CHAPTER 22

Biosynthesis of Siroheme and Coenzyme F_{430}

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Abstract

The biosynthesis of siroheme from uropoprhyirnogen III in bacteria, yeasts and plants is described. The pathway requires the *bis*-methylation of uroporphyrinogen III to generate precorrin-2, which is then oxidised to sirohydrochlorin prior to its ferrochelation. A number of structures of the various biosynthetic enzymes have been elucidated and thus the overall process is known in molecular detail. In contrast, the biosynthesis of coenzyme F_{430}, which is synthesized soley by methanogenic bacteria, is poorly understood. It is estimated that between 6 and 8 steps are required for the transformation of uroporphyrinogen III into coenzyme F_{430}, yet none of the biosynthetic enzymes have been identified and only one potential intermediate has been isolated.

Introduction

Other chapters in this book cover the biosynthesis of heme, chlorophyll, bilins and vitamin B_{12}. In this chapter we will focus on the biosynthesis of siroheme and coenzyme F_{430}, two modified tetrapyrroles that are involved in the reduction of sulphite and nitrite (see Chapter 24) and the process of methanogenesis respectively (see Chapter 23). The relationship of these compounds is outlined in the summary of the branched biosynthetic pathway shown in Figure 1. The biosynthesis of siroheme from uroporphyrinogen III requires three biosynthetic steps and is well understood, whereas the biosynthesis of F_{430} requires at least five enzyme mediated steps in a process that has not yet been characterised in any significant detail.

Siroheme Biosynthesis

Siroheme was first identified as a prosthetic group in sulphite reductase[1,2] and subsequently in nitrite reductases.[3] The structure of siroheme was determined to be that of an iron-containing isobacteriochlorin, derived from the common uroporphyrinogen III template that is characteristic of all modified tetrapyrrole.[2] The transformation of uroporphyrinogen III into siroheme requires three steps, namely the *bis*-methylation of uroporphyrinogen III into precorrin-2, the oxidation of precorrin-2 into sirohydrochlorin and the chelation of sirohydochlorin with ferrous iron to give siroheme (Fig. 2).

The biosynthesis of siroheme was first investigated in *Escherichia coli* where it was found that lesions in the *cysG* gene resulted in the loss of NADH-dependent nitrite reductase activity.[4] As the main structural genes for nitrite reductase had already been identified, it was suggested that *cysG* encoded an enzyme for the synthesis of siroheme, a known prosthetic group for this enzyme.[5] Subsequently, the gene was sequenced and shown to encode a 457 amino acid protein.[6] Sequence comparisons revealed that CysG shared similarity with the cobalamin biosynthetic methyltransferases, including CobA, CobI, CobJ, CobM, CobF and CobL (see Chapter 18) (Fig. 2).[7,8] However, the sequence similarity was shared only over the C-terminal region of CysG, from amino acids 202-457. The N-terminal region of CysG did not show sequence similarity to any other protein on the data

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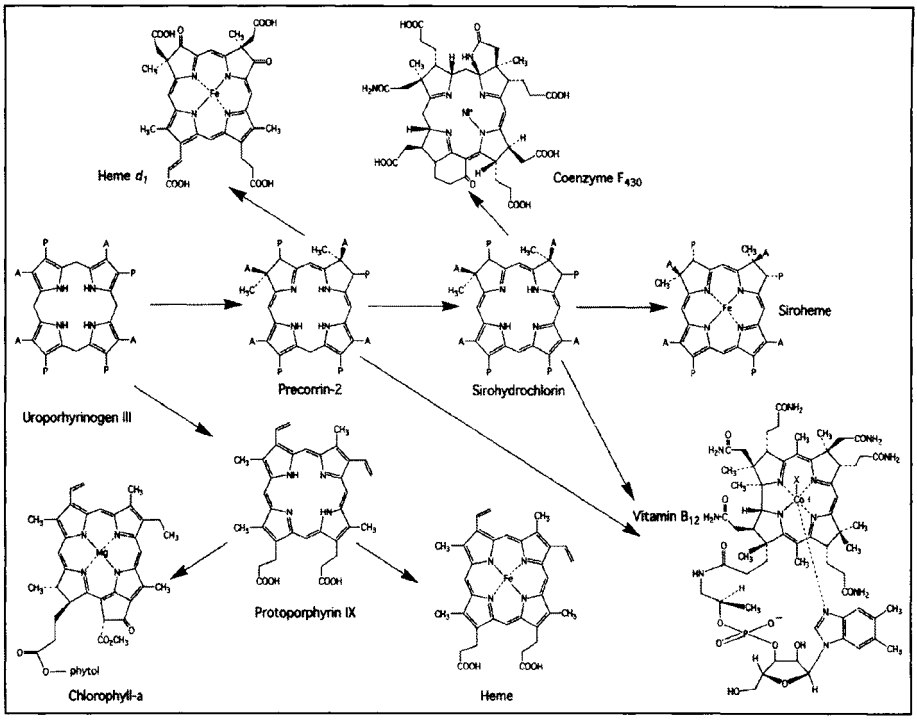

Figure 1. The branched tetrapyrrole biosynthetic pathway, highlighting how siroheme and coenzyme F_{430} are synthesised from uroporphyrinogen III via precorrin-2. (A = acetic acid side chain, P = propionic acid side chain).

bases (Fig. 2). The high level of sequence identity of the C-terminus with the cobalamin biosynthetic enzymes, and especially with CobA, the uroporphrinogen III methyltransferase that methylates at positions C2 and C7, strongly implied that *cysG* was able to catalyse a very similar reaction.[9] Indeed, it was very shortly shown that CysG did catalyse the transformation of uroporphyrinogen III into precorrin-2, by the addition of two S-adenosyl-L-methionine derived methyl groups to C2 and C7.[10] Strangely, the enzyme was able to catalyse the addition of a third methyl group to C12 to generate a trimethylpyrrocorphin.[11] However, this third methylation only occurred under conditions where a high concentration of enzyme was present, and is not thought to represent a physiological process.

Although these experiments demonstrated that CysG was able to synthesize precorrin-2, the dehydrogenase and ferrochelatase activities required to convert precorrin-2 into siroheme still had to be found. Significantly, though, no other mutants in siroheme deficiency had been located and it was thus suggested that at least one of these functions may be resident within the N-terminal region of CysG. In fact, gene dissection experiments reinforced this idea, as production of the C-terminal region of CysG gave a peptide that was still able to catalyse the synthesis of precorrin-2 from uroporphyrinogen III even though the corresponding truncated gene was not able to complement an *E. coli cysG* phenotype.[12] Moreover, within the N-terminal region of CysG a putative NAD^+ binding site (GXGXXA) was detected.[12] Finally, purified CysG was shown to catalyse the synthesis of siroheme not only by methylating uroporphyrinogen III, but also by performing an NAD^+-dependent dehydrogenation to generate sirohydrochlorin and a ferrochelation to give siroheme.[13] Thus, CysG was shown to be a multifunctional enzyme, whereby the C-terminal region of the protein is responsible for the methylation reactions and the N-terminal region is required for oxidation and metal insertion activities.

The structure of CysG was determined by X-ray diffraction studies after the *Salmonella enterica* protein had been overproduced and crystallised.[14] The protein was shown to exist as a homodimer

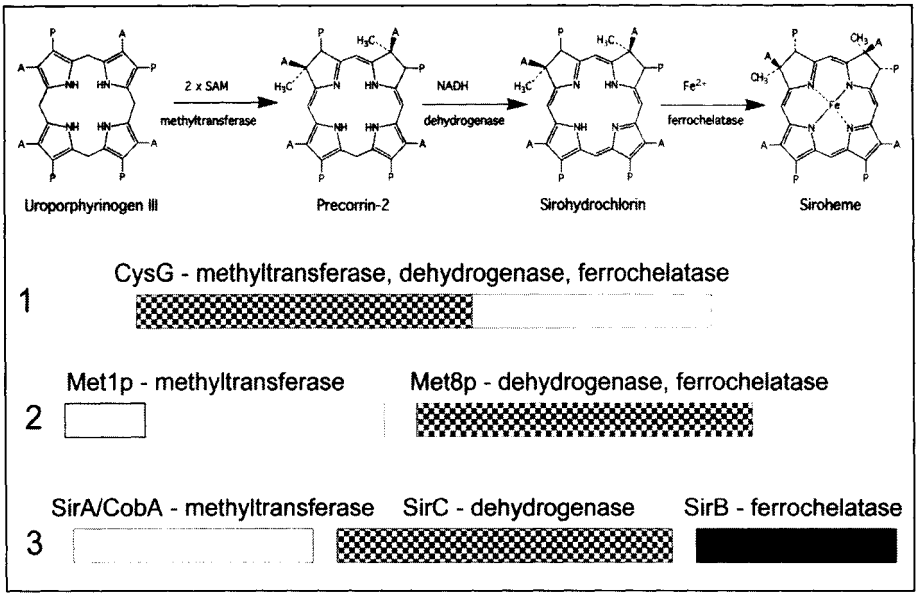

Figure 2. Synthesis of siroheme and the enzyme modules that are responsible for its synthesis. Siroheme is synthesised from uroporphyrinogen III in three steps. Initially, uorporphyrinogen III is methylated at positions 2 and 7 to yield precorrin-2. Two electrons and two protons are removed from preocrrin-2 to give sirohydrochlorin and finally sirohydrochlorin is chelated with ferrous iron to yield siroheme. Strategies to make siroheme: (1) In some enteric bacteria, siroheme is made from uroporphyrinogen III by the action of a single multifunctional enzyme called CysG. This enzyme is composed of a C-terminal methyltransferase region (diagonal shaded area) and an N-terminal dehydrogenase/chelatase region (checked area). (2) In yeast, siroheme is made by the action of two enzymes, one which carries out the methylations for the synthesis of precorrin-2 (Met1p - which displays similarity to the C-terminal region of CysG) and the other which performs the dehydrogenation and ferrochelation reactions (Met8p - and displays similarity to the N-terminal region of CysG). Finally, some bacteria have individual enzymes for each of the reactions required for siroheme synthesis. Thus, some bacteria have a separate uroporphyrinogen methyltransferase (SirA), a dehydrogenase (SirC) and a ferrochelatase (SirB).

with two structurally independent modules. The C-terminal region has a topology that is, as expected, similar to the class III methyltransferases of cobalamin biosynthesis.[14,15] The N-terminal region forms a large active site between a Rossmann fold domain and a smaller α/β domain.[14] This region of the protein contains one key catalytic residue, an aspartic acid. Although this region of the protein is able to catalyse both the dehydrogenation and chelation reactions within the one active site, it is not clear how this is achieved.[14]

The crystal structure of CysG revealed one more fascinating finding in that it was shown to be a phosphoprotein. An analysis of mutant variants of the serine residue that is post-translationally modified suggests that phosphorylation may prevent dehydrogenase activity.[14] This is significant since in many enteric bacteria, CysG acts as the source of precorrin-2 for cobalamin (vitamin B_{12}) biosynthesis as well as siroheme. Thus inactivation of the dehydrogenase activity would result in elevated precorrin-2 levels that would then be incorporated into cobalamin and represents a way that flux is controlled along a branched pathway.

In yeast, mutagenesis studies suggested that siroheme synthesis required two gene products, Met1p and Met8p (Fig. 2).[16] Sequencing of *MET1* revealed that that it encodes a protein product of 526 amino acids.[16] The first 325 amino acids of Met1p do not reveal similarity to any other protein, whereas the C-terminal 220 amino acids share a high degree of similarity with the uroporphyrinogen III methyltransferases, including the C-terminal region of CysG (Fig. 2).[16] No function for the N-terminal region of Met1p has been demonstrated but there is always the possibility that this part of the protein helps to control the activity of the enzyme. Met8p is a 274 amino acid protein that has

a high degree of similarity with the N-terminal region of CysG (Fig. 2), which is known to house the dehydrogenase and ferrochelatase activities.[17] As with CysG, Met8p also contains a putative nucleotide binding sequence in the form of a GXGXXG motif. On the basis of the sequence data, it thus appears that Met1p is a uroporphyrinogen III methyltransferase and Met8p is a precorrin-2 dehydrogenase and sirohydrochlorin ferrochelatase (Fig. 2). Indeed, yeast mutants in both *MET1* and *MET8* can be complemented by *cysG*.[16,17] Finally, the activity of Met8p was shown in vitro after the *MET8* gene was cloned and the encoded protein overproduced as a recombinant enzyme.[17] Met8p was also crystallised and its structure determined to 2.2 Å resolution.[18] The protein, as expected, has a similar topology to the N-terminal region of CysG.[14,18] Both the dehydrogenase and ferrochelatase activities are housed within a single active site, where Asp141 is thought to play an essential role in both catalytic processes.[18]

In the enteric bacteria and a range of other eubacteria, as mentioned above, siroheme is made by the action of the multifunctional enzyme CysG. However, in the bacilli, such as *Bacillus subtilis* and *Bacillus megaterium*, siroheme is synthesised by the action of three separate enzymes, which are encoded by *sirA*, *B* and *C* (Fig. 2).[19,20] These encode a uroporphyrinogen III methyltransferase (SirA, which is also known as CobA), a precorrin-2 dehydrogenase (SirC) and a sirohydrochlorin ferrochelatase (SirB) (Fig. 2). The SirA protein has a high level of sequence identity with other uroporphyrinogen III methyltransferases such as CobA and the C-terminal region of CysG (Fig. 2). SirC has sequence identity with Met8p and the N-terminal region of CysG (Fig. 2), although it has no ferrochelatase activity. It would seem likely that enzymes such as Met8p started off as straightforward dehydrogenases like SirC and then acquired chelatase activity. It is not apparent from the study of Met8p and SirC why Met8p is able to chelate metal ions into sirohydrochlorin. The final member of this bacterial siroheme pathway branch trilogy is SirB, an enzyme that has some sequence identity with the cobaltochelatases found in the anaerobic route of cobalamin (vitamin B_{12}) biosynthesis such as CbiX and CbiK.[21]

In higher plants, siroheme is made in the plastid. Uroporphyrinogen III methyltransferases have been described in both maize and *Arabidopsis* and their function determined.[22,23] The enzyme is made in the cytosol with an N-terminal extension that acts as a chloroplast transit peptide, and which is cleaved after translocation into the plastid. In maize the expression of the gene appeared to be coregulated with genes associated with nitrate assimilation, consistent with siroheme playing a role in nitrite reduction.[22] Although no precorrin-2 dehydrogenase has yet been identified in higher plants, the terminal enzyme of the siroheme pathway has recently been discovered in *Arabidopsis thaliana*.[24] The enzyme, a SirB homologue, is made as a precursor protein of 225 amino acids. The mature form of the enzyme consists of 150 amino acids, giving it a size of only about half that of the SirB orthologues found in the bacilli. In fact, the large SirB proteins appear to be made from a protein fusion of two such smaller gene products, presumably after a gene duplication event, as the N- and C-terminal region of the larger SirB proteins display a level of similarity to each other. Green fluorescent protein (GFP) tagging of the plant SirB confirmed that the protein is localised to the plastid. Surprisingly, the recombinant enzyme was found to contain a Fe_2-S_2 centre, which is very unstable. The Fe-S centre is not essential for activity and is likely to act as a redox sensor. Fe-S centres have been reported on some protoporphyrin ferrochelatases in both prokaryotes and eukaryotes, but the plant SirB is the only sirohydrochlorin ferrochelatase known to contain such a redox group. The Fe-S centre is housed in the C-terminal region of the *Arabidopsis* SirB.

Coenzyme F_{430} Biosynthesis

It has been estimated that methanogens generate about a trillion tons of methane gas per annum, of which about one third escapes into the atmosphere where it is photochemically converted into CO_2.[25-27] The concentration of methane in the atmosphere has been rising steadily over the past 300 hundred years, a highly significant finding since methane is about 50 times more potent than CO_2 as a greenhouse gas. The concentration of methane in the atmosphere is likely to increase further with the employment of intensive farming of crops such as rice and ruminant livestock. As a combustible gas, methane is also of commercial importance and through the employment of methanogens on the decomposition of organic material it represents a cheap source of energy. There are thus strong environmental and strategic arguments for a thorough understanding of the process of methanogenesis and this is covered in Chapter 23.

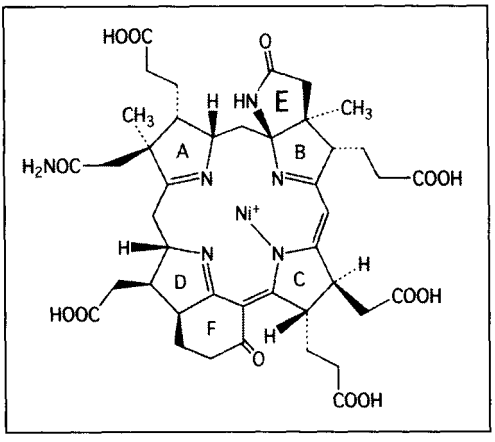

Figure 3. The structure of coenzyme F430 with the extra rings highlighted.

Methanogens are members of the *Archaea* that are highly specialised in terms of their biochemistry and are unique in being able to produce methane. Classical experiments by a number of eminent scientists have allowed much of the biochemistry of methane formation from C1 compounds such as CO_2, methanol, methylthiols and methylamines as well as C2 acetate to be elucidated.[27] Key to this process is the role of methyl-coenzyme M, which is the penultimate intermediate in methane production.[25] It is reduced by the enzyme methyl CoM reductase to give methane with the formation of a heterodisulphide between CoM and another coenzyme called coenzyme B. It is the reduction of this heterodisulphide that is coupled to ATP formation. The ability of methyl CoM reductase to catalyse the formation of methane lies in the use of a novel cofactor called coenzyme F_{430} (Fig. 3).[25] This is a modified tetrapyrrole with a centrally chelated nickel ion. Analogies with the role of the cobalt ion in vitamin B_{12} can be drawn with the change in oxidation state that nickel undergoes in the role of coenzyme F_{430}. In its active form the nickel ion at the centre of F_{430} is in a Ni(I) form, which is methylated by methyl CoM to generate a Ni(III)-CH_3 species, a transient unstable intermediate that readily reduces to Ni(II)-CH_3. This then spontaneously protonlyses to give CH_4 and Ni(II).[25]

Despite the indispensable role played by F_{430} in the process of methanogenesis and its global importance, little is known about how this remarkable cofactor is made.[26] As a modified tetrapyrrole, the synthesis of coenzyme F_{430} is based on the macrocyclic template design also observed in

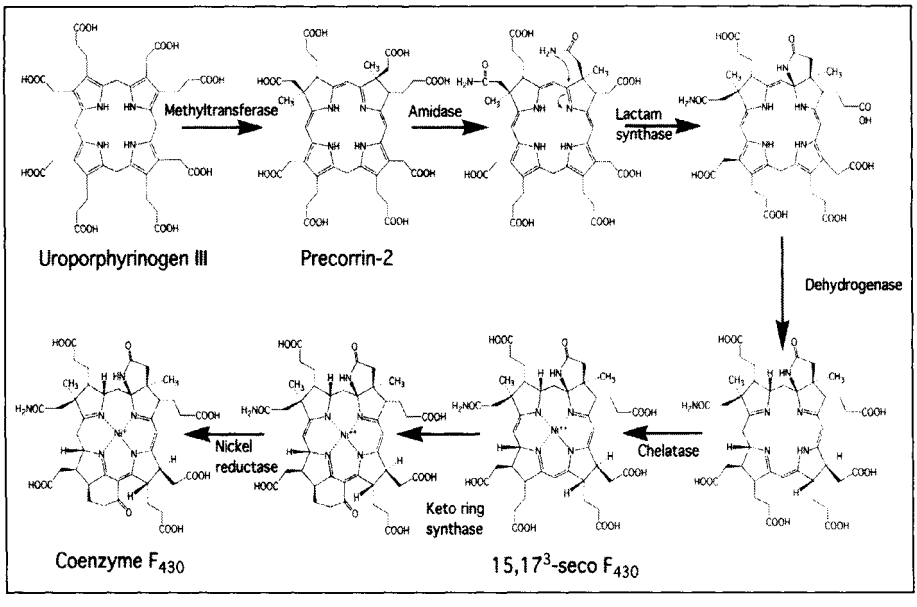

Figure 4. An outline of the biosynthesis of coenzyme F_{430} based on the synthesis from precorrin-2 and with late nickel insertion.

the hemes, chlorophylls, sirohemes, corrins and heme d_1.[26,28] However, it differs from these other modified tetrapyrroles in the nature of the centrally chelated metal ion and in the oxidation state of the macrocycle as it is a tetrahydroporphyrinogen and is therefore the most reduced member of the family. As well as the 4 pyrrole-derived rings found in all modified tetrapyrroles (labelled A-D; Fig. 3), F_{430} also contains two extra rings (E and F; Fig. 3). E is a lactam derived from the amidated acetic acid side chain attached to ring B whilst F is a keto ring derived from the propionic acid side chain on ring D. The two methyl groups found at positions 2 and 7 also suggest that F_{430} is derived from precorrin-2, an intermediate of the synthesis of cobalamin, siroheme and heme d_1. Indeed, labelling experiments clearly indicate that the biosynthesis of coenzyme F_{430} proceeds via either precorrin-2 or its oxidised relative sirohydrochlorin.[29] Moreover, under certain growth conditions, a $15,17^3$-*seco* intermediate of coenzyme F_{430}, missing ring F, can be isolated and converted into coenzyme F_{430} in the presence of ATP.[6] This is the only intermediate in the biosynthesis of F_{430} that has been isolated and characterised. Based on the observation that coenzyme F_{430} is derived from either precorrin-2 or sirohydrochlorin and proceeds via the *seco* intermediate, a biosynthetic pathway has been postulated and this is outlined in Figure 4.[26,30] There are likely to be between 6 and 8 enzymes required in the transformation of precorrin-2 into F_{430}. The steps include amidation, lactam synthesis, macrocyclic ring reduction, nickel insertion, nickel reduction, propionic acid side chain activation and ring F cyclisation.

Coenzyme F_{430} biosynthesis in a disrupted crude cell extract has been investigated more recently with the application of mass spectrometry to help in the identification of potential pathway intermediates.[31] Here, either uroporphyrinogen III or precorrin-2 was incubated with crude cell extracts from *Methanothermobacter thermoautotrophicus*. This involved the construction of a system for the rapid generation of both uroporphyrinogen III and precorrin-2, which was achieved by making plasmids encoding *hemB, C, D,* and *hemB, C, D, -cobA*. Despite the presence of these substrates in the crude cell incubation mixture, there was so significant increase in the production of coenzyme F_{430}. One reason to explain the lack of de novo synthesis of F_{430} could be the absence of a coenzyme or lack of appropriate conditions to allow relevant reactions to take place. Alternatively it may be due to the presence of a bottleneck in the pathway, but in all cases these possibilities would result in the accumulation of an earlier metabolite. A variety of methods were employed to determine the nature of any molecules that were accumulating, including an hplc method for F_{430} derivatives, isolated as their free acids. The use of this hplc method coupled to mass spectrometry provided a powerful tool for analysis of pathway intermediates. Incubation of uroporphyrinogen III and precorrin-2 with a cell free extract resulted in the isolation and identification of a range of compounds including sirohydrochlorin and nickel-sirohydrochlorin. A further compound with a mass of two units less that nickel sirohydrochlorin was also observed. It had a UV-visible spectrum similar to nickel-sirohydrochlorin although the retention time of this compound is considerably greater than nickel-sirohydrochlorin on a reverse phase column, indicating that the peripheral groups had undergone modification. Amidation of carboxyl peripheral groups of a tetrapyrrole is known to cause a dramatic increase in the retention time of a molecule. Consequently, amidation of nickel-sirohydrochlorin is the most likely cause of the shift in retention time. Mass spectral analysis of intermediates containing nickel is easy due to the isotope profile of the metal. A further compound was found in low abundance that contained nickel, with a m/z ratio of 949 that could represent a later intermediate. Based on these results it is possible that coenzyme F_{430} synthesis could proceed via sirohydrochlorin, nickel sirohydrochlorin and nickel sirohydrochlorin *a,c*-diamide as suggested in Figure 5. However, these intermediates could also be the result of mis-incoporation of nickel into the cobalamin biosynthetic pathway, which is also known to proceed via sirohydrochlorin, and thus the observed intermediates could be artifactual.

There is thus a great deal still to be learnt about coenzyme F_{430} biosynthesis, including the identification of the pathway intermediates and biosynthetic enzymes. With more archeal genomes becoming available and with the development of molecular tools for archeal genetic manipulation there is hope that some progress will be made on this very interesting biochemical pathway. What is clear from both coenzyme F_{430} and siroheme biosynthesis is that precorrin-2 plays a pivotal role as an intermediate and that this branch of the pathway is likely to represent the primordial route for modified tetrapyrrole synthesis.

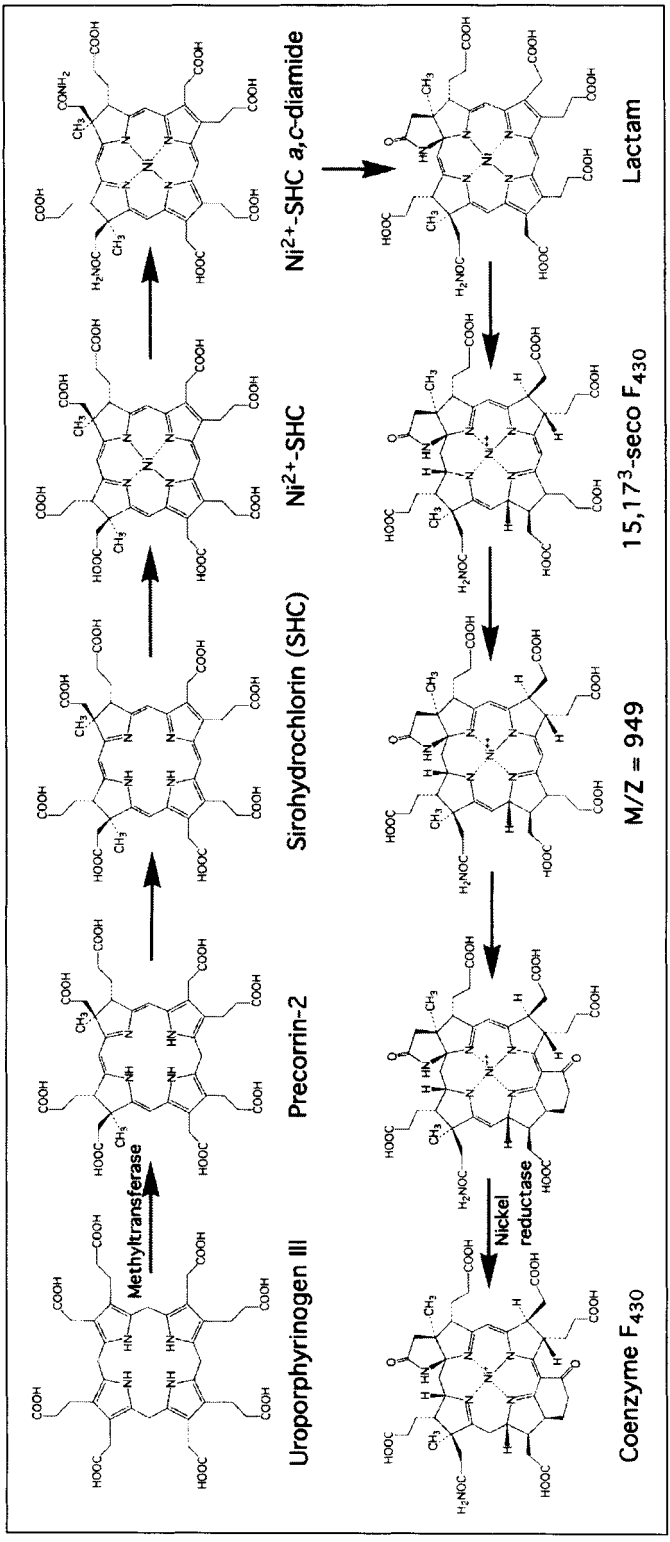

Figure 5. An alternative proposed biosynthesis of coenzyme F430, via sirohydrochlorin and with early nickel insertion.

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