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Review

Biosynthesis of cobalamin (vitamin B_{12}): a bacterial conundrum

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Abstract. The biosynthesis of cobalamin (vitamin B_{12}) is described, revealing how the concerted action of around 30 enzyme-mediated steps results in the synthesis of one of Nature's most structurally complex 'small molecules'. The plethora of genome sequences has meant that bacteria capable of cobalamin synthesis can be easily identified and their biosynthetic genes compared. Whereas only a few years ago cobalamin synthesis was thought to occur by one of two routes, there are apparently a number of variations on these two pathways, where the major differences seem to be concerned with the process of ring contraction. A comparison of what is currently known about these pathways is presented. Finally, the process of cobalt chelation is discussed and the structure/function of the cobalt chelatase associated with the oxygen-independent pathway (CbiK) is described.

Key words. Cobalamin; vitamin B_{12}; tetrapyrrole; precorrin; chelatase; pathway.

Background

Vitamin B_{12} is the 'anti-pernicious anaemia factor' that was first detected by Minot and Murphy in 1926 [1] in crude liver homogenates and subsequently purified in 1948 from liver and kidney [2, 3]. The structure of this deeply pigmented compound was famously solved by Dorothy Hodgkin in 1956 by X-ray crystallography and since then, much effort has been put into understanding its form and function. Vitamin B_{12} is found largely in one of two biological forms, either as adenosylcobalamin or methylcobalamin. Commercially, vitamin B_{12} is purchased as cyanocobalamin where the cyano group replaces the adenosyl or methyl group as a result of the extraction process. In a biological context, vitamin B_{12} acts as a coenzyme in a wide spectrum of metabolic processes, ranging from complex metabolic rearrangements and reductions such as those undertaken by methylmalonyl CoA mutase and the type II ribonucleotide reductases through to methylation processes as exemplified in methionine synthesis [4]. However, despite the versatility of the coenzyme in metabolism, the actual number of known B_{12}-dependent enzymes remains comparatively small and, therefore, most organisms require cobalamin in vanishingly small quantities. Humans require between 1–2 μg per day, which is ingested from our diet and is taken up by an elaborate absorption mechanism [5]. As will be highlighted later, cobalamin is made only by members of the Archea and certain eubacteria and it is to this biosynthesis that we will shortly turn our attention since it represents one of the most complex biosynthetic pathways known in Nature.

To place cobalamin biosynthesis in the correct context we must remember that the coenzyme forms of B_{12} are just part of a family of biological prosthetic groups,

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which, like cobalamin, adorn a diverse range of proteins, providing a wealth of colour and added functionality. The prosthetic group family are all tetrapyrrole derivatives, and include molecules such as haem, chlorophyll, sirohaem, haem *d1* and coenzyme F_{430} (fig. 1) [6]. The gem in the centre of these prosthetic groups is a metal ion, which varies from the emerald or magnesium ion in chlorophyll, the ruby or ferrous ion in haem to the amethyst or cobalt in cobalamin. Indeed, in the case of cobalamin, it is the natal chemistry of the cobalt-carbon bond that is key to its biological function Given the structural similarity between these molecules, it is not surprising to find that they are all synthesised along a branched biosynthetic pathway [7] (fig. 2). In organisms that synthesise up to four of these different modified tetrapyrroles, such as *Rhodobacter sphaeroides* [8] and *Pseudomonas aeruginosa* [9], over 50 separate proteins may be involved in the synthesis. Such complexity poses a number of interesting regulatory as well as evolutionary questions.

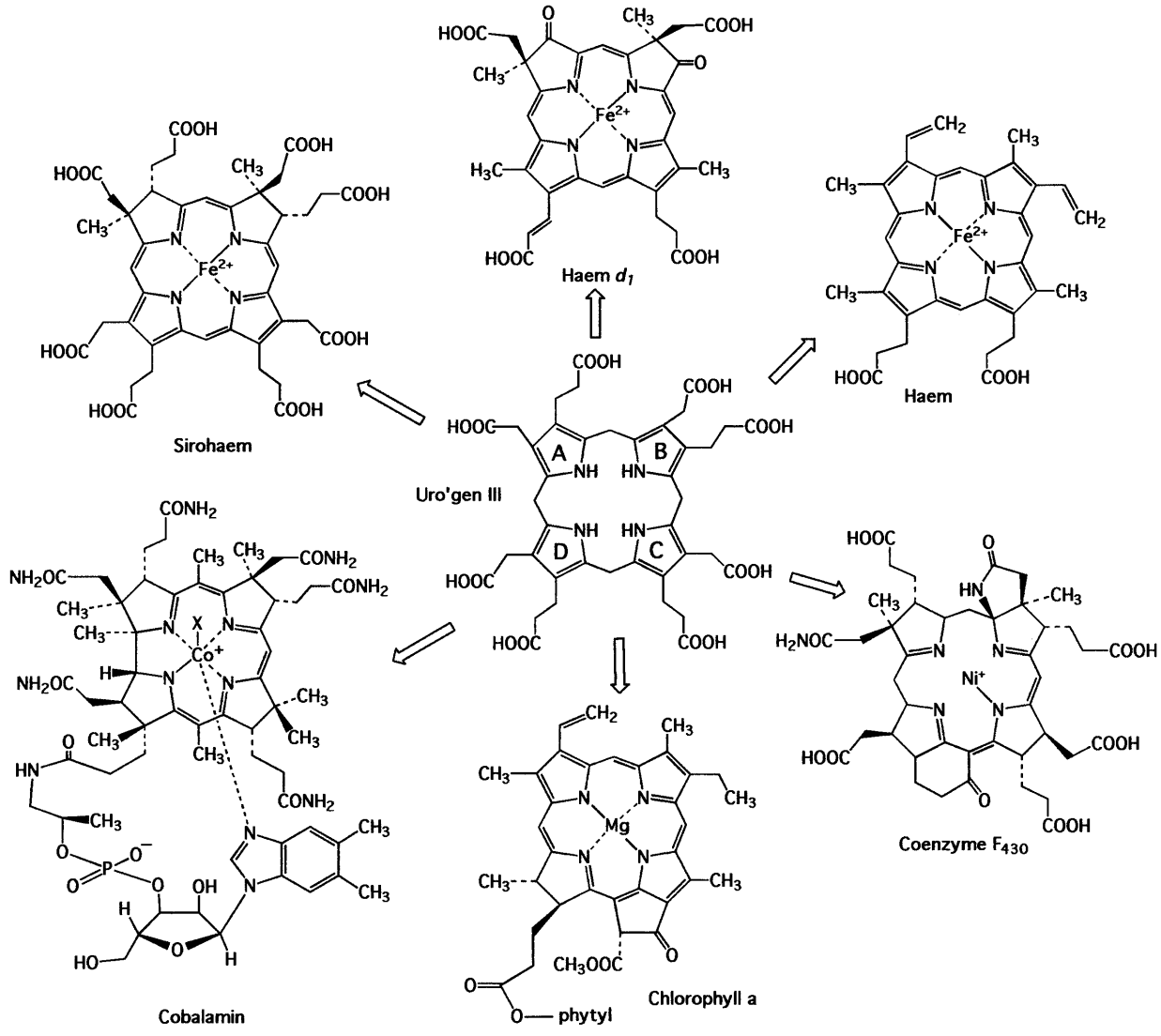


Figure 1. Modified tetrapyrroles. A range of modified macrocyclic tetrapyrroles that are found as metalloprosthetic groups in Nature, all of which are derived from the precursor uroporphyrinogen III (uro'gen III) by a branched biosynthetic pathway. Sirohaem is the prosthetic group found in assimilatory sulphite and nitrite redutases, haem *d1* is required in the denitrification of nitrate salts, haem is required in a range of electron transport systems and oxygen transport and is also a component of a range of enzymes including catalases and peroxidases, coenzyme F_{430} is required in the reduction of coenzyme M in methanogenesis, chlorophyll acts as the light-absorbing component of photosynthetic reaction centres and cobalamin acts as a coenzyme in a range of diverse rearrangement, methylation and reduction processes.

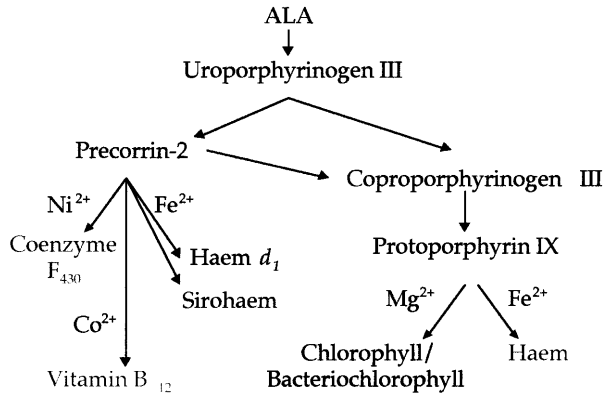


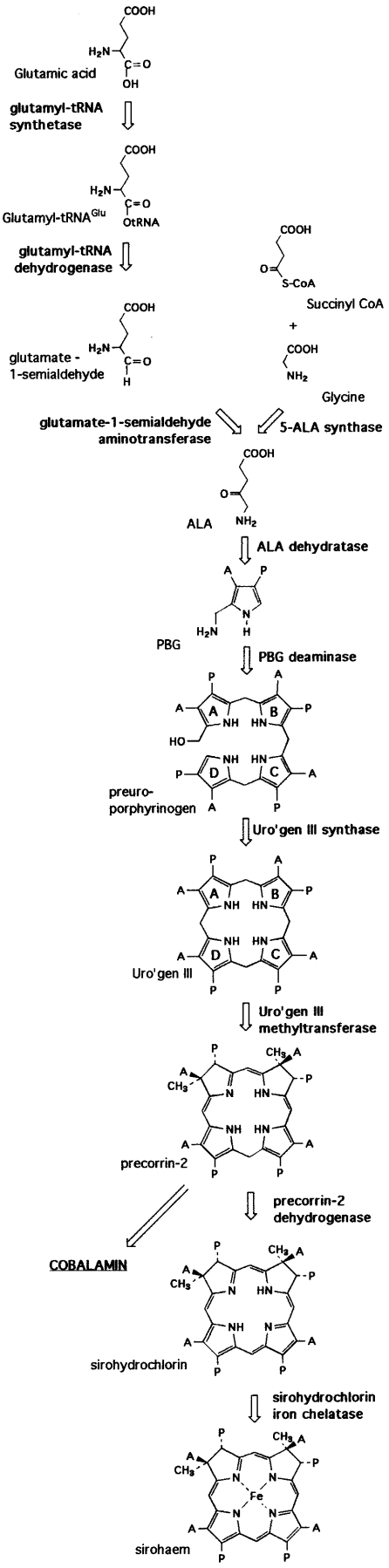
Figure 2. Branched biosynthesis of the modified tetrapyrroles. The branched biosynthesis of the modified tetrapyrroles is outlined, revealing how precorrin-2 acts as the last common intermediate for coenzyme F_{430}, vitamin B_{12} and sirohaem synthesis, while protoporphyrin acts as the last common intermediate in the synthesis of haem and chlorophyll. It is interesting to note that a pathway has recently been described for the synthesis of haem via precorrin-2, suggesting that the precorrin branch may represent a more evolutionary ancient arm of the pathway.

The tetrapyrrole component of the cobalamins is a corrin ring structure. This is a shrunken macrocycle in comparison to those found in the porphyrins and chlorins of haem and chlorophyll (fig. 1). This is due to the elimination of one of the methylene bridging carbons that links two of the pyrrole units within the structure [10], a process that occurs during its synthesis. At the centre of the corrin ring is a cobalt ion, which is ligated by the four pyrrole-derived nitrogens and by two other ligands. On the lower side, one of these bonds is mediated via the nitrogen of a modified base called dimethylbenzimidazole, which is attached to the corrin ring through a propionate side chain via an aminopropanol linker. The upper axial ligand is normally either the 5′ carbon of adenosyl (adenosylcobalamin) or a methyl group (methylcobalamin). The complexity of the structure is reflected in the biosynthesis of the coenzyme, which requires somewhere around 30 enzyme-mediated steps [11–13].

Early stages of tetrapyrrole biosynthesis

Tetrapyrrole synthesis is initiated by the synthesis of 5-aminolaevulinic acid (ALA), a comparatively stable amino ketone (fig. 2). This molecule is synthesised by one of two routes, either from the condensation of succinyl CoA and glycine or, more commonly, from the intact carbon skeleton of glutamic acid (fig. 3). The transformation of succinyl CoA and glycine into ALA is mediated by ALA synthase (EC 2.3.1.37), a pyridoxal-phosphate-dependent enzyme [7, 14]. The catalytic cycle of the enzyme and its stereochemistry have been well studied: glycine binds first to the enzyme cofactor and, after condensation with succinyl CoA, CoA, CO_2 and ALA are produced. The synthesis of ALA from glutamate is a more complex process, requiring three separate enzymes [15]. The first step in the so-called C-5 pathway is the charging of a glutamate-accepting tRNA ($tRNA^{Glu}$) with glutamate, a step identical to that observed in protein synthesis. The reaction is performed by glutamyl-tRNA synthetase (EC 6.1.1.17) and requires ATP, glutamate and $tRNA^{Glu}$. The next step is a unique reaction seen nowhere else in metabolism, the reduction of the aminoacylated-$tRNA^{Glu}$ to glutamate-1-semialdehyde (GSA). The enzyme that undertakes this reaction is a glutamate-tRNA dehydrogenase and requires NADPH as a coenzyme [16]. The final step in the synthesis of ALA from glutamate is a transamination reaction catalysed by the enzyme GSA aminotransferase (EC 5.4.3.8). The structure of this enzyme has recently been deduced through X-ray crystallography and was found to have a high degree of similarity with the amino acid transaminases [17].

The conversion of ALA into the first macrocyclic tetrapyrrolic structure is mediated by three enzymes common to all organisms that biosynthesise members of this family of compounds (fig. 3) [7]. The first of these enzymes is ALA dehydratase (ALAD) (EC 4.2.1.24), which catalyses a Knorr-type condensation reaction between two molecules of ALA to generate the pyrrolic building block porphobilinogen (PBG). The enzyme requires a metal ion for full activity. In yeast and mammals, zinc is essential for activity [18, 19], while in bacteria such as *P. aeruginosa* [20] and *B. japonicum* [21], magnesium is required. In other bacteria such as *Escherichia coli*, the enzyme is zinc dependent but is stimulated by magnesium [22]. The structure of the enzymes from yeast [23], *E. coli* [24] and *P. aeruginosa* [25] have all been solved by X-ray crystallography revealing a similar overall shape despite their different metal requirements. All the structures are based on a TIM barrel topology made up of eight identical subunits. The eight active sites point into the medium, with the yeast and *E. coli* structures harbouring a zinc ion bound through three cysteine residues in close proximity to the catalytic site. In contrast, the *P. aeruginosa* contains no bound metal at the equivalent position but does contain a magnesium ion at the subunit interface. A similar magnesium is found in the *E. coli* enzyme and it is likely that the ion has an allosteric effect in stimulating the enzyme [22]. No such magnesium stimulation is apparent with the yeast enzyme. Substrate-bound complexes have also been determined and ALA clearly binds to an active-site lysine forming a Schiff base [24, 26]. The roles of the remaining amino acids at the active



site remain to be confirmed, although site-directed mutagenesis coupled with structural studies is starting to allow greater insight into the individual roles played by these groups during the catalytic cycle of the enzyme.

The next enzyme in the pathway, PBG deaminase (PBGD; EC 4.3.1.8) [7], polymerises four molecules of PBG into a linear tetrapyrrole (fig. 3) with the concomitant release of four molecules of ammonia. PBGD is a monomeric enzyme containing a unique dipyrromethane cofactor [27, 28], made from two molecules of PBG [29]. The tetrapyrrole product is synthesised in an ordered, sequential fashion, by initial attachment of the first pyrrole unit (A) to the cofactor followed by subsequent addition of the remaining units (B-C-D) to the growing polypyrrole chain. Once all four units have been strung together, the link between the cofactor and the first pyrrole ring of the product is broken to yield the product, preuroporphyrinogen, and reactivated enzyme.

PBGD was the first enzyme of the pathway to have its atomic structure determined [30]. These studies revealed that the enzyme is folded into three distinct domains that enclose a single, large active site. There appears to be only one essential catalytic group within the active site, an aspartic acid residue that is thought to act as a general acid/base during the catalytic process [31]. The active-site cavity is lined with a number of arginine side chains which are thought to help stabilise the growing tetrapyrrole chain, with its associated negative charge, during the catalytic cycle of the enzyme [32, 33].

The final enzyme of tetrapyrrole synthesis is uroporphyrinogen III synthase (EC 4.2.1.75) [7]. This enzyme is responsible for inverting the final pyrrole unit (ring D) of the newly synthesised linear tetrapyrrole and for linking it to the first pyrrole unit (unit A), thereby generating a large macrocyclic structure called uroporphyrinogen III (fig. 3). Why Nature has opted for the type III isomer, especially since synthesis of the type I isomer would be more straightforward, is not clear. The type III isomer may represent a molecular memory of a prebiotic synthesis [34] or, since it is the only unsymmetrical isomer of uroporphyrinogen, may provide a molecular handle for the later enzymes of the pathway [35].

Figure 3. Biosynthesis of 5-aminolaevulinic acid (ALA), uroporphyrinogen III and sirohaem. The biosynthesis of ALA from either glutamate or succinyl CoA is outlined. Two molecules of ALA are condensed to form one molecule of porphobilinogen (PBG) and four molecules of the pyrrole (labelled A to D) are then attached in an ordered sequential fashion to form the linear tetrapyrrole preuroporphyrinogen. After ring inversion of the terminal pyrrole ring (D), the molecule is cyclised to given uroporphyrinogen III which after methylation, oxidation and chelation with ferrous iron gives rise to sirohaem, the simplest of all the modified tetrapyrroles.

Uroporphyrinogen III represents the first branch point of the pathway. The synthesis of haem and chlorophyll requires decarboxylation of the four acetate side chains by the enzyme uroporphyrinogen III decarboxylase to generate coproporphyrinogen III [7]. However, for cobalamin, sirohaem, coenzyme F_{430}, haem *d1* and, in certain organisms, haem synthesis the conversion of uroporphyrinogen III into precorrin-2 is the essential next step in the process [13]. This transformation is mediated by the enzyme uroporphyrinogen III methyltransferase (EC 2.1.1.107), which requires S-adenosyl-L-methionine (SAM) as a methyl donor [36, 37]. The enzyme methylates uroporphyrinogen III at position 2, generating precorrin-1, and after prototrophic tautomerisation methylates again at C-7. In the absence of a general acid or base, the enzyme catalyses the reaction by proximity, bringing the two substrates together at the active site to allow their inherent nucleo- and electrophilicity to initiate the reaction. The role of precorrin-2 in corrin synthesis as well as the methyltransferases associated with cobalamin synthesis will be discussed in more detail later.

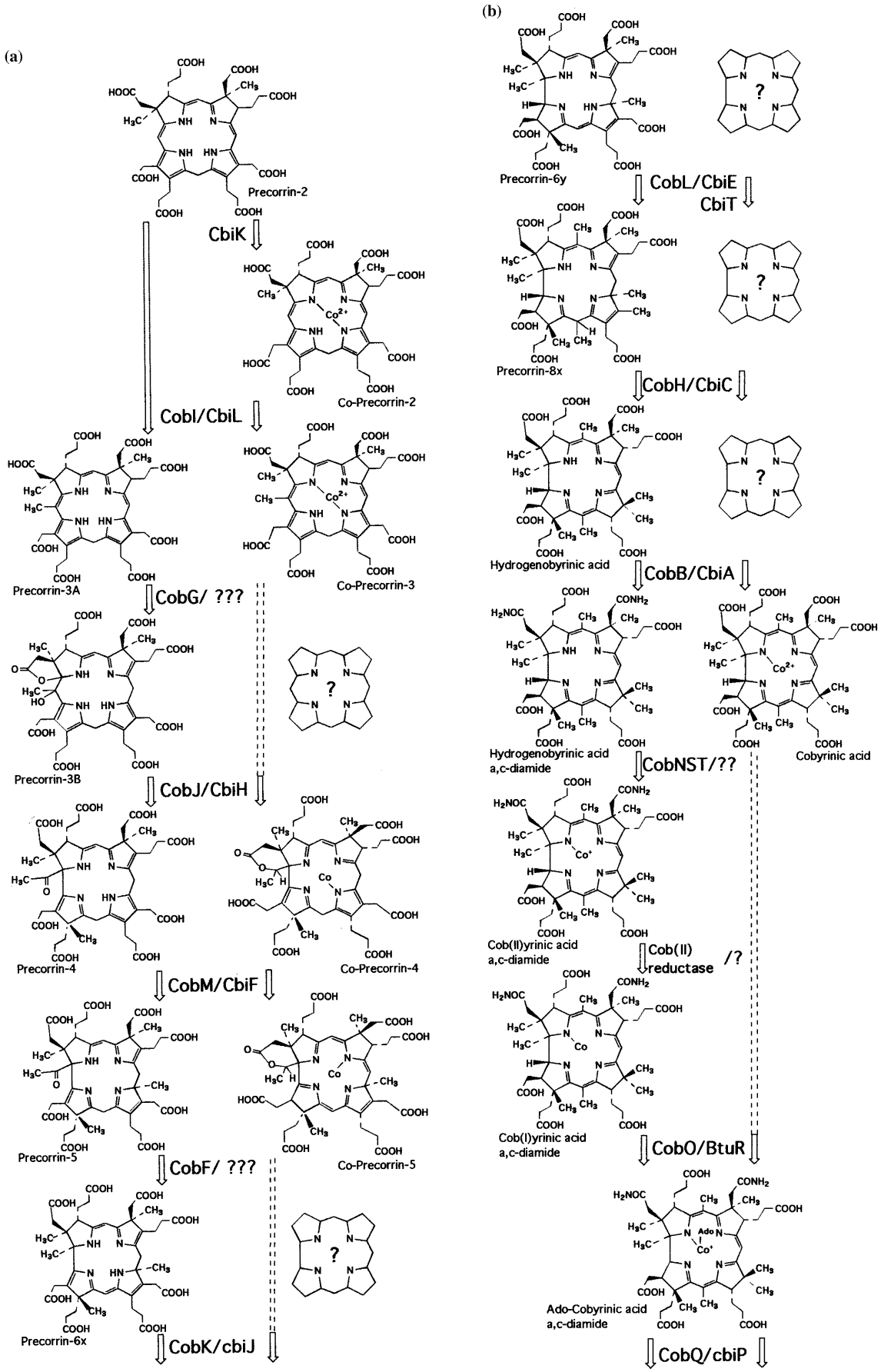
Little is known about the transformation of precorrin-2 into coenzyme F_{430} or haem *d1* but some progress has been made recently on the novel transmogrification of precorrin-2 into haem. This process requires decarboxylation of the acetate groups on rings C and D to generate 12,18-didecarboxyprecorrin-2, followed by elimination of the acetate side chains from rings A and B to generate coproporphyrinogen III (fig. 2) [38]. This remarkable series of reactions has so far only been described in *Desulfovibrio vulgaris* but provides a unique insight into what is likely to be a primitive route for the synthesis of porphyrins. This convoluted route for coproporphyrinogen III synthesis has been superseded by the more direct route of decarboxylation of uroporphyrinogen III.

For sirohaem synthesis, precorrin-2 is first oxidised to sirohydrochlorin and then chelated with ferrous iron to yield sirohaem (fig. 3) [39, 40]. Nature would appear to have evolved several different enzyme systems for this process. In *E. coli*, *Salmonella typhimurium* and *Neisseria meningitidis*, sirohaem synthesis from uroporphyrinogen III is mediated by a single multifunctional protein, sirohaem synthase or CysG. The C terminus of the enzyme contains uroporphyrinogen III methyltransferase activity while the N-terminal domain contains the dehydrogenase and chelatase functionalities [39]. In yeast, sirohaem synthesis from uroporphyrinogen III requires the action of two separate proteins, a uroporphyrinogen III methyltransferase encoded by MET1 and a dehydrogenase/chelatase encoded by MET8 [41]. In bacteria such as *Bacillus subtilis* and *Bacillus megaterium*, the transformation of uroporphyrinogen III into sirohaem requires three enzymes, YlnD, E and F [42; and unpublished results]. The uroporphyrinogen III methyltransferase activity is contained within YlnD, the dehydrogenase activity within YlnF and the chelatase within YlnE. There is little sequence similarity between YlnE, YlnF and either CysG or Met8p apart from some conservation around the putative NAD^+-binding site. Thus the synthesis of sirohaem can be mediated by a single multifunctional enzyme, by the action of two enzymes in tandem or by the concerted efforts of three independent enzymes. While on theoretical grounds, a multifunctional enzyme such as CysG could be argued to directly transfer intermediates from one active site to another, experimental evidence does not suggest that the metabolites are held within the confines of the enzyme [43]. Indeed, the lack of sequence similarity between the dehydrogenase/chelatase domains of CysG, YlnE/F and Met8p suggests that these proteins may have arisen by means of convergent evolution. However, as we discuss later, the lack of primary sequence similarity can be misleading.

Corrin synthesis

Cobalamin biosynthesis can be divided into three sections: the first deals with the synthesis of the corrin ring component, the second with the construction of the lower axial ligand and the third with the piecing together of the components to yield the final coenzyme. The genes required for the synthesis of cobalamin are also divided into three sections and are referred to as *cobI*, *cobII* and *cobIII* [12]. The *cobI* genes therefore encode the enzymes that transform uroporphyrinogen III into cobinamide, the *cobII* genes encode enzymes that synthesise the α-ribazole, while the *cobIII* genes encode enzymes that synthesise adenosylcobalamin. The genes for cobalamin biosynthesis were originally isolated from two main sources, *P. denitrificans* [11] and *S. typhimurium* [12]. Since then, the *cobI* genes from *B. megaterium* [44] have been reported and a host of other corrin biosynthetic genes have subsequently been identified on the basis of similarity from a range of other bacteria as a result of genome sequencing projects.

The complete step-by-step biosynthesis of adenosylcobalamin has so far only been described in one organism, *P. denitrificans*, an industrial strain which probably belongs to the alpha subgroup of the purple bacteria. The enzymes and intermediates involved in cobalamin biosynthesis were overproduced through the application of recombinant DNA technology allowing the scientists at Rhone-Poulenc, the world's largest industrial producer of vitamin B_{12}, and the laboratories of Prof. Sir Alan Battersby and Prof. Ian Scott to provide a final description of the pathway [11, 45–47]. The biosynthe-


(a)
Precorrin-2
CbiK
Co-Precorrin-2
CobI/CbiL
Precorrin-3A
Co-Precorrin-3
CobG/ ???
Precorrin-3B
CobJ/CbiH
Precorrin-4
Co-Precorrin-4
CobM/CbiF
Precorrin-5
Co-Precorrin-5
CobF/ ???
Precorrin-6x
CobK/cbiJ
(b)
Precorrin-6y
CobL/CbiE
CbiT
Precorrin-8x
CobH/CbiC
Hydrogenobyrinic acid
CobB/CbiA
Hydrogenobyrinic acid a,c-diamide
Cobyrinic acid
CobNST/??
Cob(II)yrinic acid a,c-diamide
Cob(II) reductase
/?
Cob(I)yrinic acid a,c-diamide
CobO/BtuR
Ado-Cobyrinic acid a,c-diamide
CobQ/cbiP

sis of cobalamin in *P. denitrificans* produced a number of surprises, notably that it requires molecular oxygen and that cobalt is added to the corrin ring at a comparatively late stage. Researchers quickly realised that there must be at least two routes for cobalamin synthesis since some bacteria, such as *S. typhimurium* and *P. shermanii*, were able to make cobalamin in the absence of oxygen, and cobalt was added early in the biosynthetic sequence [48, 49]. The cobalamin biosynthetic pathway from *P. denitrificans* is outlined in figure 4 and

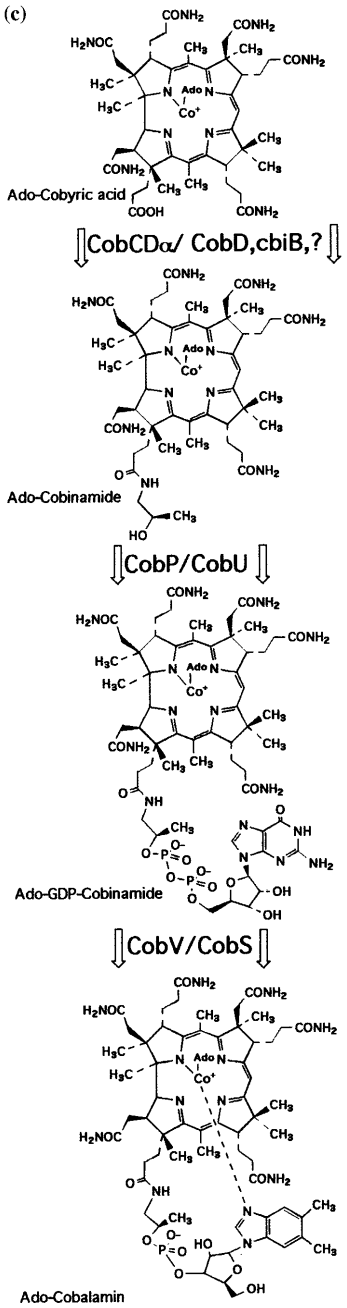


Figure 4. Comparative synthesis of cobalamin. The conversion of precorrin-2 into adenosylcobalamin is shown diagrammatically. The fully elucidated oxygen-dependent pathway (left-hand side) is compared with the structures that have been identified in the oxygen-independent pathway. The major differences occur around the point of ring contraction where the 'aerobic' process requires molecular oxygen, and also in the timing of cobalt insertion: the oxygen-dependent route requires the insertion of cobalt at a comparatively late stage while the oxygen-independent route inserts cobalt at the level of precorrin-2. The two pathways rejoin at the level of adenosylcobyric acid a,c-diamide. The enzymes required at the various stages are highlighted, with the oxygen-dependent enzymes given the prefix Cob and the oxygen-independent enzymes generally given the prefix Cbi.

Figure 5. Arrangement of cobalamin biosynthetic genes in a number of different eubacteria. Genes that are specific to the oxygen-dependent pathway are highlighted in blue, genes specific to the oxygen-independent pathway are highlighted in yellow and genes that are found in both pathways are highlighted in red. The strains marked with an asterisk are considered to represent the oxygen-dependent route and the genes are largely prefixed *cob*. The other strains are considered to contain the oxygen-independent route and their genes are prefixed *cbi*. No cobalamin biosynthetic genes are shown from members of the Archae, since their genes are not organised into operons and are generally dispersed individually throughout the genomes.

compared to what is known about the oxygen-independent route. The genes of the two pathways are given different prefixes which makes understanding the literature somewhat confusing. In general, the genes for corrin synthesis from the oxygen-independent pathway are given the prefix *cbi* while those from the oxygen-dependent pathway are prefixed *cob*. The letters following the prefix refer to the gene order within the cobalamin biosynthetic operons and not to the order in which the gene products are used in the biosynthetic pathway. The biosynthetic intermediates of corrin synthesis are generally referred to as precorrins, but are also given a number between 1 and 8, which refers to the number of methyl groups that have been added to the tetrapyrrole framework in accordance with the nomenclature described in Uzan et al. [50].

In the oxygen-independent pathway, the reaction that directs precorrin-2 solely towards corrin synthesis is the insertion of cobalt by CbiK to generate cobalt-precorrin-2 [51, 52]. This then acts as the substrate for the C-20 methyltransferase, CbiL. Interestingly, in *Clostridium difficile*, CbiK and CbiL are fused together as a multifunctional protein [53] (see fig. 5). In the oxygen-dependent route, methylation of precorrin-2 is mediated by CobI, which generates the corrin-committed intermediate precorrin-3a [54–56]. The ring contraction process in the oxygen-dependent pathway is then orchestrated by the action of CobG, a mono-oxygenase-containing an iron-sulphur centre [45, 57]. The enzyme generates a γ-lactone with the acetate side chain on ring A to give precorrin-3b. The intermediate next undergoes contraction during methylation at C-17 by CobJ, with the resulting extruded methylated C-20 fragment left attached as an acyl group at C-1 [45]. In the oxygen-independent route, the process of ring contraction remains poorly understood but CbiH, the C-17

methyltransferase, is known to use cobalt-precorrin-3 as a substrate to generate a tetramethylated δ-lactone [58]. However, this compound is probably not the true intermediate since it is not incorporated in high yields into cobalamin by cell-free extracts [59].

In the oxygen-dependent route, precorrin-4 is methylated at C-11 by the action of CobM to give precorrin-5 (fig. 4) [60]. The extruded acyl group is removed in the subsequent step, which also sees a methyl group added at C-1 in a reaction catalysed by CobF [60, 61]. The product of the reaction, precorrin-6a, is reduced by an NADPH-dependent reductase to give precorrin-6b [62, 63]. The next enzyme is a bifunctional protein in that it catalyses both the methylation of precorrin-6Y at positions 5 and 15 and also the decarboxylation of the acetate side chain located on ring C [64]. This generates precorrin-8, which is subsequently converted into hydrogenobyrinic acid by an amazing enzyme that moves the methyl group from C-11 to C-12 [65]. Hydrogenobyrinic acid now contains all the major framework alterations associated with corrin synthesis. For the oxygen-independent route, very little is known about the mechanism by which cobalt-precorrin-4 is converted into the cobalt-containing form of hydrogenobyrinic acid, cobyrinic acid. We can only guess which enzymes are involved, based largely on sequence similarity.

The conversion of hydrogenobyrinic acid into adenosylcobyric acid is mediated initially by CobB, which amidates the acetate side chains on rings A and B using glutamine as the amido donor (fig. 4) [66]. The product of the reaction, hydrogenobyrinic acid a,c-diamide, acts as the substrate for the cobalt chelatase complex, which is made from three polypeptide components, CobN, S and T [67]. This heterotrimeric enzyme inserts one molecule of Co^{2+} into the corrin ring structure but requires ATP. The resultant cob(II)yrinic acid a,c-diamide is reduced to the Cob(I) form by a flavin-requiring reductase [68]. After adenosylation by CobO [69], the remaining side chains are amidated by CobQ, which, like CobB, uses glutamine as an amido source [70]. While the conversion of cobyrinic acid into adenosylcobyric acid in the oxygen-independent pathway has not been studied ex vivo, it is likely to be undertaken by the homologues of the oxygen-dependent pathway, CbiA, P and BtuR.

The synthesis of adenosylcobyric acid represents the point at which the oxygen-dependent and -independent routes rejoin. Attachment of the aminopropanol side chain to the remaining acetate side chain found in ring D, and its subsequent connection to the α-ribazole generates adenosylcobalamin [11]. Synthesis of the nucleotide loop of cobalamin by the CobC, U, S and T enzymes from *S. typhimurium* has recently been demonstrated in vitro [71] and the crystal structures of CobU and T have also been reported [72–74].

The differences between the oxygen-dependent and -independent routes therefore largely relates to the use of molecular oxygen for ring contraction and the timing of cobalt insertion. The differences in the genetic requirements for these processes can often be used to identify which pathway an organism uses for corrin synthesis [13, 75]. Thus the presence of *cobG*, which encodes the mono-oxygenase, and *cobN*, *S*, *T*, which encode the heterotrimeric cobalt chelatase complex, can be used as indicators of the oxygen-dependent pathway. This pathway also uses another protein, encoded by *cobF*, that deacetylates the ring-contracted macrocyle and also methylates at C-1.

Conversely, in the oxygen-independent route, proteins encoded by *cbiD* and *G* are essential components of the pathway although their roles have not been described. The early cobalt chelation event is catalysed by an enzyme encoded by either *cbiK* or *X* [76]. On this basis, the type of pathway within any bacterial species may be predicted, although as we will describe shortly, the situation is more complicated than previously thought.

Genome studies

A quick study of current genome sequencing projects reveals that B_{12} biosynthetic genes are found in just over one-third of the bacteria sequenced so far. An example of the gene organisation in a selected variety of these bacteria is shown in figure 5. There is no genetic evidence that any eukaryote can make cobalamin de novo, an observation that makes cobalamins unique among the vitamins. The presence of B_{12} biosynthetic genes in the prokaryotes is difficult to interpret. For example, *S. typhimurium* can make B_{12} de novo yet *E. coli* is missing the entire CobI pathway [77, 78]. Similarly, *B. subtilis* [79] contains none of the B_{12} biosynthetic machinery yet *Bacillus stearothermophilus* [80] and *B. megaterium* [44] harbour some or all of the cobalamin biosynthetic genes. Another particularly interesting aberrant is observed with *Porphyromonas gingivalis*, an obligate anaerobe associated with gum disease, which appears to contain all the necessary genes to convert precorrin-2 into cobalamin yet does not possess the genetic software to encode the enzymes for precorrin-2 synthesis (fig. 5) [81; unpublished results].

The genome data also allow us to divide the bacteria into those that contain aerobic and anaerobic pathways. This can be done in one of two ways, either by looking for proteins specifically associated with each of the individual pathways as described above, e.g. CobG, CobF or CobN, or CbiD, G or X/K, or, alternatively, by undertaking a phylogenetic analysis of the methyltransferase genes found in the individual organisms,

since these appear to fall into classes that recognise cobalt-precorrins or cobalt-free precorrins [13]. Such analysis suggests that the oxygen-independent pathway is more prevalent but it also highlights a number of further interesting points. For example, *Rhodobacter capsulatus* has an aerobic-like pathway and indeed contains CobN and CobF. However, the bacterium does not harbour CobG (fig. 5) and we have shown that it can make cobalamin efficiently when grown aerobically or anaerobically [unpublished results]. Likewise, *P. aeruginosa* also contains an aerobic-like pathway with CobG and CobN, but it also contains CbiD and G (fig. 5), i.e. it contains the hallmarks of both the oxygen-dependent and -independent pathways. We have also demonstrated that this bacterium is able to make B_{12} de novo aerobically and anaerobically [unpublished results]. In both *R. capsulatus* and *P. aeruginosa*, the bacterium is likely to make cobalamin with cobalt-free precorrins. Both must have novel mechanisms for anaerobic ring contraction, differing from the mechanism outlined in this review. Clearly, a great deal still has to be learnt about B_{12} synthesis. In this respect, we believe it is easier to refer to the cobalamin biosynthetic pathways in terms of pathways utilising cobalt-containing and cobalt-free intermediates. A simple description of two pathways clearly does not cover all the variations that are observed. There are also differences within the oxygen-independent route. In *B. megaterium*, there is an absolute requirement for a precorrin-2 dehydrogenase, which is not required in the *S. typhimurium* pathway [41, 82]. This variation is probably related to differences in CbiD activity, since the *S. typhimurium* and *B. megaterium* CbiD are not interchangeable. Furthermore, in the Archae, cobalt chelatase has not been identified and the CobII pathway is different to that found in the eubacteria.

The genome sequencing data also reveal a number of gene fusions that give rise to multifunctional proteins (fig. 5). This was initially demonstrated with CobL of *P. denitrificans*, since this gene product not only methylates at positions 5 and 15 but also decarboxylates the acetate side chain on ring C. In *S. typhimurium*, the equivalent protein is found encoded by two separate gene products called *cbiE* and *T*. Interestingly, these genes are again fused in *B. megaterium*—*cbiET*. Other examples of gene fusion are observed with the uroporphyrinogen III synthase and the uroporphyrinogen III methyltransferase, *hemD-cobA* [42]. This multifunctional protein would allow the synthesis of precorrin-2 from preuroporphyrinogen within a single protein entity. Other multifunctional proteins that have been observed include fusions between *cbiK* and *L*, *cbiG* and *F*, *cbiJ* and *D*, and *cbiG* and *H*. Since these fusions appear to occur between consecutive enzymes, they suggest roles for *cbiG* and *D* around the ring contraction stage and C-1 methylation level, respectively.

Structural studies on the CobI enzymes

To date, the structures of only two CobI enzymes have been determined and both are from the oxygen-independent pathway. The first of these structures is the corrin biosynthetic methyltransferase, CbiF [83, 84]. In total, there are six methyl transferases required for corrin ring synthesis, which insert eight methyl groups as part of the peripheral decoration of the macrocycle (see figs 3, 4). All the methylation events require SAM as a methyl donor, which is converted to S-adenosyl-L-homocysteine (SAH) during the transferase reaction. SAH acts as a powerful feedback inhibitor of these biosynthetic methyltransferases, especially the uroporphyrinogen methyltransferases, and thus plays a role in the control and regulation of the pathway. The structure of CbiF, which

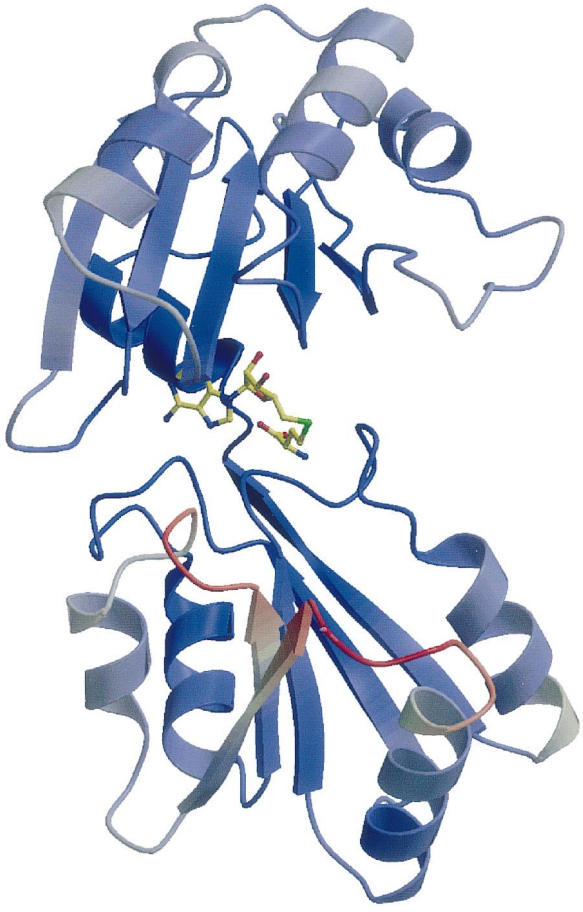


Figure 6. Structure diagram of CbiF coloured by temperature factor. Well-ordered regions of the structure are dark blue and more flexible regions are coloured red. SAH is bound to the active site and just below it is the substrate-binding cleft containing a flexible region, divergent in sequence among the individual transmethylases, which may be associated with the different substrate selectivities.

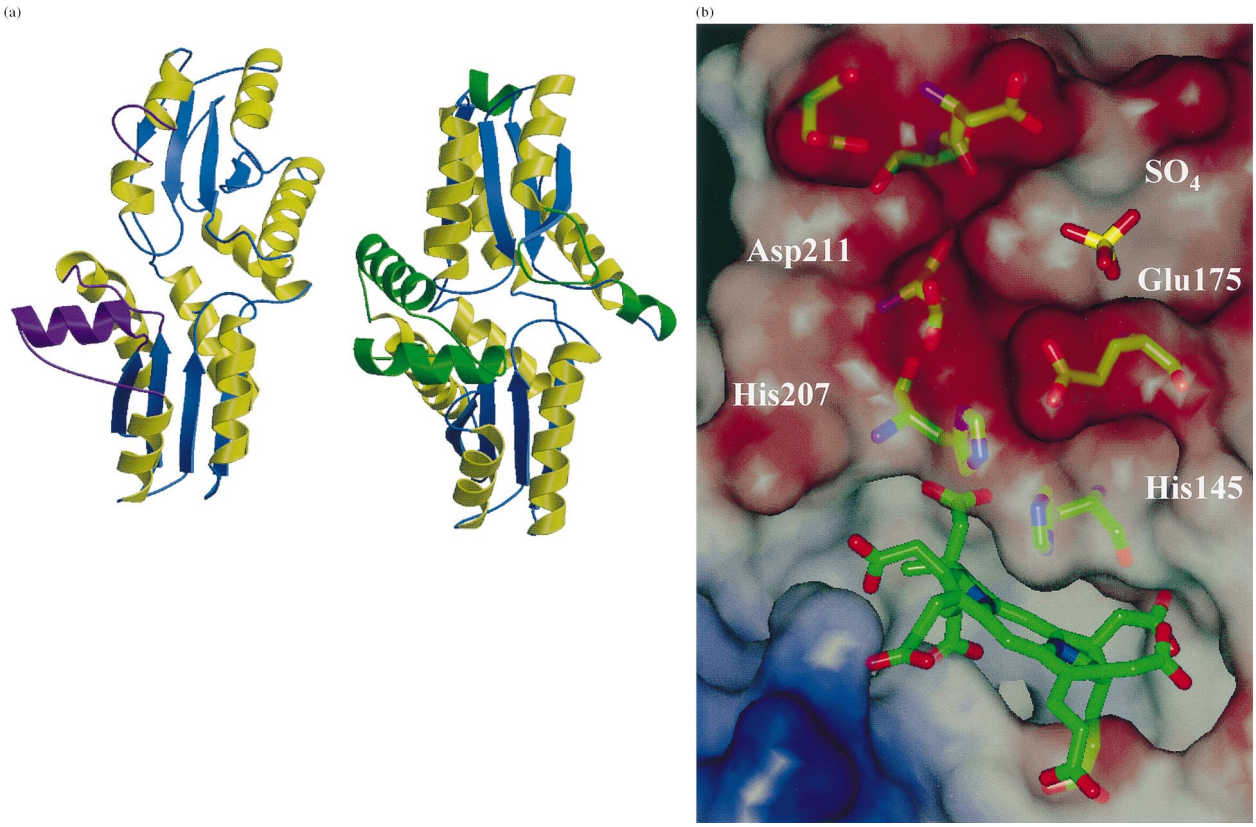


Figure 7. Structure and function of CbiK. (*a*) Comparison of the three-dimensional structures of the anaerobic cobalt chelatase, CbiK (left), and the protoporphyrin IX ferrochelatase (right). Despite a lack of any significant amino acid sequence similarity, the two structures are remote divergent homologues that have acquired insertions to the polypeptide chain in different topological locations but similar three-dimensional locations (purple in CbiK and green in protoporphyrin ferrochelatase). (*b*) Electrostatic surface and catalytic residues surrounding the active site. A precorrin-2 molecule has been modelled into the active-site cleft such that a cobalt ion, ligated through the two histidines (His145 and His207), could be catalytically transferred to the pyrrole nitrogen of the corrin ring. Asp211 and Glu175 are conserved amino acids that lie above the active site to form an anionic pocket. A sulphate ion from the crystallization conditions was identified in this region, and lies in an identical position to a bound magnesium ion in a substrate-bound complex of ferrochelatase [87]. These residues may contribute to the funnelling of a cobalt ion down to the active site and serve as a hydrogen-bonding network for removal of the protons abstracted from the pyrrole ring during catalysis. Glu89 lies below the modelled precorrin-2 in this picture.

catalyses the conversion of cobalt-precorrin-4 to cobalt-precorrin-5, was determined in the presence of SAH and reveals that the protein contains two domains, which hinge to form the active site [84] (fig. 6). SAH is seen bound in a contorted configuration which, if applied to SAM, may further favour the transfer of the methyl group to the precorrin substrate. A comparison of all the cobalamin biosynthetic methyltransferases reveals significant sequence similarity, suggesting that they have all evolved from a common ancestral enzyme and are therefore likely to adopt a similar overall topology. This structural insight into CbiF is providing a molecular perception of the evolution of this family of enzymes, where small changes in the primary structure have resulted in altered substrate specificity.

The second enzyme of the pathway whose structure was determined was the cobalt chelatase, CbiK. There are two very distinct enzymes associated with cobalt chelation. The enzyme found within the oxygen-dependent route is a heterotrimeric enzyme made from CobN, S and T, and is similar to the complex required for Mg^{2+} chelation within chlorophyll synthesis (ChlH, I, D) [85]. Both these heterotrimeric complexes require ATP for metal insertion, although once inserted, the metal is difficult to remove. In the oxygen-independent route, cobalt is inserted at the level of precorrin-2 by the enzyme CbiK. This enzyme, which was initially identified on the basis of functional complementation, has subsequently been overexpressed, purified and crystallised [51, 52]. The X-ray-derived structure of CbiK

reveals that the protein has a similar topology to the haem biosynthetic enzyme protoporphyrin ferrochelatase, HemH, despite a lack of sequence similarity [86] (fig. 7a). The structural similarity has implications for understanding the evolution of these separate branches of the tetrapyrrole pathway, where the same enzyme design has been utilised for the fulfilment of similar biological function.

CbiK is a bilobal enzyme containing two a/b domains, which generate an active site with a deep rectangular cleft at their interface. The active site contains a mixture of charged and neutral residues to interact with the charged substrate. The main catalytic groups are formed from two histidines, 145 and 207, and two glutamic acid residues, 89 and 175, as well as an aspartic acid residue, 211. Site-directed mutagenesis has shown that the two histidines are involved in metal binding and metal specificity, while the acidic acid residues contribute to a large charge disparity between the two domains of the enzyme. The enzyme functions as a chelatase by binding the precorrin-2 substrate in a puckered conformation such that the cobalt ion, held between histidines 145 and 207, is transferred to the waiting pyrrolic nitrogens of the tetrapyrrole (fig. 7b). This would likely induce a more linear conformation within the tetrapyrrole, favouring release of the cobalt-precorrin-2 from the active site.

The cobalamin biosynthetic pathway is one of the most complex biosynthetic pathways found in Nature, requiring around 30 enzyme-catalysed reactions for its complete de novo synthesis. The situation is made more complex by the appearance of several related though distinct variants of the pathway, which reflect differences in the way Nature has tackled the problem of ring contraction. Insights into these differences are being explored at the genetic, enzyme and structural levels. What is clear is that there is still much to be learnt about cobalamin biosynthesis in terms of chemistry, regulation and evolution. The many genome sequencing projects are providing a major resource for studying the majestic construction of cobalamin. Many questions concerning the biosynthesis still remain to be answered, including how cobalamin is made anaerobically, how its synthesis is controlled and regulated, and why the ability to make vitamin B_{12} de novo has not been transferred to eukaryotes.

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