Structural diversity in metal ion chelation and the structure of uroporphyrinogen III synthase

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Abstract

All tetrapyrroles are synthesized through a branched pathway, and although each tetrapyrrole receives unique modifications around the ring periphery, they all share the unifying feature of a central metal ion. Each pathway maintains a unique metal ion chelatase, and several tertiary structures have been determined, including those of the protoporphyrin ferrochelatase from both human and Bacillus subtilis, and the cobalt chelatase CbiK. These enzymes exhibit strong structural similarity and appear to function by a similar

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mechanism. Met8p, from *Saccharomyces cerevisiae*, catalyses ferrochelation during the synthesis of sirohaem, and the structure reveals a novel chelatase architecture whereby both ferrochelation and NAD$^+$-dependent dehydrogenation take place in a single bifunctional active site. Asp141 appears to participate in both catalytic reactions. The final common biosynthetic step in tetrapyrrole biosynthesis is the generation of uroporphyrinogen III synthase, whereby the D ring of hydroxymethylbilane is flipped during ring closure to generate the asymmetrical structure of uroporphyrinogen III. The recently derived structure of uroporphyrinogen III synthase reveals a bi-lobed structure in which the active site lies between the domains.

**Introduction**

All physiologically relevant tetrapyrrole-derived cofactors, such as haem, sirohaem, cobalamin (vitamin B$_{12}$) and coenzyme F$_{430}$, contain a specific metal ion at the centre of a tetrapyrrole ring. Tetrapyrrole biosynthesis begins with three shared enzymic steps culminating with the formation of uroporphyrinogen (uro‘gen) III. The last step of this process requires the cyclization of the linear tetrapyrrole hydroxymethylbilane into the physiologically relevant asymmetric tetrapyrrole ring (Scheme 1) [1–3]. This reaction is catalysed by uro‘gen III synthase (U3S). From this branch point, the only other biosynthetic reaction that each cofactor shares is that of metal ion chelation. In this respect, each branch of the pathway contains a specific metal ion chelatase that catalyses the insertion of a particular bivalent metal ion into the centre of the tetrapyrrole ring (Scheme 1). This review will cover recent structural advances that are assisting our understanding of how the processes of ring cyclization, isomerization and metal ion chelation occur.

**Structure of U3S**

The last shared reaction in tetrapyrrole cofactor biosynthesis is the formation of uro‘gen III by U3S. The enzyme catalyses the inversion of ring D and the simultaneous closure of the linear tetrapyrrole, hydroxymethylbilane, to form an asymmetric macrocyclic ring (Scheme 1). Biochemical studies suggest that the reaction involves a spirocyclic intermediate consisting of a single-carbon attachment of pyrrole ring D to the bridge carbons of both rings A and C [4]. A synthetic spirolactam was shown to be a strong

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**Scheme 1**

**Tetrapyrrole biosynthesis**

The formation of all tetrapyrrole cofactors begins with three shared biosynthetic steps that culminate in the formation of uro‘gen III from the linear tetrapyrrole hydroxymethylbilane. Two steps downstream of uro‘gen III, HemH inserts ferrous ion into protoporphyrin IX to form haem. Alternatively, after methylation of uro‘gen III by CobA/CysG to form precorrin-2, CbiK inserts cobalt to form co-precorrin-2 and initiate cobalamin synthesis. Finally, Met8p catalyses the NAD$^+$-dependent dehydrogenation of precorrin-2 to generate sirohydrochlorin, followed by ferrochelation to form sirohaem. Enzyme names are shown in boxes. Groups: A, acetate; P, propionate; M, methyl; V, vinyl.

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inhibitor of the enzyme, perhaps by mimicking the transition state [5,6]. It has been postulated that the reaction may be triggered by the nucleophilic attack on the hydroxy group attached to ring A, but the details of the enzyme-assisted reaction are largely unknown.

Insights into how a comparatively small enzyme (U3S has a molecular mass of \( \sim 28 \text{kDa} \)) can promote such a complex reaction have been investigated through the structural determination of the human enzyme. U3S consists of a bi-lobed structure in which the two domains are separated by a two-stranded \( \beta \)-sheet (PDBcode 1JR2; Figure 1) [7]. A large cleft between the two domains contains most of the conserved and invariant residues that are surface exposed, and is likely to be the location of the active site. Modelling studies indicate that the active site is larger than the product uro'gen III, which suggests that a large conformational change may be required during catalysis. Mutation of all the surface-exposed conserved residues (mostly serine and threonine residues), and of several other residues in the putative active-site cleft, did not reveal any single residue that was absolutely required for catalysis, suggesting that the reaction mechanism does not involve a general acid or base [7].

Due to the direct involvement of U3S in the mammalian haem biosynthetic pathway, inherited genetic mutations in the U3S gene can result in a disease known as congenital erythropoietic porphyria, a severe recessive form of porphyria where patients have to keep away from direct sunlight [8]. To date, 16 disease-causing mutations have been identified in patients with congenital erythropoietic porphyria around the world. These resulting amino acid changes are found on the surface of U3S, and predictions have been made as to their expected catalytic effect [7]. Most mutations disrupt the packing of the hydrophobic core of the protein, leading to an unstable or misfolded enzyme. Only two of the mutations are on the active-site surface, consistent with the recessive nature of the disorder, since mutations that abolish catalytic activity would be lethal.

### Metal ion chelation

Each tetrapyrrole cofactor contains a specific metal cation: haem and sirohaem contain iron, cobalamin (vitamin B\(_{12}\)) contains cobalt, chlorophyll contains magnesium, and coenzyme F\(_{430}\) contains nickel. The metals are inserted in their bivalent ionization state. Despite differences in the metal ion identity and the oxidation state of the macrocyclic porphinoid ring, the various chelation reactions are thought to proceed in a similar fashion. Thus two protons extending from pyrrole nitrogens are abstracted from the tetrapyrrole-derived substrate to allow for electron orbital overlap with the incoming metal ion [9,10]. To facilitate this reaction, the enzymes induce a distortion in the tetrapyrrole through puckering of the structure. Resonance Raman studies have suggested that the puckering may take the form of a dome where all four pyrrole nitrogens are oriented in one direction, or a semi-dome where two pyrrole rings are more puckered than the other two [11–13]. There is additional evidence that the ring puckering only occurs when all three enzymic participants, i.e. enzyme, tetrapyrrole and metal ion, are combined to form a tertiary complex [12]. A naturally distorted tetrapyrrole, \( N \)-methylmesoporphyrin, is a potent inhibitor of protoporphyrin IX ferrochelatase, and is thought to mimic the enzymic transition state [14,15].

To be fully functional, each tetrapyrrole-derived cofactor must contain a specific metal ion, and significant metal ion cross-reactivity does not occur in vivo. In contrast, in vitro, the chelatase enzymes show a wide degree of cross-reactivity towards alternative metal ions. For instance, the widely studied protoporphyrin IX ferrochelatase inserts Fe\(^{2+}\), Zn\(^{2+}\) and Co\(^{2+}\) into protoporphyrin IX at significant rates. Two factors are thought to contribute to the high metal ion selectivity in vivo: (1) free metal ion concentrations are believed to be...
vanishingly small in vivo, in contrast with the micromolar amounts used in the in vitro reactions; and (2) subcellular compartmentalization may restrict access of certain metal ions to the chelatases. Both of these considerations suggest that specific metal ion chaperones may exist to transport metal ions from storage locations within a cell to the proper chelatase ([16] and refs therein). This metal ion shuttling is crucial in bacterial and plant systems, where multiple metal ion chelatases may be functioning within the same organism.

Metal ion chelatases can be segregated into two classes based on their structural architecture. Class 1 chelatases are heteromultimeric enzymes that require three gene products for efficient catalysis [17]. Although the exact subunit stoichiometry is not known, the large porphyrin-binding subunit (110–150 kDa) [18] is thought to be activated by a heterodimer formed by a member of the AAA ATPase family in complex with a second regulator. The chelation reaction subsequently requires ATP hydrolysis for catalysis [19]. Enzymes in this class include the chlorophyll/bacteriochlorophyll magnesium chelatase (ChlHID/BchHID), the aerobic cobalt chelatase (CobNST) and the coenzyme F$_{430}$ nickel chelatase. Class 2 chelatases are small (30–40 kDa) monomeric or dimeric proteins, and include the protoporphyrin IX ferrochelatase (HemH), the anaerobic cobalt chelatase (CbiK), and the ferrochelatases involved in sirohaem synthesis (CysG/Met8p). Tertiary structures have been solved for each member of this class (Figure 2) [15,20–23].

The tertiary structures of the Bacillus subtilis protoporphyrin IX ferrochelatase (HemH) and the anaerobic cobalt chelatase (CbiK) share a great deal of structural similarity (PDBcodes 1AK1 and 1QGO respectively; Figures 2A and 2B) [20,22]. This similarity was initially surprising, since search algorithms were unable to detect significant sequence similarity. Structural alignment corrected the gaps in the sequence alignment, and the two were revealed to share ~14% identity. Both enzymes adopt a two-domain structure in which the topological folds of each domain are similar – a four-stranded b-sheet flanked by four or five a-helices. The active site lies in a cleft between these domains, and conserved charged residues lie on one face of the active site – two histidines in CbiK and a histidine and a glutamic acid acid in ferrochelatase. The human protoporphyrin IX ferrochelatase also adopts a similar structure, although it functions as a homodimer, contains an (Fe$_2$S$_2$) iron–sulphur centre and is embedded in the matrix side of the mitochondrial inner membrane (Figure 2C) [21]. The intact Fe$_2$S$_2$ cluster is required for full functionality of the chelatase, but the iron within the cluster is not the source of ferrous iron for the chelation reaction [24].

Recently, the novel structure of the ferrochelatase involved in sirohaem synthesis in Saccharomyces cerevisiae, Met8p, has been determined [23]. The enzyme is responsible for the final two biosynthetic reactions in the production of sirohaem: an NAD$^+$-dependent dehydrogenation of precorrin-2 to produce sirohydrochlorin, and subsequent ferrochelation to produce sirohaem (Scheme 1). Met8p is a homodimer in which each polypeptide can be described in terms of three functional domains: an N-terminal NAD$^+$-binding domain, a central dimer-interface domain and a helical C-terminal domain (Figure 2D). NAD$^+$ is bound to the surface of the NAD$^+$-binding domain in a conformation typical for such nucleotides, interacting with the signature nucleotide binding motif, GxGxxG. Unfortunately, the $\beta$-nicotinamide ring is not visible in the electron density, prompting the selection of a representative NAD$^+$ molecule from the protein databank to be modelled into the binding pocket.

The modelled $\beta$-nicotinamide lies adjacent to an invariant residue, Asp-141, and a cleft formed between the NAD$^+$-binding domain and the interface domain. The cleft between the NAD$^+$-binding and interface domains most likely contains both the dehydrogenase and ferrochelatase activities in a novel bifunctional active site. This proposal is supported by the observations that all of the invariant residues among Met8p bacterial homologues map to the cleft, and that the cleft maintains an overall positive electrostatic potential that might help to offset the $-8$ charge held by the substrate, precorrin-2.

Structure-based site-directed mutations were designed in order to disrupt the NAD$^+$-binding site and test the role of Asp-141 in the catalytic mechanism. NAD$^+$ binding is abolished when the second glycine of the GxGxxG sequence, Gly-22, is changed to Asp. The resultant mutant is completely inactive as a dehydrogenase, but functional as a metal ion chelatase [23]. These results suggest that the NAD$^+$ is not required for the chelation reactions, and that the dehydrogenase and ferrochelatase reactions are not linked mechanistically. Mutation of Asp-141 to an alanine resulted in a protein that was devoid of both dehydrogenase and ferrochelatase activities,
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thereby implicating the carboxylate-containing amino acid in both catalytic activities.

During both reactions catalysed by Met8p, protons are removed from nitrogens in the pyrrole rings (Scheme 1). We propose a mechanism in which dehydrogenation proceeds by proton abstraction from a pyrrole nitrogen, subsequent bond rearrangement and hydride transfer from a bridge carbon to NAD^+. In the second step, two additional protons are removed from pyrrole nitrogens and a ferrous ion is inserted into the ring. It is therefore possible that Asp-141 functions to abstract protons during both dehydrogenation and chelation [23].

Three metal-bound chelatase structures have provided conflicting information as to the location of the metal-binding site. The most logical position for a metal-binding site is presented in the gold- and cadmium-bound structures of the \textit{B. subtilis} ferrochelatase. The metals are potent inhibitors of the ferrochelatase and are positioned within ~2.0 Å of the invariant histidine residue at the active site, His-183 [20]. Two other metal-bound structures are less informative. Met8p crystals soaked in cobalt did not reveal a distinct metal-binding site, but a manganese soak revealed a strong Mn^{2+}-binding site within the C-terminal helical domain involving a histidine residue [23]. Mutation of this non-conserved histidine did not affect catalytic activity \textit{in vitro}, and therefore this residue is unlikely to play a prominent role in the catalytic cycle. Finally, a cobalt soak of human protoporphyrin IX ferrochelatase revealed a strong cobalt-binding site on the back of the enzyme, among non-conserved residues [21]. Mutagenesis studies support a role for this histidine in catalysis, and it has been suggested that this site represents the beginning of a channel through the protein for substrate metal ions that would otherwise have difficulty accessing the active site, which is buried in the periplasmic membrane [25]. This proposal conflicts with the conclusions of Lange et al. [16], who suggested that ferrous ion must reach the enzyme via the mitochondrial inner membrane and that ferrous ion from the matrix pool is not a substrate for ferrochelatase.

Perhaps the most revealing evidence that the charged residues above the active site are involved in metal ion selection can be deduced from work with the anaerobic cobalt chelatase. CbiK can complement a \textit{cysG} deletion strain by functioning as a ferrochelatase during sirohaem synthesis [26]. Addition of exogenous cobalt to the growth medium results in the selective insertion of cobalt instead of ferrous ion by CbiK, and inhibited complementation of the \textit{DcysG} strain. Mutation of either invariant histidine within the CbiK active site reduced the catalytic efficiency of the chelation reaction, although sufficient activity was maintained to complement the ferrochelatase deficiency. Surprisingly, the histidine mutants were not inhibited by the addition of exogenous cobalt during complementation, suggesting that the

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**Figure 2**

**Structural diversity in metal ion chelation**

Shown are the X-ray crystal structures of (A) \textit{B. subtilis} protoporphyrin IX ferrochelatase, (B) the anaerobic cobalt chelatase CbiK, (C) one monomer of the dimeric human protoporphyrin IX ferrochelatase, and (D) Met8p, the bifunctional dehydrogenase and ferrochelatase involved in sirohaem synthesis. Each structure is displayed with dark β-strands and light α-helices, and unique regions within the first three structures are shown in mid-grey. Invariant residues involved in the catalytic mechanisms, and the β-NAD^+ cofactor of Met8p, are shown in white ball-and-stick representation. A disordered loop in Met8p is shown as a light grey coil.
mutants had lost the ability to selectively insert cobalt over ferrous ions [22].

Although Met8p differs significantly in tertiary structure from CbiK and bacterial and mammalian HemH, there are similarities between their active sites that support the proposal that all four enzymes share a common catalytic mechanism. Examination of the known chelatase structures defines an active-site cleft that contains charged side chains on one side and hydrophobic residues on the other. In a complex between N-methylmesoporphyrin and the B. subtilis ferrochelatase, the tetrapyrrole ring packs against a hydrophobic tyrosine and the pucker of the distorted tetrapyrrole points towards the charged side chains of a catalytic chelatase antibody and of the cobalt chelatase supports this proposal. Even the diverse toporphyrin IX ferrochelatase and the cobalt chelatases all utilize a similar catalytic mechanism.

In conclusion, it is proposed that the class 2 ferrochelatases all utilize a similar catalytic mechanism.

Conclusions

In conclusion, it is proposed that the class 2 ferrochelatases all utilize a similar catalytic mechanism. The significant structural similarity between protoporphyrin IX ferrochelatase and the cobalt chelatase supports this proposal. Even the diverse structures of a catalytic chelatase antibody and of Met8p reveal similarities in the details of their active sites. Further understanding of the chelation reaction is expected to result from knowledge of tertiary structures containing enzyme, porphyrin and free metal ion.

References


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