Finding the final pieces of the vitamin B₁₂ biosynthetic jigsaw

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Ever since Dorothy Hodgkin solved the structure of vitamin B₁₂ some 50 years ago, researchers have been puzzling over how this amazing molecular jigsaw is pieced together. In essence, vitamin B₁₂ is a modified tetrapyrrole (corrinoid ring) to which is attached either an upper adenosyl or methyl group and a lower base, usually dimethylbenzimidazole (DMB) (Fig. 1). Although significant success has been achieved toward an understanding of the construction of the corrin ring component of the coenzyme, there has been a paucity of information concerning the biosynthesis of DMB. In a recent issue of PNAS, Campbell *et al.* (1) identified an enzyme that appears to play a key role in the transformation of flavin into DMB.

Vitamin B₁₂ is best known as the anti-pernicious anemia factor, which was first identified 80 years ago in crude liver extracts (2) before its subsequent purification in 1948 (3) and structure determination in 1955 (4). There are two features that make vitamin B₁₂ stand out in comparison with all other nutrients. First, it is made only by microbes (5, 6) (seemingly its biosynthetic software never made the prokaryotic to eukaryotic transition), and second, there is the sheer complexity of its synthesis, which requires ≈30 enzyme-mediated steps (7). The latter part of the 20th century saw a major effort to elucidate the biosynthesis of this remarkable cofactor. A multidisciplinary approach involving microbiology, genetics, recombinant DNA technology, NMR, and chemistry saw the dissection of this complex metabolic pathway (8–10). Of course, things were more complicated than anticipated. It was found that there were two distinct pathways for cobalamin biosynthesis, representing aerobic and anaerobic routes (11). Although the aerobic route for corrin ring assembly was elucidated, the secrets of the anaerobic process have yet to be determined. The attachment of the lower nucleotide loop to the corrin ring also has been well established (12). However, despite all this research, little information was forthcoming on the synthesis of the lower nucleotide base, DMB.

The majority of early work on the synthesis of the DMB moiety of vitamin B₁₂ was based on studies with the obli-

gate anaerobe *Eubacterium limosum* (13). Here, labeling revealed that the DMB framework was synthesized anaerobically from erythrose, glycine, formate, glutamine, and methionine. However, as with the synthesis of the corrin ring, it also was noted that some organisms synthesized DMB aerobically and required molecular oxygen to allow its synthesis (14). In the aerobic pathway, it was shown that riboflavin is transformed into DMB through FMN (15) (Fig. 1). This amazing transformation, for which no precedent exists in chemistry, sees the C-2 carbon of DMB derived from the C1 of the ribose moiety of FMN (16, 17). Significantly, however, no genes or enzymes involved in either the aerobic or anaerobic biosynthesis of DMB have been identified so far.

There appears to be a certain amount of serendipity in the isolation of Campbell *et al.*'s *bluB* strain.

Campbell *et al.* (1) were not actually trying to investigate B₁₂ biosynthesis. They were studying aspects of polysaccharide synthesis in Rhizobia, the soil bacteria that live in specialized root nodules of leguminous plants. As part of their symbiotic relationship with the plant, the bacteria produce fixed nitrogen in return for carbon compounds. During the invasion of the plant nodules, the bacteria produce an acidic exopolysaccharide, termed succinoglycan. They devised a rapid genetic screen for *Sinorhizobium meliloti* that allowed them to identify bacteria that were either deficient in the production of succinoglycan or able to accumulate this high-molecular-weight polymer. One of the variants from their screen that accumulated succinylglycan was found to be inactivated in a gene that displayed a high level of similarity to *bluB*, a gene previously identified as causing a vita-

min B₁₂ deficiency in the photosynthetic bacterium *Rhodobacter capsulatus* (18). Campbell *et al.* (1) demonstrate that their *S. meliloti bluB* mutant strain is also a B₁₂ auxotroph but, more specifically, that it is deficient in the biosynthesis of DMB. There appears to be a certain amount of serendipity in the isolation of their *bluB* strain, because there is no obvious connection between a requirement for B₁₂ and the production of succinylglycan. The *blu* prefix refers to the fact that the *blu* genes are required to make an aerobic culture of *R. capsulatus* bluish (i.e., produce photosynthetic pigments) after reduction of the pO₂. This earlier research demonstrated that a *bluB* strain could be corrected by the addition of vitamin B₁₂ but not by cobinamide (i.e., vitamin B₁₂ missing the lower base). This finding suggests two things: (i) that *bluB* is somehow involved in either the synthesis of DMB or its attachment to the corrin ring framework, and (ii) that the biosynthesis of vitamin B₁₂ somehow disturbs the biosynthesis of bacteriochlorophyll. In the case of the latter, strong evidence was subsequently presented to demonstrate that vitamin B₁₂ may well be required for the biosynthesis of the isocyclic ring of bacteriochlorophyll (19).

Perhaps we should not be surprised to find that *BluB* is involved in DMB biosynthesis, because evidence that *BluB* was involved in DMB biosynthesis was presented in a patent application, which claimed that overproduction of *BluB* led to raised levels of DMB (20). This finding is significant because DMB is normally limiting in the biosynthesis of vitamin B₁₂. Thus, before the work by Campbell *et al.* (1), there was evidence that *BluB* was required for the aerobic synthesis of the lower axial base. However, what Campbell *et al.* were able to do was to show that a mutant in *bluB* was able to grow in the presence of either vitamin B₁₂ or DMB but not in the presence of cobinamide. They also showed that *bluB* mutants accumulated GDP-cobinamide, the immediate precursor of vitamin B₁₂.

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Fig. 1. Transformation of FMN into DMB and its subsequent incorporation into vitamin B₁₂. The portion of FMN that is transformed into DMB is highlighted in blue. This transformation is likely to require BluB and molecular oxygen. The incorporation of DMB into vitamin B₁₂ requires the actions of three enzymes, CobT, C, and S.

Although the evidence is very strong in supporting a role for BluB in the biosynthesis of DMB, a note of caution has to be added. It had been suggested that CobT (NaMN:DMB phosphoribosyltransferase) synthesized DMB (21), although this hypothesis was subsequently shown not to be the case. Moreover, within the B₁₂ pathway, mutational inactivation of one gene sometimes can apparently affect other parts of the pathway. So, for instance, the activity of CbiD (a methyltransferase) requires the presence of the amidases CbiA and P (22). Thus, to confirm truly the role of BluB in the biosynthesis of DMB, direct

biochemical proof is required to demonstrate that it catalyzes a reaction associated with DMB formation. Because BluB belongs to the NADH/FMN oxidoreductase family, it is plausible that it could catalyze the oxygenation of FMN, a prerequisite to the fragmentation of the flavin. The abiotic conversion of FMN to DMB has been shown (16, 17), although, contrary to the suggestion in Campbell *et al.* (1), it was never claimed that that this abiotic process is the physiological source of DMB. Moreover, it is not clear whether the transformation of FMN into DMB will require one or several steps (Fig. 1). The chemistry of the

process suggests a retro-aldol condensation sandwiched between two 2-electron oxidations (13, 16). This number of steps seems too much to be catalyzed by a single (small, 15-kDa) enzyme. So are other enzymes involved, such as the poignantly named *bluF* gene (23)? And what does BluB actually do? Moreover, some organisms such as *Salmonella enterica* also make DMB from FMN but do not appear to have a *bluB* orthologue, so how do they catalyze this reaction? These are the really interesting questions that need to be addressed next as the enigmatic and furtive biosynthesis of cobalamin is further unraveled.

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