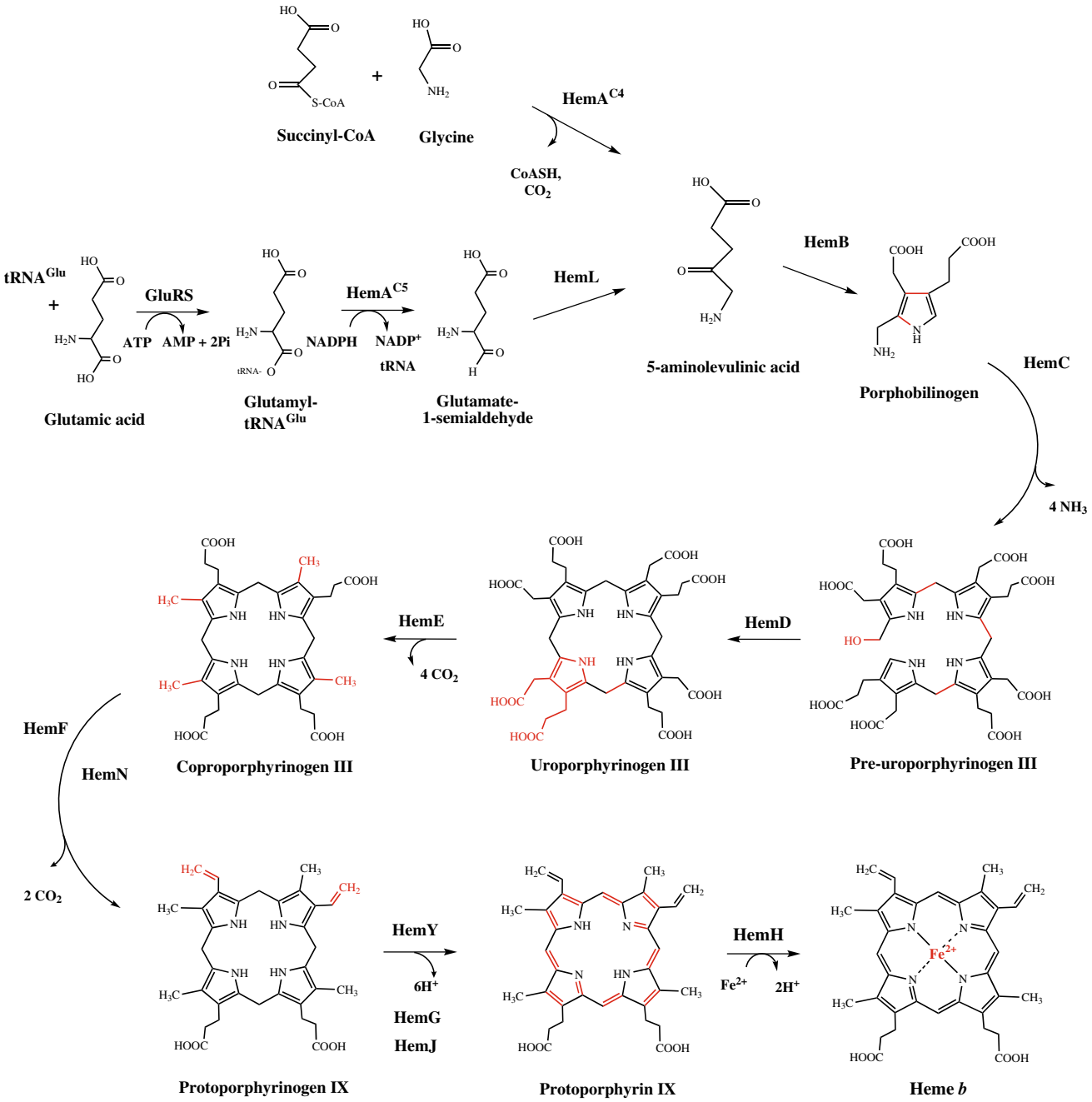

Fig. 3 Overview of the classical heme biosynthesis pathway

succinyl-CoA. The requirement of glycine for porphyrin synthesis was shown by Shemin through digestion of ^{15}N-labeled glycine, which resulted in the isotopic labeling of his own heme [36]. The second source of carbon atoms, succinyl CoA, was identified through labeling studies using radioactive acetate [45]. The enzyme mediating this condensation reaction is the pyridoxal-5′-phosphate (PLP) dependent 5-aminolevulinic acid synthase (ALAS), encoded by *hemA*^{C4}, which was first isolated from *Rhodobacter sphaeroides* [37]. The catalytic cycle and stereochemistry of the enzyme have been well studied. ALAS is highly specific for its substrate glycine and does not recognize any other amino acid [46]. After the binding of glycine to the enzyme, succinyl-CoA is incorporated with the release of coenzyme A and CO_2 prior to the release of the product ALA [47]. The first and only crystal structure of a full-length ALAS has been solved from the bacterium *Rhodobacter capsulatus* [48]. The protein forms a homodimer with each monomer consisting of an N-terminal-, catalytic-, and C-terminal domain. The homodimer

symmetrically binds two molecules of the cofactor PLP through a lysine residue that is conserved in all ALASs.

C-5 pathway

It took another 25 years before the 'C_5 pathway' was discovered. In contrast to the C-4 pathway here, the initial substrate is not glycine but glutamyl tRNA. It was found that ^{14}C labeled glutamate, glutamine, and α-ketoglutarate were incorporated into ALA in plants and a number of bacteria [38]. Subsequently, it was shown that it is the intact carbon skeleton of glutamate that is incorporated into ALA [49]. The C-5 route is a more complex process requiring the action of three separate enzymes.

During the first reaction, glutamyl-tRNA synthetase (GluRS), encoded by *gltX*, charges a glutamate-accepting tRNA ($tRNA^{Glu}$) with glutamate in an ATP-dependent reaction [10, 50]. This reaction proceeds in the same manner as observed in protein synthesis. In the following step, glutamyl-tRNA reductase (GluTR), encoded by $hemA^{C5}$, catalyzes the reduction of the glutamic acid residue of the $tRNA^{Glu}$ to glutamate-1-semialdehyde (GSA) [10, 51, 52]. This reaction is an NADPH-dependent reaction, which with the role of the tRNA, has no parallel in any other metabolic process. The third and final reaction is the transamination of GSA into ALA catalyzed by the PLP-dependent glutamate-1-semialdehyde-2,1-aminomutase (GSAM), encoded by *hemL* [10, 53, 54].

GSA is a highly reactive aldehyde. In order to ensure efficient synthesis of ALA without any loss of substrate, substrate channeling of GSA between GluTR and GSAM has been suggested. The structures of GluTR [55] and GSAM [54] have been solved and analysis revealed indeed structural complementarity leading to the model of a GluTR/GSAM complex [56]. Existence of this complex could further be confirmed by showing the interaction of the two enzymes in vivo [57, 58]. Substrate channeling has also been suggested to occur during the synthesis of cobalamin, where some of the intermediates are highly sensitive to oxygen [59]. It is further interesting to note that GSAM is structurally related to ALAS, suggesting that ALAS may have evolved from GSAM [56].

The biosynthesis of uroporphyrinogen III

The last common intermediate, and the first branch point in the biosynthesis of all modified tetrapyrroles, is uro'gen III (Fig. 1). The first macrocyclic intermediate of the pathway is made from eight molecules of ALA, which involves the action of three different enzymes.

The first enzyme ALA dehydratase (ALAD) or porphobilinogen synthase (PBGS), encoded by *hemB*, condenses two molecules of ALA asymmetrically to yield the pyrrole derivative PBG [60]. Most ALADs require metals to be fully functional though the existence of metal-independent enzymes has been reported [61]. ALADs share a high degree of sequence similarity although enzymes from different organisms display differential metal dependency. In yeast and mammals, the enzyme contains two zinc ions [62] while the enzyme from bacteria, such as *Escherichia coli,* contains one active site zinc ion but is further stimulated by magnesium [63]. Bacteria such as *Pseudomonas aeruginosa* require magnesium for their activity [64] and the same is the case for ALADs from plants [65]. Structural studies on the enzymes from yeast [66] and *E. coli* [67] have helped dissect the role of the two active site lysines that assist in the binding and catalysis of the ALA molecules through a Knorr type condensation. The quaternary structure reveals that the enzyme generally forms homo-octamers, although the reason for such a large macromolecular assembly is not clear.

In the proceeding reaction, four molecules of PBG are linked consecutively by porphobilinogen deaminase (PBGD or hydroxymethylbilane synthase) to produce the linear tetrapyrrole pre-uroporphyrinogen (or hydroxymethylbilane) [68]. This enzyme, which is encoded by *hemC*, uses a unique dipyrromethane cofactor that is itself derived from two molecules of PBG [69, 70]. The covalently linked cofactor serves as a primer for the oligomerization of four PBG molecules, which proceeds in a sequential fashion. Starting with ring A, the subsequent addition of the remaining units (B–C–D) to the growing polypyrrole chain results in the formation of a protein-bound linear hexapyrrole with four molecules of ammonia being released concomitantly [68]. After the addition of ring D, the tetrapyrrole is cleaved from the cofactor and pre-uroporphyrinogen is released leaving reactivated enzyme [71]. The structure of the enzyme reveals how the cofactor is tethered via a thioether linkage to a conserved cysteine residue within the active site that is formed between the three domains of this monomeric enzyme [72].

The final reaction is catalyzed by uro'gen III synthase (UROS), encoded by *hemD*. The enzyme mediates the inversion of ring D of the linear pre-uroporphyrinogen followed by closure of the macrocyclic ring through formation of a link between the first (ring A) and fourth (ring D) pyrrole units generating uro'gen III [73].

In the absence of UROS, pre-uroporphyrinogen spontaneously cyclizes to the isomer uroporphyrinogen I. Uro'gen I is the symmetrical isomer of uro'gen III as ring D is not inverted. Even though uro'gen I is not further involved in the pathway of tetrapyrrole biosynthesis [74], its oxidized form, uroporphyrin I, is found as a cofactor in the rubredoxin oxidoreductase of *Desulfovibrio gigas*, a terminal oxidase involved in reduction of oxygen into water [75].

The structure of the uro'gen III synthase has been solved, revealing a bi-domain structure. The enzyme has comparatively few conserved residues and the mechanism of ring inversion is still not fully understood, even though an enzyme–product complex has been determined by protein crystallography [76]. The most likely mechanism for this remarkable reaction involves a spiro intermediate, especially as a spiro-lactam analogue has been shown to act as a competitive inhibitor.

Completion of heme synthesis in the classical pathway

Uro'gen III represents the branchpoint at which the classic and alternative heme biosynthetic pathways diverge. Along the classical route, heme is produced through modifications of the side chains of uro'gen III, followed by oxidation of the macrocycle and is completed by the insertion of iron.

The first reaction starts with the successive decarboxylation of the four acetate side chains of uro'gen III in clockwise direction starting at ring D, followed by A, B, and C, forming coproporphyrinogen III [77]. This reaction is catalyzed by uro'gen decarboxylase (UROD), which is encoded by *hemE*, and releases four molecules of CO_2 [78]. UROD is an unusual decarboxylase, as it does not require any cofactor for its activity but instead employs the pyrrole rings as electron sinks to help facilitate catalysis. The structure of this homodimeric enzyme shows how the substrate is accommodated within the active site and suggests that the substrate has to leave the site and re-orientate itself to allow for each subsequent decarboxylation event [79, 80].

In the next step, the two propionate side chains of coproporphyrinogen III on rings A and B are decarboxylated in an oxidative manner to yield vinyl groups under the release of two molecules of CO_2, thereby generating protoporphyrinogen IX. This reaction is mediated by one of two structurally and mechanistically unrelated coproporphyrinogen oxidases (CPO) [10]. The distinction is made on whether molecular oxygen is required as terminal electron acceptor for the oxidative decarboxylation, which proceeds via the same stereochemistry in both cases. The oxygen-dependent enzyme HemF is mainly found in Eukaryotes [42], and the oxygen-independent HemN is present in most bacteria [78].

HemF is metal-independent [81], and through the formation of the reaction intermediate harderoporphyrinogen decarboxylates the propionate at ring A prior to that on ring B [82–84]. The structures of *Saccharomyces cerevisiae* [85] and human [86] HemF could only be obtained in the absence of tetrapyrrole. However, through mutagenesis study of the human CPO it has been suggested that one highly conserved aspartate and two arginine residues play an essential role in substrate binding and catalysis [87]. So far, only putative reaction mechanisms for HemF have been proposed [88, 89].

Due to similarities to enzymes involved in the alternative heme and heme d_1 synthesis, the enzyme HemN is discussed in greater detail. HemN is a radical *S*-adenosyl-L-methionine (SAM) enzyme and was the first of this class to have its structure determined [90]. Radical SAM enzymes catalyze a diverse range of biochemical transformations by activation and functionalization of unreactive C–H bonds [91, 92]. They require SAM and a reductant for their activity and are generally involved in anaerobic processes. Enzymes of this family contain an [4Fe–4S] center in which three iron atoms are coordinated by three conserved cysteine residues following a CX_3CX_2C motif [93]. The fourth iron atom is ligated by one molecule of SAM. During one reaction cycle, the iron sulfur cluster is reduced by a reductase. The electron is transferred onto SAM, which is then homolytically cleaved, generating a [4Fe–4S]$^{2+}$-methionine intermediate and a 5′ deoxyadenosyl radical, which can subsequently abstract a hydrogen atom from the substrate. The mechanism of the reaction has been determined through isotopic labeling revealing that after formation of the highly reactive 5′ deoxyadenosyl radical, a hydrogen atom is abstracted from the propionate side chain of the substrate forming a 5′-deoxyadenosine and an allylic substrate radical initiating the elimination of a CO_2 [94]. For HemN, the reductase and final electron acceptor have not been identified, although it was found that two molecules of SAM are cleaved during one reaction cycle.

It is worth noting that some annotations for HemN enzymes actually encode proteins not directly involved in heme biosynthesis. The protein present in bacteria, plants, and animals has therefore been named HemW [95]. For HemW from *Lactococcus lactis*, which has ~50 % similarity to *E. coli* HemN, it was demonstrated that the protein has no CPO activity but binds heme and was therefore proposed to be involved in heme transport [95].

The penultimate step of heme biosynthesis, the oxidation of protoporphyrinogen IX into protoporphyrin IX, gives rise to a fully conjugated, aromatic and planar macrocycle. During the reaction, which is catalyzed by protoporphyrinogen IX oxidase (PPO), six electrons and six protons are abstracted (from two of the four imino groups of the pyrrole and from the four bridging carbons C_5, C_{10}, C_{15}, and C_{20}) [78]. So far, three distinct enzymes have been identified to catalyze this reaction, including those encoded by *hemY* [96], *hemG* [97], and *hemJ* [98].

HemY is a flavo-dinucleotide-dependent enzyme utilizing molecular oxygen as terminal acceptor for the electrons abstracted from protoporphyrinogen IX resulting in the generation of H_2O_2. In most organisms, HemY is membrane-associated (bound to the inner mitochondrial membrane in Eukaryotes and to the cytoplasmic membrane in

Prokaryotes) [96, 99] but was also found to be soluble in the cytoplasm as in *Bacillus subtilis* [100, 101]. The structures of several HemYs have been solved by protein crystallography, revealing how the flavin binds in proximity to the porphyrinogen binding site [102–104]. It is not clear if the mechanism involves reorientation of the substrate during the reaction to help facilitate the removal of the protons and electrons.

In enteric γ-proteobacteria such as *E. coli* and *Salmonella enterica*, HemY is replaced by the flavo-mononucleotide-dependent HemG [105]. The enzyme has been reported to couple heme biosynthesis to the electron transport chains for energy generation [106]. Here, the six-electron abstraction does not result in the production of H_2O_2 but is coupled to ATP generation, depending on availability, employing oxygen, fumarate, or nitrate as terminal electron acceptors.

The third PPO that has been reported is encoded by *hemJ*. The gene was identified through comparative genomic analysis and occurs in the genomes of almost all bacteria, which produce heme via the classical pathway but whose genomes lack a *hemY* or a *hemG* [98]. Its role as a PPO was verified through complementation of a *hemJ* mutant in *Actinobacter baylyi*. It has been suggested that the enzyme could employ oxygen as a final electron acceptor [107] though further experimental evidence is needed to support this view. So far, a functional enzyme has not been recombinantly produced and purified and there is evidence that the enzyme is dependent on an additional component for activity [98].

Heme biosynthesis along the classical pathway is completed by the insertion of Fe^{2+} into protoporphyrin IX generating heme *b*, the precursor to hemes *a*, *c*, and *o*. During the reaction catalyzed by ferrochelatase or HemH [108], the planar macrocycle has to be distorted by the enzyme to allow for metal chelation. In Eukaryotes, HemH is associated with the matrix side of the inner mitochondrial membrane and in many cases is found to contain a [2Fe–2S] center [109]. Membrane-association and the presence of an iron–sulfur cluster are also observed in some prokaryotic ferrochelatases [108]. Nevertheless, many ferrochelatases do not contain iron–sulfur clusters and can be soluble, such as the enzyme from *B. subtilis* [110]. The exact function of the Fe–S center is unknown. The structures of the *B. subtilis*, *S. cerevisiae*, and human enzymes have revealed that the enzymes promote chelation through a distortion of the porphyrin ring, although there is a debate as to the route the metal ion follows to the active site. The ferrochelatases appear to share a common structure with chelatases associated with some siroheme synthases and the cobalatochelatase of the anaerobic cobalamin pathway, indicating that they are all derived from a common ancestral source [111].

It has been reported that in bacteria employing the soluble form of HemY heme synthesis is dependent on the action of an additional protein, HemQ [112]. The exact role of HemQ remains unclear, though it appears to be essential in these organisms as it is required for HemY and HemH activity.

Biosynthesis of siroheme

Although this review focuses on the biosynthesis of heme and heme d_1, an understanding of the biosynthesis of siroheme is required, as this pathway and its intermediates are key to understanding the alternative heme biosynthesis pathway.

In comparison to other tetrapyrroles, the synthesis of siroheme is relatively simple and proceeds from uro'gen III in a three-step process (Fig. 4): (1) addition of two methyl groups from SAM at positions C2 and C7 forming precorrin-2, (2) oxidation of precorrin-2 to sirohydrochlorin, and (3) chelation of iron to form siroheme. Different organisms can use a variety of enzymes for the synthesis of siroheme. *Bacillus megaterium* contains three separate enzymes for the formation of siroheme, a SAM-dependent uro'gen III methyltransferase (SUMT) SirA, a precorrin-2 dehydrogenase SirC and a ferrochelatase SirB (an enzyme that shares structural similarity to HemH, as described above) [113]. *Saccharomyces cerevisiae* uses only two enzymes, the SUMT Met1p and the bi-functional precorrin-2 dehydrogenase and ferrochelatase Met8p [114]. *Escherichia coli* on the other hand has one multifunctional enzyme, CysG, that catalyzes all three steps from uro'gen III to siroheme [115]. CysG contains two domains termed $CysG^A$ and $CysG^B$. $CysG^A$ is homologous to SirA and Met1p, and $CysG^B$ is responsible for precorrin-2 decarboxylation and iron chelation (Fig. 4).

Interestingly, the siroheme pathway shares intermediates of cobalamin synthesis with the aerobic and anaerobic pathways branching at different points. Precorrin-2 represents the branch point for the aerobic pathway where cobalamin synthesis proceeds with the production of precorrin-3 by CobI [116]. For the anaerobic pathway precorrin-2 is converted to sirohydrochlorin and chelation of cobalt by CbiK or CbiX directs the intermediate to cobalamin production instead of siroheme [113].

In some organisms capable of heme d_1 synthesis, production of siroheme is yet to be characterized. Almost all of the organisms that make heme via the alternative pathway contain siroheme-dependent sulfite and nitrite reductases and as such contain an intact siroheme synthesis pathway. The *nir* gene cluster contains a SUMT (NirE) but lacks genes encoding enzymes capable of converting precorrin-2 to siroheme. This may be due to the requirement of

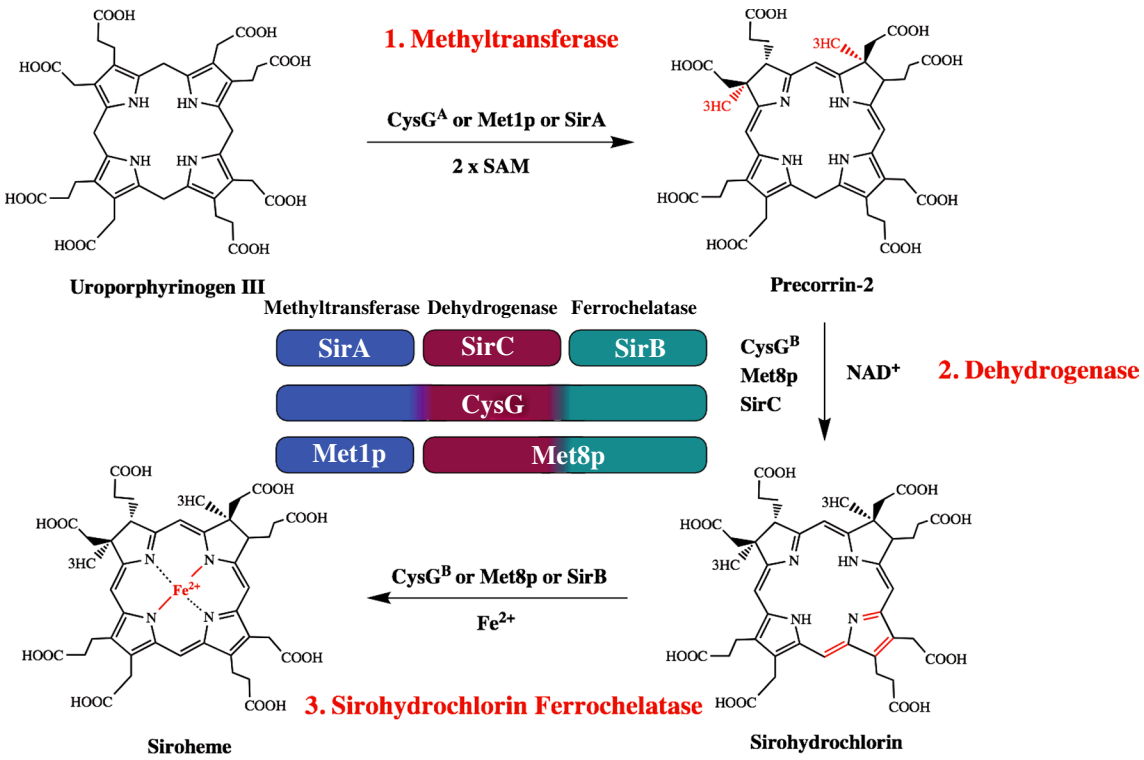

Fig. 4 Biosynthesis of nature's simplest tetrapyrrole, siroheme. Siroheme is synthesized from the common tetrapyrrole precursor uroporphyrinogen III in three consecutive enzymatic steps via the intermediates precorrin-2 and sirohydrochlorin. In *E. coli*, multifunctional siroheme synthase (CysG) catalyzes all the three reactions, which include the SAM-dependent methylation of uroporphyrinogen III, NAD^+-dependent dehydrogenation of precorrin-2 and the iron insertion into sirohydrochlorin. In yeast, the methyltransferase Met1p and the bifunctional dehydrogenase/ferrochelatase Met8p are responsible for synthesis of siroheme and in *B. megaterium* three monofunctional enzymes exist for siroheme formation, SirA (*methyltransferase*), SirC (*dehydrogenase*), and SirB (*chelatase*). The *center* shows a functional alignment that does not represent the amino acid sequence homologies

precorrin-2 in other pathways as when not in denitrifying conditions transcription of NirE would not occur, therefore making precorrin-2 unavailable. In fact, denitrifying bacteria appear to contain multiple SUMTs, suggesting that each tetrapyrrole pathway has its own dedicated SUMT with the other genes (i.e., homologues of *sirB* and *sirC*, *cysG*, or *met8p*) found elsewhere in the genome. Some denitrifiers contain a copy of *cysG*, which is indispensable for heme d_1 synthesis in *Ps. aeruginosa* [117]. All of the denitrifiers also have one or more copies of chelatase genes (e.g., *sirB* or *cbiX*), which may be involved in iron chelation in these organisms, although this is yet to be verified.

As with heme d_1-synthesizing organisms, complete synthesis of siroheme in most bacteria and archaea that synthesize heme via the alternative pathway is yet to be unraveled. Many archaeal genomes appear to have a SUMT, although annotations are varied between *cobA*, *cysG-1*, *cysG-2*, *cysG*, *uroM*, or *hemX*. Likewise, the gene encoding precorrin-2 dehydrogenase is referred to as *sirC*, *hemX*, *cysG*, or *cysG1* [118]. A unique siroheme ferrochelatase appears to be absent from archaeal genomes but it is conceivable that the short cobaltochlatase $CbiX^S$ found in archaea may have a duel role in both iron and cobalt chelation into sirohydrochlorin. Indeed it was demonstrated that $CbiX^S$ from *M. barkeri* and *Methanobacter thermoautotrophicum* are capable of chelating iron into sirohydrochlorin, although at a lower rate to that of cobalt insertion [119]. *Desulfovibrio vulgaris* possesses a bi-functional uro'gen III synthase/methyltransferase (HemD-CobA) and a precorrin-2 dehydrogenase and is therefore capable of synthesizing sirohydrochlorin [120]. Again, a unique siroheme ferrochelatase is absent in the genome but the organism contains two putative CbiK cobaltochelatases ($CbiK^C$ and $CbiK^P$), which are capable of chelating both cobalt and iron into sirohydrochlorin [121]. The genes encoding these proteins are not restricted to *D. vulgaris* but are in fact present in the genomes of other deltaproteobacteria including the groups *Desulfovibrio*, *Desulfobulbus*, *Desulfatibacillum*, and *Desulfobacterium* [122].

Biosynthesis of heme d_1

The elucidation of the biosynthesis of heme d_1 has proven challenging due to difficulties in identifying the pathway

intermediates and their enzymes. These proteins function under anaerobic conditions and both their potential substrates and the products can be unstable in laboratory conditions due to temperature, light, and oxygen sensitivity.

Heme d_1 is structurally closely related to siroheme (Fig. 1), as it carries the same combination of methyl and acetate side chains at positions C2 and C7. In siroheme, the methyl groups are derived from SAM [123]. Indeed, stable isotope labeling experiments in *Ps. aeruginosa* demonstrated that this is also the case in heme d_1 [124]. This result suggested that the synthesis of heme d_1 may proceed via precorrin-2 after which possibly five enzymatic steps may be required for the formation of heme d_1 including; (1) the conversion of propionate side chains at positions C3 and C8 to keto groups, (2) the introduction of a double bond into the propionate side chain attached to C17, (3) the oxidation of the tetrapyrrole macrocycle to yield an isobacteriochlorin, (4) the decarboxylation of acetate side chains at positions C12 and C18 in the southern half of the tetrapyrrole to give methyl groups, and finally, (5) the insertion of iron into the macrocycle. The order of these chemical transformations was difficult to predict, although it was thought that metal ion insertion would be the last step during biosynthesis, owing to the potential cyto-toxicity of the metal-chelated intermediates and the analogy to heme biosynthesis where the ferrochelatase is the terminal enzyme. After the biosynthesis of heme d_1 is completed, the molecule has to be transported across the membrane to the periplasm where it, along with heme *c*, is inserted into *apo*-cytochrome cd_1 [125].

Analysis of the of *nirS* loci from diverse species of denitrifying bacteria and some archaea revealed the presence of a set of contiguous genes downstream of *nirS* that were found to be essential for heme d_1 biogenesis [126, 127]. Depending on the organism, these loci consist of eight or nine open reading frames (ORFs). In *Ps. stutzeri*, the downstream region harbors the genes *nirFDLGHJE*. In *Paracoccus pantotrophus* and *Paracoccus denitrificans*, these genes are organized in the operon *nirECFD-LGHJN* and their transcription activated in the presence of nitric oxide [14].

Mutagenesis of the *nirS* downstream regions in *Ps. stutzeri* and *Ps. aeruginosa* resulted in the synthesis of semi *apo* cytochrome cd_1 (i.e., cytochrome cd_1, which lacks heme d_1) [128, 129]. In analogous studies of *nirD*, *nirL*, *nirG*, and *nirH* mutational analysis in *Ps. stutzeri* and *Ps. aeruginosa*, the indispensable nature of *nirF*, genes during heme d_1 biogenesis has been shown [28, 130]. *Paracoccus denitrificans* mutant strains were generated by disrupting the genes *nirS*, *nirE*, *nirC*, and *nirF* and all these mutants strains were reported to be unable to synthesize heme d_1 [131]. In addition, heterologous expression of *nirS* in organisms lacking cytochrome cd_1 yields an inactive form of NirS without heme d_1 [130]. However, it was reported that an active form of NirS, (i.e., a *holo*-cytochrome cd_1 with both *c* and heme d_1) was produced in *E. coli* when transformed with a plasmid including *nirS* from *P. denitrificans* and about 10 kbp of its downstream region [132]; it would be valuable if this work were to be confirmed and extended.

A comparison of the genetic organization of *nir* clusters from some of the well-studied denitrifiers is shown in Fig. 5. It is worth noting that *Ps. stutzeri* and *Ps. aeruginosa* harbor two separate genes *nirD* and *nirL*, which are fused encoding a single protein NirDL in *Paracoccus* species as well as in several other denitrifying bacteria. Many denitrifying bacteria additionally have a *nirM* gene encoding cytochrome c_{551}, the physiological electron donor for NirS within the *nir* cluster. However, in *P. denitrificans*, NirM is absent but it has been shown that NirS has two periplasmic physiological electron donors; cytochrome c_{550} and copper-containing pseudoazurin [32]. In addition, *nirC*, which is present in the *nir* cluster of most of these organisms, also encodes a small periplasmic cytochrome *c*, which in some cases is able to

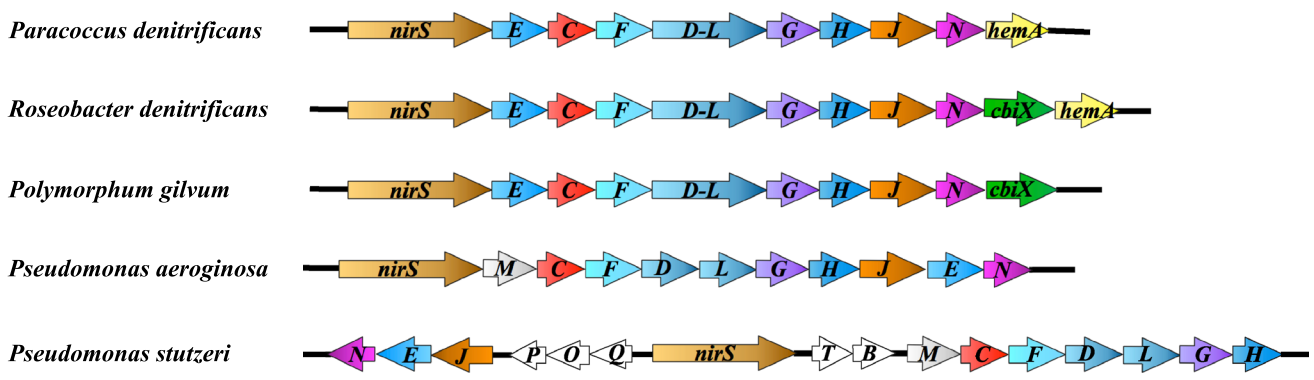

Fig. 5 Genetic organization of the *nirS* loci in different denitrifiers. Clusters were generated from the "Microbial Genome Database for Comparative Analysis" (http://mbgd.genome.ad.jp)

transfer electrons to NirS. However, in the case of *Paracoccus* species, this appears not to apply in vivo, as a double mutant strain lacking both cytochrome c_{550} and *pseudoazurin* was unable to reduce nitrite, an observation indicating another role for the product of the *nirC* gene. There was a report that *nirC* is required for biogenesis of the d_1 heme cofactor in *Paracoccus* (Van Spanning, pers. comm.) but further work, for example complementation studies, is needed to confirm this proposal.

Another interesting observation is the presence of *cbiX* in the *nir* gene clusters of *Roseobacter denitrificans, Dinoroseobacter shibae*, and *Polymorphum gilvum*. The gene *cbiX* is also present in *P. denitrificans* and some other denitrifiers, though elsewhere in the genome, but it is absent in the genomes of *Ps. aeruginosa* and *Ps. stutzeri*. This suggests that CbiX could act as a ferrochelatase for iron insertion during the heme d_1 biosynthesis in these organisms.

Biosynthesis of heme d_1: structure and role of individual Nir proteins

Unfortunately, there are no reports on the accumulation of heme d_1 precursors in the aforementioned mutant strains. The classical approaches of solving a biosynthetic pathway by blocking the pathway at one stage in order to accumulate intermediates have failed in the case of heme d_1 biosynthesis. Furthermore, until recently, very little was known about the individual roles of the nine Nir proteins (i.e., NirE, NirC, NirF, NirD, NirL, NirG, NirH, NirJ, and NirN) in heme d_1 biogenesis.

Out of all Nir proteins, to date NirE is the most well characterized and shares a very high sequence homology to known SUMTs including the C-terminal region of CysG, CobA (methyltransferase of the cobalamin pathway), SirA, and Met1p [133]. NirE from *P. pantotrophus* has been shown to catalyze the conversion of uro'gen III to precorrin-2 in vitro; for *Ps. aeruginosa*, the same has been demonstrated both in vitro and in vivo [14, 134].

As with other SUMTs, NirE from *Ps. aeruginosa* exhibits substrate inhibition at high uro'gen III concentrations and product inhibition by *S*-adenosyl-L-homocysteine [134]. In organisms with overlapping tetrapyrrole biosynthesis pathways, such regulation could ensure direction of common intermediates towards the appropriate pathways. However, it is interesting to note that substrate inhibition by uro'gen III is not observed for NirE from *P. pantotrophus* [14].

Pseudomonas aeruginosa has both CysG and CobA, which carry out the same catalytic activity as NirE. However, the coordinated co-transcription of the *nirE* gene with the other heme d_1 biosynthesis genes, under denitrifying conditions, has been proposed to ensure that the cells can cope with the increased demands for heme d_1 intended for anaerobic respiration. Therefore, NirE is suggested to be specifically required for heme d_1 biosynthesis in organisms where precorrin-2 is precursor to various other tetrapyrroles. The crystal structure of NirE revealed that the protein is a dimer with a similar active site to that of CysG and CobA suggesting a conserved reaction mechanism [135].

Role of NirC, NirN, and NirF

NirC and NirN are *c*-type cytochromes that harbor N-terminal Sec signal sequences. Recombinant expression in *E. coli* demonstrated that both NirN and NirC are targeted to the periplasm via the Sec translocon [14]. Genomic deletion of either *nirN* or *nirC* have established that both proteins are not essential for the formation of heme d_1 [14, 136] and several proposals for their physiological role have been made as discussed above for NirC.

NirN can bind heme d_1 at neutral to low pH to produce a stoichiometric complex due to a C-terminal binding domain [14]. Therefore, it has been suggested that the protein could act as a chaperon for newly synthesized heme d_1 in the periplasm before its capture by the semi *apo*-cytochrome cd_1. This would prevent nonspecific transport out of the cell by porins located in the outer membrane. It has been shown in vitro that heme d_1 can be transferred from *Paracoccus* NirN to semi *apo* NirS (i.e., containing the *c*-type heme only) [14]. It was also proposed that NirN could serve as an electron donor to cytochrome cd_1, a signal transducer element or a catalyst for the degradation of excess heme d_1. NirC can act as an electron donor to both the NirN-heme d_1 complex and to cytochrome cd_1 in vitro [14, 136] though it is unlikely in vivo as the oxidation/reduction potential of NirC is not optimal for the transfer of electrons from the cytochrome bc_1 complex to NirS [14]. Therefore, the physiological roles of NirN and NirC remain unclear, as both are not essential for heme d_1 biogenesis in at least some organisms [14, 28]. There are nevertheless indications that at least under some circumstances the absence of NirN is deleterious. Thus, in a recent study with *P. aeruginosa* [137], there are indications for production of at least a fraction of a modified version of heme d_1 in the absence of NirN.

NirF is a protein of about 43 kDa and like NirN harbors a C-terminal heme d_1 binding domain though neither NirF nor NirN can function as nitrite reductases [28, 127]. NirF is essential for the production of heme d_1. Addition of purified periplasmic NirF to heme d_1 causes a large shift in the UV–Vis spectrum, confirming binding [125]. Mutagenesis identified an amino acid (His41, *P. pantotrophus* NirF) as essential for in vitro binding of heme d_1 and in vivo formation of *holo*-cytochrome cd_1 [125]. However, transfer of heme d_1 to either NirS or NirN could not be shown. Sequence analysis

of all known *nirF* genes has revealed the presence of an N-terminal Sec targeting signal sequence with the exceptions for *nirF*s from *Ps. aeruginosa* and *Magnetospirillum magneticum*. NirF from *P. pantotrophus* has been confirmed to be periplasmic by both cell fractionation work and signal sequence deletion/replacement studies in vitro as well as in vivo [125]. As the location of NirF is in the periplasm, and given the non-essential nature of the two other known periplasmic proteins, NirC and NirN, it seems sensible to assume that the last step of heme d_1 synthesis is catalyzed in the periplasm. Interestingly, it was previously suggested that NirF may act as a dehydrogenase in the terminal steps of heme d_1 synthesis due to the presence of a glycine-rich region resembling a Rossman fold motif ($GXGX_2G$) [125, 138]. This motif is also found in several dehydrogenases that are involved in the siroheme biosynthesis pathways [133]. However, mutagenesis studies established that the glycine residues are not essential for heme d_1 biosynthesis, so the role of NirF also still remains elusive.

The absence of a periplasmic targeting sequence in NirF from *P. aeruginosa* has had a disproportionate influence, leading to the widespread belief that NirF is a cytoplasmic protein. A dual location, periplasmic in some organisms and cytoplamsic in others, was rendered unlikely by the finding that a N-terminally truncated form, and thus cytoplasmic form, of *P. aeruginosa* NirF was unable to complement a *Paracoccus* mutant that lacked its endogenous NirF protein [139]. Furthermore, a recent study has shown that the globular domain of *P. aeruginosa* is exposed to the periplasm and that the protein is a lipoprotein, a feature that anchors it to the membrane [137]. Hence, it is now safe to say that NirF is involved in the last step of heme d_1 production, presumably through acting as an enzyme to catalyze a chemical transformation. There is, however, little clue as to what that reaction might be. For the moment, it can be considered that the synthesis of heme d_1 is completed in the periplasm and thus that contrary to expectation it is not heme d_1 itself but rather an immediate precursor that is transported from the cytoplasm to the periplasm.

It is clear that, at the time of writing, the roles of the NirF and NirN proteins are enigmatic, although both have been shown to bind specifically heme d_1 in vitro. A recent study, based mainly on the results of immunoprecipitation studies, has shown that NirS can interact not only with NirN but also with NirF, leading to the conclusion that the optimal final step in formation of *holo* NirS involves a complex of the three proteins [137].

Role of NirDL, NirG, and NirH

As stated earlier, in several denitrifying bacteria, the genes *nirD* and *nirL* are fused to give a single protein. In *P. pantotrophus*, NirDL (~37 kDa), NirG (~17 kDa), and NirH (~18 kDa) are all cytoplasmic proteins. They share very high sequence homology to each other (~40 % similarity for N-terminal part of NirDL and NirG, and ~42 % similarity for the C-terminal part of NirDL and NirH). Despite the extensive sequence homology, functional redundancy does not exist as all four proteins were shown to be essential for nitrite reductase function [127, 129]. There is also a charge complementarity between these proteins, which suggests that pairs of them might be involved in forming an $\alpha\beta$–$\alpha'\beta'$ multimeric protein complex that may participate in symmetrical reactions of heme d_1 biosynthesis such as decarboxylation of acetate side chain at position C12 and C18 or conversion of propionate side chains at C3 and C8 to oxo side chains [129].

Due to the experimental evidence for precorrin-2 being an intermediate in both the siroheme and heme d_1 pathways it, was proposed that sirohydrochlorin might be a potential intermediate during heme d_1 biosynthesis, even though no gene encoding a precorrin-2 dehydrogenase is located in the *nir* cluster. When total cell lysates of *E. coli* overexpressing a different combination of Nir proteins (e.g., NirDL or NirDLGH from *P. pantotrophus*) were incubated with sirohydrochlorin, a new bis-decarboxylated intermediate, 12,18-didecarboxysiroheme (DDSH), was produced [44]. Further analysis revealed that the true substrate for NirDLGH is not sirohydrochlorin but the iron-containing siroheme. All four proteins were shown to be involved in catalyzing a symmetrical decarboxylation at C12 and C18 of siroheme. The details of these decarboxylation reactions were not reported in the study but a probable reaction mechanism for the chemical transformation was discussed (Fig. 6) [44]. This result furthermore explains the lack of a dedicated ferrochelatase encoding gene in the *nir* cluster.

It has been reported that in *Ps. stutzeri* the maturation of active cytochrome cd_1 requires an intact Tat transport system because a deleterious mutation of *tatC*, encoding one component of the Tat transport system, leads to the formation of an inactive nitrite reductase that lacks heme d_1 [140]. The Tat (twin-arginine translocation) system is a bacterial protein transport pathway that transports folded proteins across the cytoplasmic membrane into the periplasm. This suggests that at least one protein involved in the biosynthesis/assembly of heme d_1 is translocated to the periplasm via the Tat system. It was also concluded that NirD from *Ps. stutzeri* is a periplasmic protein, since a twin arginine motif was found in the N-terminal region of NirD sequence [140]. However, homologues in other organisms do not have this sequence motif and now that a function has been assigned to NirD it seems very unlikely that just NirD, and not NirLGH, is periplasmic. Nevertheless, if variants of *P. stutzeri* lacking a functional Tat system cannot make *holo* NirS, this result still requires explanation.

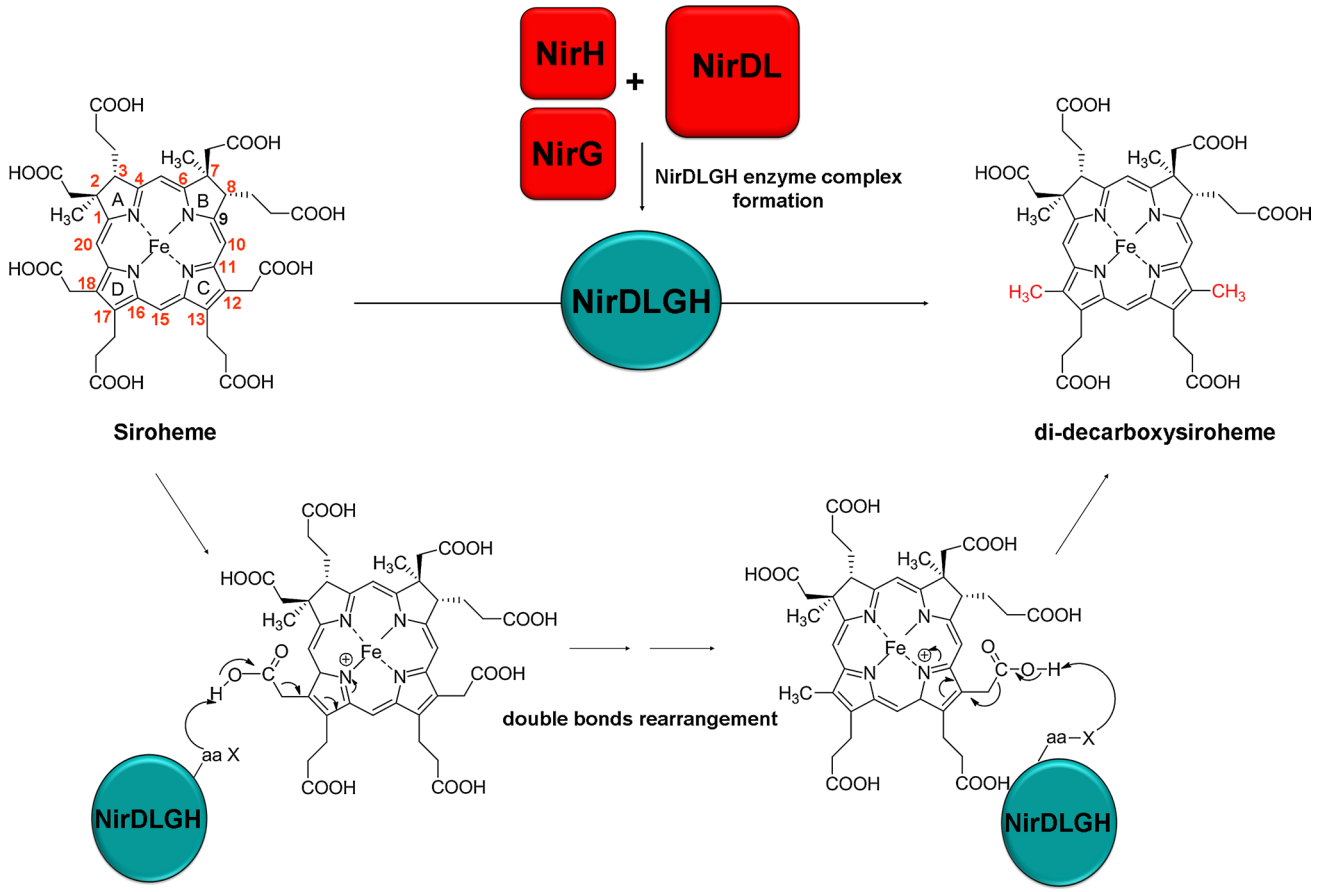

Fig. 6 Potential reaction mechanism of the NirDLGH-catalyzed double decarboxylation. The double decarboxylation of siroheme is catalyzed by the NirDLGH and occurs via the formation of an iminium ion. Here, ring D is shown to undergo decarboxylation at position C18 prior to the decarboxylation of the acetate side chain at ring C. The actual order of these transformations is not known

One possibility is that it may be a secondary consequence of the absence of a functional cytochrome bc_1 complex as export of the Rieske FeS protein depends on the Tat machinery. For example, it was shown that transcription of the *nir* genes for heme d_1 synthesis required nitric oxide, which presumably binds to the transcription factor *nnr* (or *dnr* in some organisms) [34]. Clearly, if electron transfer is blocked because of an inactive bc_1 complex, then the necessary nitric oxide cannot be produced, and thus heme d_1 biosynthesis is also blocked.

The sequences of NirD, NirL, NirG, and NirH reveal similarities to the AsnC/Lrp family of transcriptional regulators. Leucine Responsive Protein (Lrp) homologues are mostly involved in the regulation of genes relating to amino acid metabolism [141]. It was demonstrated that NirL from *Heliophilum fasciatum* is able to bind DNA, specifically the putative promoter of the *nir* operon in this organism [142], confirming that NirL is likely to be a transcriptional regulator as previously annotated. As these proteins also have enzymatic functions [44] it suggests they may play both a catalytic and regulatory role in heme d_1 synthesis.

Role of NirJ

NirJ (~45 kDa) is a monomeric cytoplasmic protein [129] harboring two conserved cysteine-based motifs. The N-terminal motif (CX_3CX_2C) corresponds exactly to the signature motif of the 'radical SAM' superfamily such as the aforementioned HemN that is involved in classic heme biosynthesis [143, 144]. The presence of an iron sulfur cluster was confirmed by electron paramagnetic resonance (EPR) spectroscopy [145]. NirJ also contains a C-terminal cysteine-rich motif (CX_2CX_5C) that is suspected to bind a second iron–sulfur cluster although this was not detected in the EPR analysis [28, 145]. However, it can be difficult to resolve and assign individual centers in proteins with more than one [4Fe–4S] cluster, and the obtained spectra could have been the sum of resonances emerging from two [4Fe–4S] clusters.

The NirJ protein shares a high sequence similarity with radical SAM enzymes, in particular PqqE involved in pyrroloquinoline quinone biosynthesis, MoaA involved in molybdopterin cofactor biosynthesis, and NifB

participating in iron-molybdenum cofactor biosynthesis in nitrogenase [28]. All these proteins contain a second, C-terminal cysteine-rich region in addition to their [4Fe–4S] binding N-terminal CX_3CX_2C motif. The crystal structure of MoaA shows that $[4Fe–4S]^{2+}$ clusters are bound, both at the N-terminal radical SAM motif and the C-terminal $CX_2CX_{12}C$ motif, each with a non-cysteinylated site [146, 147], though the role of the latter cluster is unclear. Most importantly, there is a recent report on the true enzymatic activity of the second [4Fe–4S] cluster from AlbA protein, which is involved in generating the three thioether bonds by radical-based chemistry during subtilosin A maturation [148]. Based on this result and due to the conservation of this second potential [4Fe–4S] binding motif in NirJ sequences, it is possible that both these motifs may be important for the NirJ-catalyzed chemical transformation of the heme d_1 biosynthesis pathway.

It has been suggested that NirJ could catalyze the elimination of propionate side chains at position C3 and C8 of DDSH by activating the alkyl leaving groups in a radical mechanism [44]. However, the source of the oxygen atoms in the resulting carbonyl groups is not known. Interestingly, there are recent examples of radical SAM enzymes with multiple [4Fe–4S] clusters that are involved in two-electron oxidation/dehydrogenation reactions on the organic substrate or on cognate proteins [149–152]. In particular, BtrN, an enzyme involved in the biosynthesis of antibiotic butirosin B, catalyzes the two-electron oxidation of the C3 alcohol of 2-deoxy-scyllo-inosamine to give amino-2-scyllo-inosose [149]. Another interesting aspect of this conversion of an alcohol to a ketone is that the protein was not reported to utilize any of the usual cofactors such as flavin-, pyridine-, or quinone-containing nucleotide metabolites [149]. Therefore, it is possible that NirJ also catalyzes the two-electron oxidation of the propionate side chain at position C17 of DDSH to yield acrylate functionality of heme d_1 molecule in a similar fashion as that of BtrN. However, so far there is no evidence to support the presence of these activities.

Biosynthesis of heme d_1: pathway intermediates and their transport to the periplasm

To transform DDSH into heme d_1, at least two chemical steps are required. These steps include (1) the removal of the propionate side chains at positions C3 and C8 of DDSH and their replacement by carbonyl groups and (2) the dehydrogenation of the propionate group at C17 to generate a double bond. The order in which these steps occur is not known, but it is speculated that NirJ carries out the transformation at C3 and C8 of DDSH to generate "pre-heme d_1″, which could then be converted into heme d_1 by the action of NirF [44]. NirF is the only essential periplasmic enzyme during heme d_1 biosynthesis and it has been shown to bind heme d_1. It is also possible that NirJ catalyzes both chemical transformations to convert DDSH to heme d_1 in a novel, yet unidentified radical-based chemistry.

It is still unclear how the intermediate pre-heme d_1 (if it is the di-oxo intermediate that acts as the substrate for NirF in the periplasm) and or heme d_1 is transported across the membrane to reach the site of cytochrome cd_1 assembly. Furthermore, it is not known which enzyme is responsible for the insertion of heme d_1 into semi *apo* cytochrome cd_1 or if insertion occurs spontaneously in the periplasm.

NirN has been shown to capture heme d_1 and this NirN-heme d_1 complex could transfer the heme d_1 to semi *apo*-cytochrome cd_1 in vitro [14]. However, the rate at which the NirN-heme d_1 complex is made is faster than the rate at which transfer of heme d_1 to semi *apo*-cytochrome cd_1 takes place and also NirN is not essential during heme d_1 biosynthesis as shown by mutagenesis study in *P. pantotrophus* [14]. This suggests that heme d_1 insertion might be a spontaneous process, unless the transfer is catalyzed by either NirF or some other yet unidentified protein under the physiological conditions. NirF has been shown to bind heme d_1 to give a stoichiometric complex, but this complex was unable to transfer the heme d_1 to semi *apo*-cytochrome cd_1 under the experimental conditions [44]. There are no obvious enzymes/transporters in the genomes of denitrifying bacteria that could be assigned the function of heme d_1 transport from cytoplasm to periplasm.

Discovery of the alternative heme biosynthesis pathway

As discussed earlier, some organisms contain heme but lack the later enzymes of the classical heme pathway that are required to convert uro'gen III into protoheme. The following sections outline the discovery of the alternative heme biosynthesis pathway and the enzymes involved.

Early biochemical evidence for an alternative heme biosynthesis pathway

Evidence for the existence of an alternative heme biosynthesis pathway was discovered some 20 years ago by serendipity [153]. During an experiment to determine redox potentials of cytochrome c_3 from the sulfate-reducing bacterium *D. vulgaris* Miyazaki F, it was noted that labeled methionine was incorporated not only into the protein structure but also into the bound heme prosthetic group. It was found that the methyl groups at positions C2 and C7 in heme *c* were not derived from ALA, as would be the case for synthesis via the classical pathway, but were in

fact from methionine, presumably via SAM. As previously mentioned, the two methyl groups at carbons C2 and C7 of precorrin-2 and sirohydrochlorin are derived from SAM [154] and the same has been demonstrated for the methyl groups at C2 and C7 of heme d_1 [124]. Combined with this knowledge and the fact that sulfate reducers contain siroheme and also sirohydrochlorin, it was considered likely that either precorrin-2 or sirohydrochlorin was an intermediate of the alternative heme biosynthesis pathway.

Further to these initial findings, cultures of *D. vulgaris* were supplemented with deuterated L-methionine-methyl-d_3 and porphyrins produced were extracted in an attempt to detect labeled intermediates of the proposed alternative pathway [43]. Several compounds containing deuterated methyl groups at positions C2 and C7 were discovered which included sirohydrochlorin, coproporphyrin III, and protoporphyrin IX as well as a previously unknown hexacarboxylic acid termed 12,18-didecarboxysirohydrochlorin. Based on these results, a new pathway was devised deviating from the classical pathway at uro'gen III to precorrin-2, but then later re-joining the classical pathway with the formation of coproporphyrinogen III, which then proceeds to heme. The steps of the alternative pathway starting at uro'gen III were suggested as follows: (1) methylation of C2 and C7 positions of uro'gen III to form precorrin-2, (2) decarboxylation of C12 and C18 positions to form 12,18-didecarboxyprecorrin-2, (3) elimination of acetate groups from C2 and C7 positions to form coproporphyrinogen III, (4) decarboxylation of propionate side chains on C3 and C8 positions to vinyl groups forming protoporphyrinogen IX, (5) oxidation of ring system to form protoporphyrin IX, (6) insertion of ferrous iron to form heme. As aforementioned, iron chelation was accepted to be the final stage of heme synthesis in due to the potential cyto-toxicity of the metal-chelated intermediates. Since the majority of these reactions are homologous to reactions of previously characterized pathways, the search for similar enzymes began.

Genetic evidence for an alternative heme biosynthesis pathway

The constantly growing number of sequenced genomes of bacteria and archaea allowed for detailed genetic analysis of the alternative heme biosynthesis pathway. Several independent studies have been able to show that this pathway is not restricted to sulfate-reducing bacteria as once believed [42, 118, 155].

It was discovered that some bacteria and archaea only contain the genes corresponding to early heme biosynthesis up to uro'gen III (i.e., *hemA*, *hemL*, *hemB*, *hemC*, and *hemD*) and lack the necessary genes to convert uro'gen III to heme via the classical pathway (*hemE*, *hemF/N*, *hemY/G/J*, and *hemH*, Fig. 3) [42, 155]. This information showed that completion of heme biosynthesis in these organisms must proceed via an alternative route which may occur via precorrin-2 and sirohydrochlorin. At the same time, this also meant that the latter stages of the proposed pathway, from coproporphyrinogen III, could not occur as they lacked homologues of the genes mediating these steps. Therefore a unique set of enzymes that catalyzed the conversion of precorrin-2 to heme must exist in these organisms.

The archeon *Methanosarcina barkeri* was shown to be one of the organisms lacking the latter half of the classical pathway and similar labeling studies were used as with *D. vulgaris* to analyze the incorporation pattern of deuterated methionine in heme [156]. These experiments revealed that, like *D. vulgaris*, two methyl groups in heme from *M. barkeri* are also derived from SAM and not ALA, therefore suggesting that they use the same pathway to synthesize heme.

Further genomic analysis of archaea showed that many species (47 out of 59 studied) only contained the *hem* genes for the production of uro'gen III and not the late *hem* genes needed for the completion of heme biosynthesis [118]. It was also noted that the early *hem* genes were often clustered with genes encoding SUMT and precorrin-2 dehydrogenase enzymes, required for the conversion of uro'gen III to sirohydrochlorin via precorrin-2, supporting the utilization of sirohydrochlorin as a precursor in the alternative heme pathway. Furthermore, it was found that homologues of the *nir* genes (*nirD*, *nirH*, and *nirJ*) were also present in many of these gene clusters, suggesting involvement in the alternative pathway as only three of 27 of the species containing these genes also contained a potential cytochrome cd_1.

The same observation was made in *D. vulgaris*, showing it too only contained the *hem* genes for the production of uro'gen III [120]. Furthermore, it was noted that it contained the necessary genes to convert uro'gen III to sirohydrochlorin and homologues of *nirD* and *nirJ*, despite the fact that it too does not produce cytochrome cd_1. As these gene products are not involved with heme d_1 synthesis, they will be subsequently referred to as the *ahb* genes (**a**lternative **h**eme **b**iosynthesis) *A*, *B*, *C*, and *D*, which are homologous to *nirD*, *H*, and *J*, respectively (with *nirJ* being homologous to both *ahbC* and *D*) (Fig. 7a).

Given the homology to the heme d_1 biosynthesis enzymes and their previously suggested functions [129], a new pathway was proposed. Decarboxylation of the acetate side chains at C12 and C18 positions of precorrin-2 were attributed to AhbA and AhbB, as was suggested for NirDLGH. Next, the removal of acetate side chains at carbons C2 and C7 was assigned to AhbC; this had been

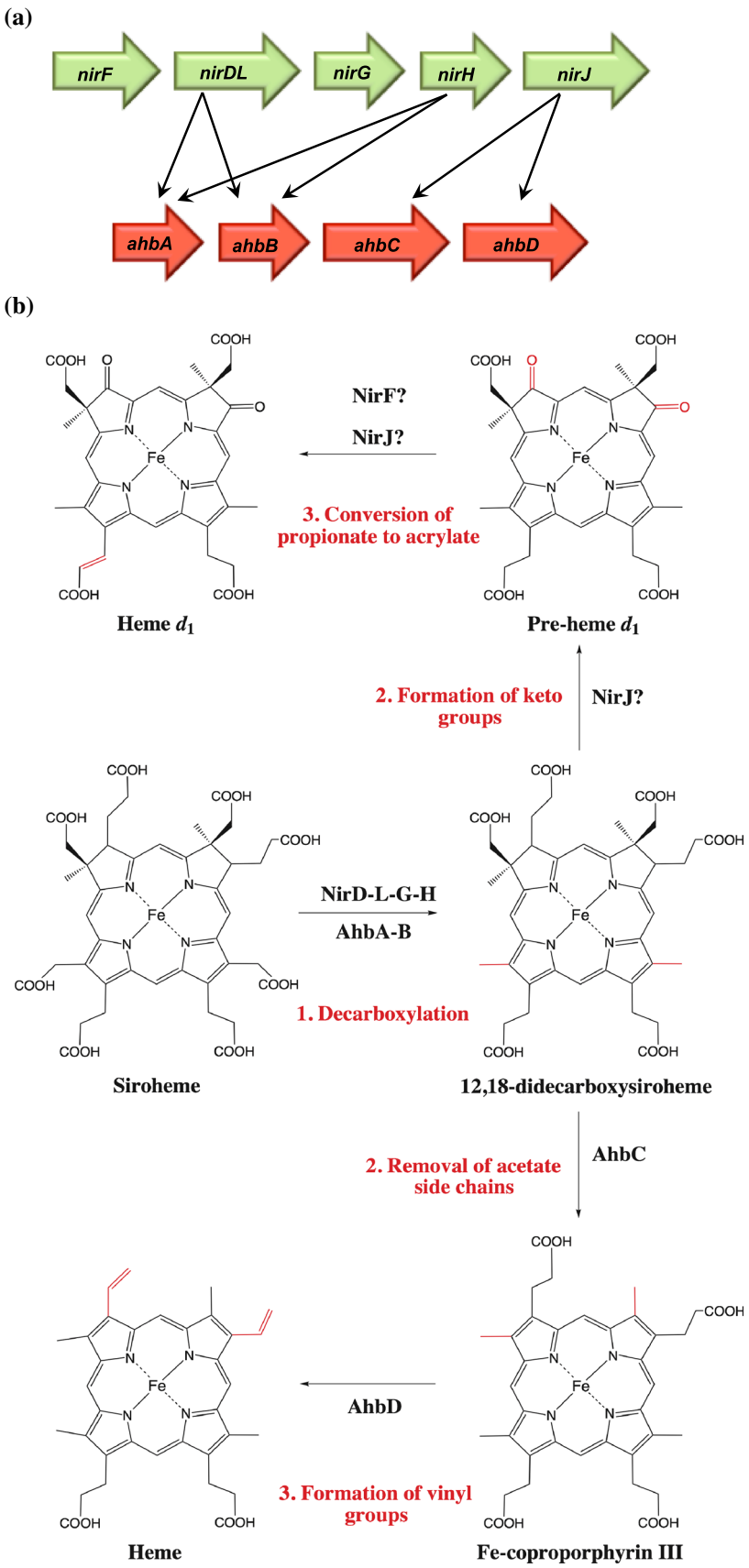

Fig. 7 **a** Representation of genes involved in heme d_1 (*nir*) and alternative heme (*ahb*) biosyntheses and their homology. **b** The alternative heme and proposed heme d_1 biosynthesis pathways starting from siroheme (chemical modifications are highlighted in *red*)

suggested previously in regard to a NirJ-like protein from *D. vulgaris* [120], which is now known to be AhbC in this organism [44]. Finally, a reaction not required for heme d_1 synthesis, the oxidative decarboxylation of propionate side chains on carbons C3 and C8 to vinyl groups, was suggested to be catalyzed by AhbD. This reaction occurs in the classical heme biosynthesis pathway and is catalyzed by HemF or HemN. AhbD is from the radical SAM enzyme family, as is HemN, and it was therefore a suitable candidate for the formation of the vinyl groups.

Although potential biosynthesis enzymes had been highlighted and their function suggested, no activity studies had been performed and no obvious ferrochelatase was known to be involved in the alternative pathway, which was believed to be the terminal step in analogy to the classical heme synthesis pathway [10].

Finding the missing link in the alternative heme biosynthesis pathway

As was the case for the Shemin/C_5 ALA synthesis routes, the alternative heme biosynthesis pathway was discovered and characterized some 20 years after its counterpart. For many years it had been proposed that sirohydrochlorin was the substrate for the alternative pathway but recently the true substrate for the Ahb enzymes was discovered and a surprising link between the alternative heme and the heme d_1 biosynthesis pathways was found.

As mentioned earlier, the substrate for the first step in heme d_1 synthesis is in fact siroheme and not sirohydrochlorin as previously thought [44]. The heme d_1 pathway enzymes NirDLGH unexpectedly formed DDSH from siroheme. In the same study, incubations of AhbA and B from *D. vulgaris* and *D. desulfuricans* with purified siroheme yielded the same result, showing that this reaction was also a conserved step in the alternative heme biosynthesis pathway. Furthermore, the conversion of siroheme to heme was demonstrated using cell lysates of *D. vulgaris* incubated with purified siroheme. Heme was detected as well as intermediates of the pathway including monodecarboxysiroheme, DDSH, and monovinyl iron coproporphyrin.

By utilizing siroheme as a substrate, a chelatase unique to the alternative heme biosynthesis pathway is not required, and hence explains why one was not discovered in early genomic analysis. Previous work had found that *D. vulgaris* could chelate iron into sirohydrochlorin using homologues of CbiK, $CbiK^P$, and $CbiK^C$, which were capable of chelating both iron and cobalt into sirohydrochlorin [121]. As mentioned previously, siroheme is the prosthetic group of sulfite and nitrite reductases, making it an important molecule in sulfate-reducing bacteria. Siroheme is also found in sulfite reductases in archaea [157], again suggesting a prevalence of this molecule in these organisms and an existing biosynthetic pathway for its formation. In fact, in a previous study of archaeal genomes, only four organisms (*Aeropyrum pernix* K1, *Ignicoccus hospitalis* KIN4/I, *Methanosphaerula palustris* E1-9c, and *Hyperthermus butylicus* DSM 5456) were shown to contain *ahbA*, *B*, *C*, and *D* homologues but did not have evidence of a siroheme-containing sulfite or nitrite reductase or lacked cobalamin biosynthesis genes [118].

This finding revealed a new branch point in tetrapyrrole biosynthesis where the heme d_1 and alternative heme biosynthesis pathways diverge at the point of DDSH.

Roles of the Ahb enzymes

Since the discovery of the true substrate for the alternative heme biosynthesis pathway and the enzymes involved, the biosynthetic steps can be summarized as follows (Fig. 7b).

The first reaction in the alternative heme biosynthesis pathway involves decarboxylation of acetic acid side chains at positions C12 and C18 of siroheme to form DDSH [44]. This is catalyzed by AhbA (~20 kDa) and AhbB (~18 kDa), although it is unknown if these enzymes function simultaneously or if they sequentially decarboxylate siroheme. A monodecarboxylated form of siroheme has been detected, although the order of decarboxylation was not determined [44]. It is interesting to note that this same reaction is catalyzed by 3–4 indispensable proteins (NirDLGH) in heme d_1 biosynthesis as opposed to just two in the alternative heme pathway. The relevance of this is yet to be explained, although it could be linked to the regulatory roles of NirDLGH mentioned earlier.

In the next step, DDSH is converted to iron coproporphyrin III, catalyzed by AhbC (~44 kDa). This reaction involves oxidative loss of the northern acetic acid side chains at carbons C2 and C7. AhbC is a member of the radical SAM family, suggesting that the reaction proceeds via adenosyl radicals that activate the leaving groups on the substrate. AhbC contains two cysteine-rich motifs, which are characteristic of Fe–S centers, although it is currently unknown if both centers are required for formation of radicals and enzyme activity.

The final step of the alternative pathway is the conversion of iron coproporphyrin III to heme by the oxidative decarboxylation of the propionate groups at positions C3 and C8 to vinyl groups. This is catalyzed by AhbD (~41 kDa) most likely using a similar mechanism to HemN to transform coproporphyrinogen III to protoporphryinogen IX in the classical pathway [158].

Regulatory requirements of the Ahb pathway

Recent advances in the understanding of the alternative heme and heme d_1 biosynthesis pathways have revealed the increased importance of two intermediates, precorrin-2 and sirohydrochlorin, in tetrapyrrole biosynthesis as they stand as the branch points between cobalamin, coenzyme F_{430}, siroheme, heme, and heme d_1 synthesis (Fig. 8). Therefore, regulation of these biosynthesis pathways must be highly coordinated to direct the common intermediate toward the appropriate pathway.

For example, *D. vulgaris* produces siroheme, cobalamin (via the anaerobic pathway), and heme (via the alternative pathway). Cobalamin and siroheme formation may be regulated at the stage of cobalt or iron chelation into sirohydrochlorin. However, there must also be regulation of the Ahb enzymes to prevent use of siroheme as a substrate. To compound matters, it has been shown that in *D. desulfuricans*, iron coproporphyrin III is used as a unique cofactor in bacterioferritin, again requiring a further point of regulation on the alternative heme pathway in this organism [159].

Sequence alignments of $CysG^B$ from *Salmonella enterica* and *Paenibacillus macerans* as well as Met8p from *S. cerevisiae* and SirC from *B. megaterium* revealed a conserved hydroxyl group in the form of a serine or threonine [135]. This residue may serve as a phosphorylation site for the regulation of these enzymes. Phosphorylation potentially alters ferrochelatase activity allowing regulation of siroheme or cobalt-sirohydrochlorin production or may affect precorrin-2 dehydrogenation, allowing conservation of precorrin-2 for aerobic synthesis of cobalamin or coenzyme F_{430} synthesis, this is as yet unclear. Therefore, it can be envisaged that this regulatory mechanism may be present in homologues of these enzymes in organisms containing the heme d_1/alternative heme pathway and a coenzyme F_{430} and/or cobalamin synthesis pathway. It is also apparent that there is a trend in heme d_1/alternative heme-synthesizing organisms to have one or more cobaltochelatases, some of which have been shown to chelate iron into sirohydrochlorin [119, 121]. This may represent another level of regulation of the sirohydrochlorin pool in the cell, allowing direction to either cobalamin or siroheme synthesis. This being said, further regulation would be required to control the use of siroheme as a substrate for the production of heme/heme d_1 in organisms that require siroheme as a cofactor.

It was referred to earlier that NirD, L, G, and H may play a regulatory role in heme d_1 synthesis as transcription factors. Similar to NirD, L, G, and H, both AhbA and AhbB are annotated as transcription factors belonging to the Lrp/AsnC family. As mentioned before, NirL was shown to bind DNA containing the putative promoter of the *nir* operon in *Heliophilum fasciatum*, and as such it could be expected that AhbA and AhbB function in a similar manner. It was also observed that heme and iron induces transcription of *nirL* in *P. aeruginosa* [160]. This evidence points towards the possibility that AhbA and AhbB not only play a catalytic role in the alternative biosynthesis of heme but they may also serve as transcriptional regulators to the pathway. It is possible that a feedback mechanism exists allowing the reduction of transcription of *ahb* genes in the presence of high levels of heme, therefore allowing for the

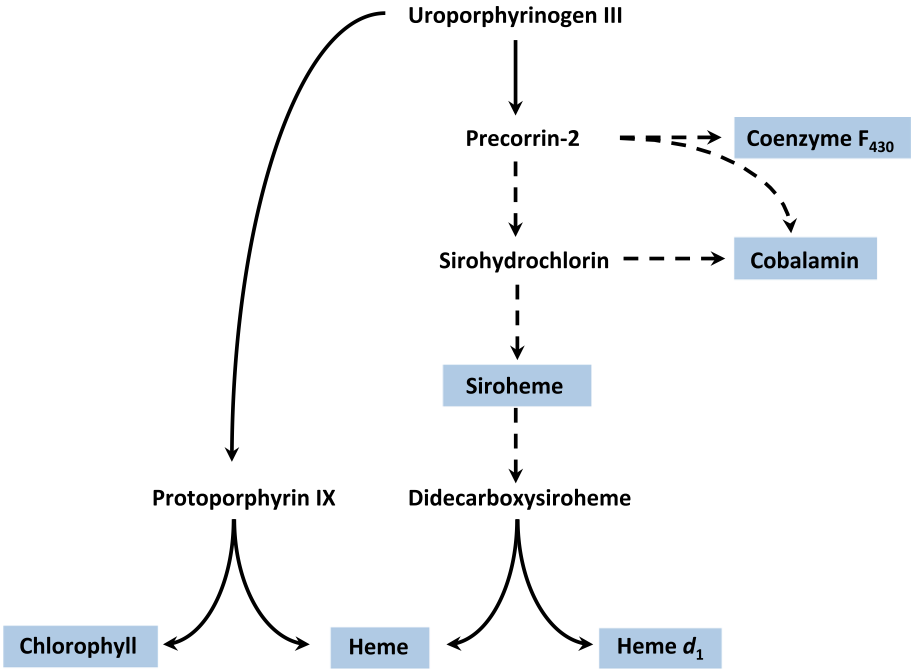

Fig. 8 The known branch points of important tetrapyrrole biosynthesis pathways starting from uro'gen III, potential points of regulation between coenzyme F_{430} cobalamin, siroheme heme, and heme d_1 are displayed as *dashed lines*

careful regulation of siroheme availability in the cell, while maintaining heme production when required, although this is yet to be experimentally verified.

Evolutionary questions

The recent advances in the research aimed at unraveling alternative heme and heme d_1 biosynthesis raise several interesting evolutionary questions. Which of the modified tetrapyrroles among heme, heme d_1, siroheme, cobalamin/B_{12}, or cofactor F_{430} is evolutionarily the oldest? Did siroheme evolve first to be the intermediate on the biosynthetic pathways for making heme and heme d_1, and then found itself hijacked for use as a cofactor, in the nitrite and sulfite assimilatory pathways? Or could it have been the other way round? There is also an interesting aspect of gene regulation here. An organism such as *P. denitrificans* can assimilate nitrite under aerobic and anaerobic conditions provided ammonium is not available as the nitrogen source. Thus, the production of siroheme in such an organism is presumably inhibited under aerobic conditions in which nitrite assimilation is not required, but is activated when assimilation is required. However, under anaerobic conditions, siroheme production must be activated under all conditions, as siroheme production is obligatory in order to sustain the nitrite reductase step of the respiratory denitrification pathway.

In the past, it has been envisaged that cobalamin is possibly the oldest modified tetrapyrrole since it is involved in ribonucleotide reduction and contains a nucleotide loop that may represent a remnant of the RNA world [161, 162]. However, it has been suggested that the main function of cobalamin was to produce an adenosyl radical that could be used to initiate a number of rearrangement reactions, which involve degradation of ethanolamine, propanediol, and glycerol to name a few [163–165]. It has also been shown that cobalamin is required for the anaerobic formation of chlorophyll in some Gram-negative bacteria [166]. Another important function of cobalamin is in methylation reactions, as seen in case of the methionine synthase where methylcobalamin transfers a methyl group to homocysteine. However, both the methyl transfer and adenosyl radical involving chemical transformations are also carried out by "radical SAM" enzymes [91, 144] and therefore there was no evolutionary driving force that particularly required cobalamin formation in the earliest life forms.

Coenzyme F_{430} is the nickel-containing cofactor found in methyl coenzyme M reductase, an enzyme involved in methanogenesis. Due to its highly reduced ring structure (Fig. 1), which is a prerequisite in prebiotic life and its involvement in methanogenesis, it has also been suggested to be a candidate [167].

The argument that heme is an ancient tetrapyrrole is weakened by the fact that not all species of archaea, which are believed to be the evolutionary older organisms, possess heme [118]. The genetic information to make heme might have been acquired by lateral gene transfer from species of bacteria, such as the sulfate-reducing bacteria, that possess the alternate heme biosynthesis pathway involving siroheme. Interestingly, it has recently been found that archaea and sulfate-reducing bacteria also share a common system for attaching heme to proteins during maturation of *c*-type cytochromes, as this type of cytochrome is not common in archaea, this suggests that gene transfer could have occurred here, too [168]. There are indications that reduction of nitrite by cytochrome cd_1 may have preceded the appearance of oxygen, whereas assimilation of sulfite and nitrite may have occurred only after oxygen appeared on earth [169]. This might suggest that heme d_1 arose before siroheme and therefore, siroheme potentially has acted as a precursor to other tetrapyrroles, before being utilized as a prosthetic group in assimilatory sulfite and nitrite reductases.

However, in most archaea and sulfate-reducing bacteria, heme is made via the newly discovered alternative route involving siroheme. Since the biosynthesis of heme d_1 is also shown to proceed via siroheme, it is very tempting to conclude that siroheme may be the most ancient tetrapyrrole. It is structurally the simplest of all modified tetrapyrroles and enzymatically the easiest to make [133]. Furthermore, some obligate anaerobes lack cytochromes but can synthesize siroheme [170]. All these facts together promote the antiquity of siroheme in the early biotic life.

Given the close relationship between the syntheses of cobalamin, siroheme, coenzyme F_{430}, and heme d_1, it is conceivable that the alternative heme biosynthesis pathway actually represents the original route for heme synthesis and the classical pathway may have emerged later. Conversion of uro'gen III to heme via the classical pathway utilizes only two molecules of SAM in comparison to the six required by the alternative. This lower-energy consumption is likely to be the driving force behind the successful integration of the classical pathway throughout all kingdoms of life.

Conclusions

In conclusion, as summarized in Fig. 7b, the chemical reactions involved in alternative heme and heme d_1 biosynthesis have been outlined. Siroheme has firmly been established as an intermediate in both pathways, as has DDSH, which represents a new branch point in tetrapyrrole biosynthesis.

The functions of the Ahb enzymes have been established and the intermediates of the pathway are known, although

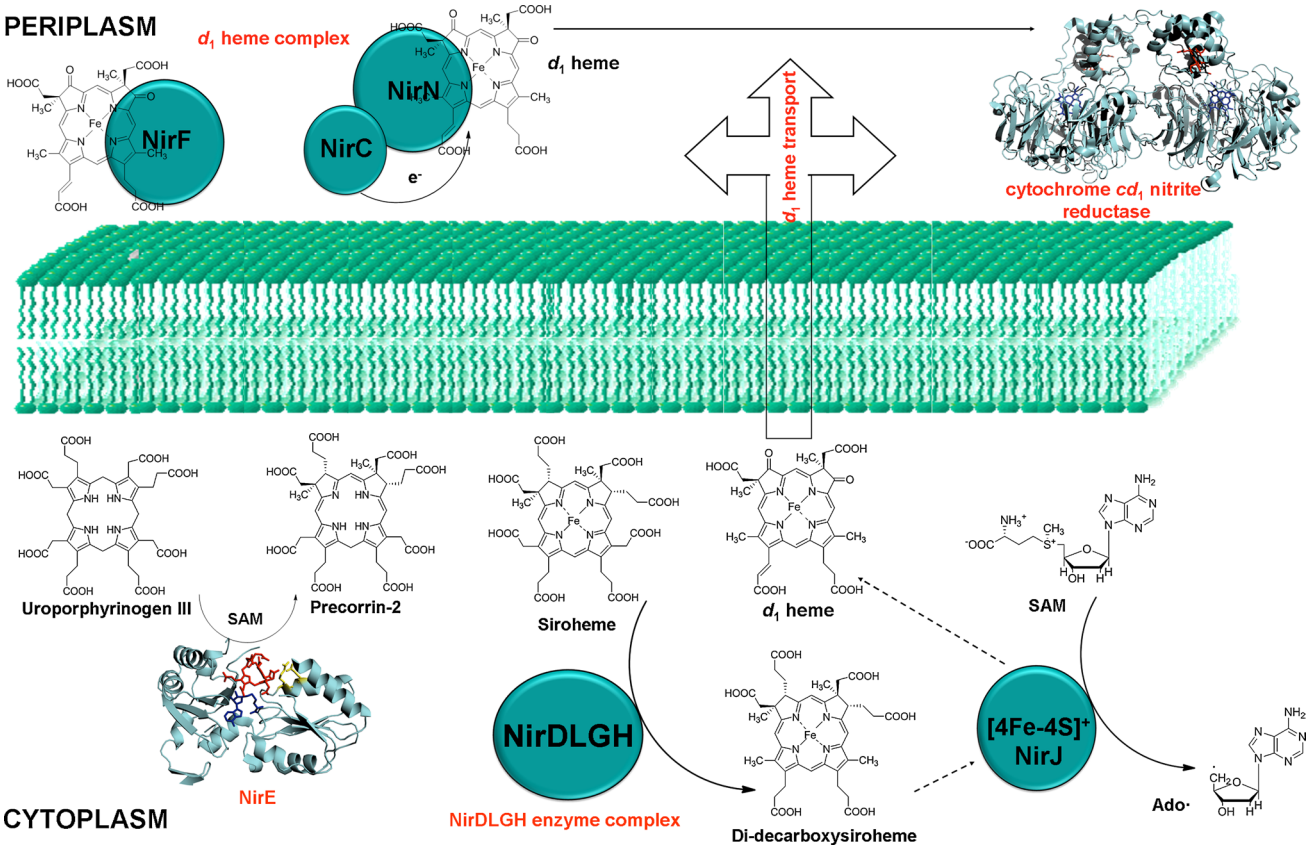

Fig. 9 Summary of the heme d_1 biosynthesis pathway. Schematic representation of all known proteins of the heme d_1 biosynthesis pathway and the reactions they catalyze. Please note that cytochrome cd_1 nitrite reductase is a homodimeric protein and requires two molecules of heme d_1 per molecule of protein

the order of decarboxylation of acetate groups of siroheme and the reaction mechanisms of AhbC and D are yet to be determined.

As with alternative heme synthesis, the mechanism of siroheme decarboxylation and exact roles of NirD, L, G, and H are yet to be established. Questions about NirC, N, and F also remain unanswered. The periplasmic location of NirC, NirN, and NirF suggests that their roles must be at the final stages of heme d_1 production. The exact enzymatic roles of NirF and NirJ still await experimental evidence. As there are more enzymes involved in the synthesis of heme d_1 than heme even though the same amount of chemical transformations are required from the point the two pathways diverge, those proteins are most likely to be responsible for the transport of heme d_1 to the periplasm as well as insertion into nitrite reductase. A summary of the proposed heme d_1 biosynthesis pathway is shown in Fig. 9.

Integration of siroheme in alternate heme and heme d_1 biosynthesis pathways represents a beautiful example of patchwork model of pathway evolution. In this model, pathways evolve in the forward direction due to the promiscuity of the enzymes and undeveloped regulation mechanisms of the system producing multiple end products.

With the discovery of a new branch point in tetrapyrrole biosynthesis there are also many questions still to be answered relating to the regulation of overlapping metabolic pathways within some organisms (Fig. 8), with the potential of the early Ahb and Nir proteins playing a novel role as both catalytic enzymes and transcriptional regulators.

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