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MINIREVIEW

Fine-Tuning Our Knowledge of the Anaerobic Route to Cobalamin (Vitamin B_{12})[▽]

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Three recent papers (by Roessner et al. [12], Kajiwara et al. [5], and Santander et al. [14]) report the use of a combination of genetic engineering, enzymology, bio-organic chemistry, and high-resolution NMR spectroscopy to help fine-tune what is known of the anaerobic pathway to cobalamin. They present new experiments that firmly establish the structures of two intermediates of the pathway and confirm the functions of 5 more of the 13 *cbi* gene-encoded enzymes that contribute to the conversion of precorrin-2 to adenosyl-cobinamide (Fig. 1). (For the purpose of this minireview, the anaerobic pathway between uroporphyrinogen III and adenosyl-cobinamide involving the *cbi* gene products only is shown. For the two most recent reviews of cobalamin biosynthesis, please see references 16 and 19. The complete aerobic and anaerobic pathways from uroporphyrinogen III to adenosylcobalamin may be seen and downloaded at http://people.tamu.edu/~c-roessner.)

Roth et al. (13) first published in 1993 the sequence of a single large *cob* operon containing most of the genes for the anaerobic biosynthesis of cobalamin in *Salmonella enterica* serovar Typhimurium, including the 13 *cbi* genes that encode the enzymes necessary for the conversion of precorrin-2 to adenosyl-cobinamide (Cbi). The functions of CbiA, -B, -C, -D, -E, -T, -F, -G, -H, -J, -K, -L, and -P for this part of the anaerobic pathway were assigned, in large part, based on similarity to counterparts in the corresponding aerobic (oxygen-dependent) pathway of *Pseudomonas denitrificans*, which had been more extensively characterized (1, 2, 3, 7, 10, 17). However, experimental evidence existed for the functions of only 4 (CbiB, -H, -K, and -L) of the 13 catalytic Cbi proteins. (There are 17 *cbi*-encoded proteins in the operon, but CbiM, -N, -Q, and -O are believed to compose a cobalt transport system.) Biochemical evidence has shown that CbiK is a chelatase that inserts cobalt into precorrin-2 (9), that CbiL is the C-20 methyltransferase for the conversion of cobalt-precorrin-2 to cobalt-precorrin-3 (11), and that CbiH catalyzes methylation at C-17 of cobalt-precorrin-3, resulting in the formation of the ring-contracted, lactonized cobalt-precorrin-4 (15). (In some organisms, CbiK is replaced by either CysG or CbiX as the cobaltochelatase. There also may be some variation in the oxidation state of the di- and trimethylated intermediates, since factor II and factor III, the oxidized forms of precorrin-2 and precorrin-3, can also be used as substrates in the in vitro systems described in reference 5.) In addition, genetic evidence (8) has suggested that CbiB is involved in the coupling of the aminopropanol group to adenosyl-cobyric acid to form adenosyl-cobinamide, but the functions of the remaining nine Cbi enzymes between precorrin-2 and adenosyl-cobinamide have proved elusive. Especially intriguing are two enzymes, CbiD and CbiG, whose activities could not be predicted because of lack of similarity to any other proteins of known function. The three recent papers mentioned above now provide experimental evidence for the functions of CbiA, CbiD, CbiF, CbiG, and CbiT and reveal the structures of two new intermediates in the pathway, cobalt-precorrin-5A and -5B.

CbiF METHYLATES COBALT-PRECORRIN-4 TO FORM COBALT-PRECORRIN-5A

Roessner et al. predicted in 1992 (11) that CbiF (and, thus, CobM of the aerobic pathway) was the C-11 methyltransferase necessary for the transformation of cobalt-precorrin-4 to cobalt-precorrin-5 because of its ability to mismethylate precorrin-3 at C-11. However, the mismethylation activity of CbiF and the extraordinary sensitivity of the CbiF methylation products to oxidation (14) greatly complicated the isolation of intermediates subsequent to cobalt-precorrin-4. Kajiwara et al. (5) were able to synthesize, purify, and characterize cobalt-precorrin-5A (Fig. 1), the product resulting from the CbiF-catalyzed methylation of cobalt-precorrin-4 at C-11, by the careful elimination of oxygen not only from the in vitro incubations containing cobalt, precorrin-3, CbiH, and CbiF but also during the isolation procedures.

CbiG OPENS THE LACTONE RING AND DEACYLATES COBALT-PRECORRIN-5A TO AFFORD COBALT-PRECORRIN-5B

Unlike the equivalent precorrin-5 of the aerobic pathway, cobalt-precorrin-5A still carries the δ-lactone that formed as a consequence of C-17 methylation and ring contraction. The deacylated product, cobalt-precorrin-5B (Fig. 1), was observed by Kajiwara et al. (5) only when CbiG was included in the incubation mixture described above. Thus, the function of CbiG can now be assigned as catalyzing both the opening of the lactone ring and the extrusion of the two-carbon fragment (deacylation) derived from C-20 and its associated methyl

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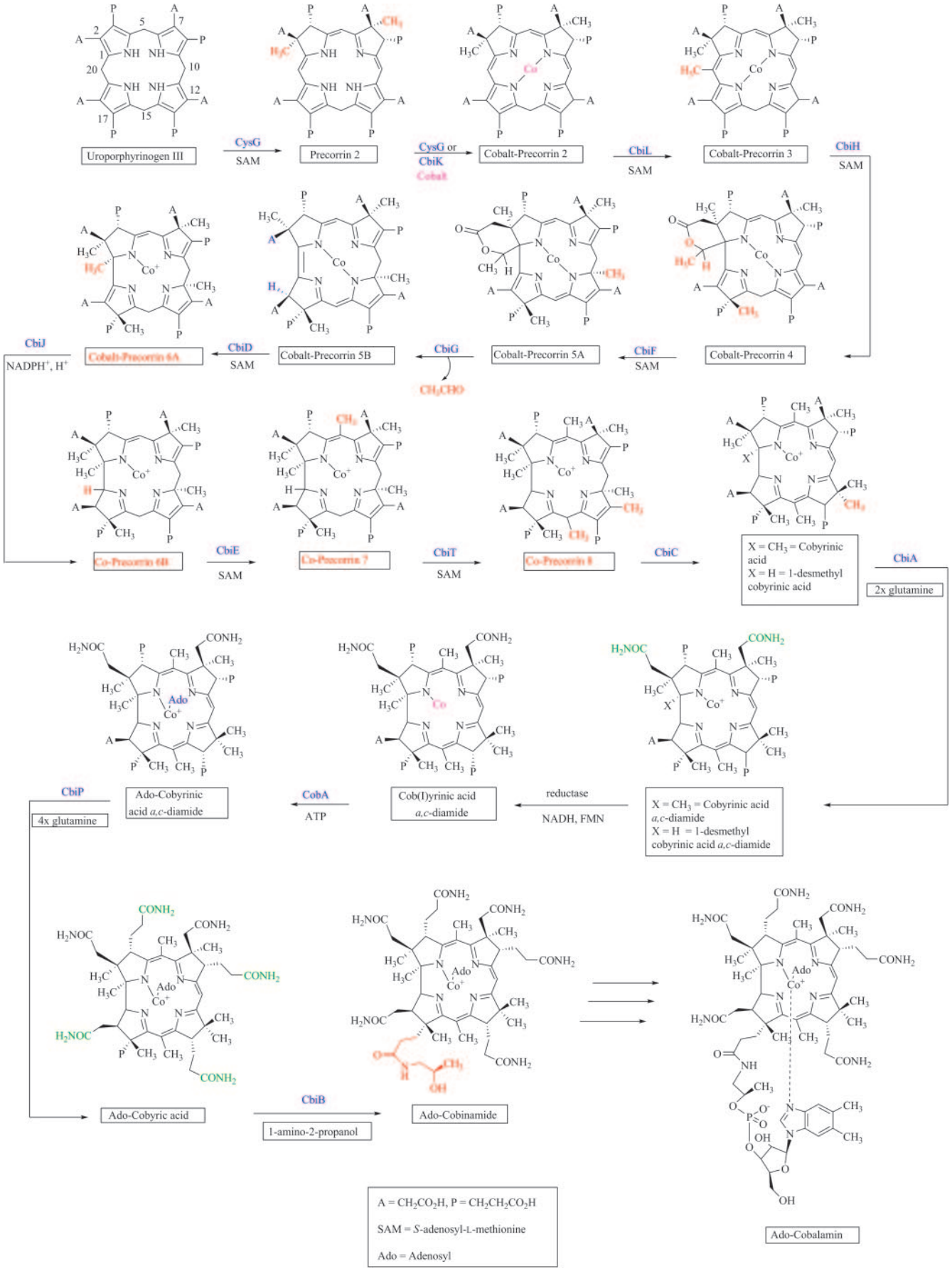
Uroporphyrinogen III
CysG
SAM
Precorrin 2
CysG or
CbiK
Cobalt
Cobalt-Precorrin 2
CbiL
SAM
Cobalt-Precorrin 3
CbiH
SAM
Cobalt-Precorrin 4
CbiF
SAM
Cobalt-Precorrin 5A
CbiG
CH_3CHO
Cobalt-Precorrin 5B
CbiD
SAM
Cobalt-Precorrin 6A
CbiJ
$NADPH^+$, H^+
Co-Precorrin 6B
CbiE
SAM
Co-Precorrin 7
CbiT
SAM
Co-Precorrin 8
CbiC
X = CH_3 = Cobyrinic acid
X = H = 1-desmethyl cobyrinic acid
CbiA
2x glutamine
X = CH_3 = Cobyrinic acid *a,c*-diamide
X = H = 1-desmethyl cobyrinic acid *a,c*-diamide
reductase
NADH, FMN
Cob(I)yrinic acid *a,c*-diamide
CobA
ATP
Ado-Cobyrinic acid *a,c*-diamide
CbiP
4x glutamine
Ado-Cobyric acid
CbiB
1-amino-2-propanol
Ado-Cobinamide
Ado-Cobalamin
A = CH_2CO_2H, P = $CH_2CH_2CO_2H$
SAM = *S*-adenosyl-L-methionine
Ado = Adenosyl

group. The acyl group has been shown to be eliminated as acetaldehyde (18).

The observation that CobE of the aerobic pathway (whose function is unknown) shows some similarity to the carboxyl terminal of CbiG suggests that it, too, may be involved in opening of the lactone ring or deacylation. CobE was not required for the in vitro biosynthesis of precorrin-6A (10), but the deacylation of precorrin-5 was the least efficient step in the multienzyme synthesis of precorrin-6A. It may be that CobE facilitates this process.

CbiD IS NECESSARY FOR C-1 METHYLATION

In the aerobic pathway, deacylation is concomitant with C-1 methylation, catalyzed by CobF. The anaerobic pathway has no methyltransferase similar to CobF, but the paper by Roessner et al. (12) provides evidence that CbiD is required for C-1 methylation and thus may be a nonorthologous methyltransferase that takes the place of CobF in the anaerobic pathway. In this work, a strain of *Escherichia coli* was genetically engineered to contain the 12 *S. enterica* genes believed to be required for the biosynthesis of cobyric acid (all of the *cbi* genes except *cbiB*). This strain accumulated cobyrinic acid *a*,*c*-diamide (Fig. 1). However, a mutant of the strain constructed to lack only the *cbiD* gene accumulated a similar product that was still protonated, rather than methylated, at the C-1 position (1-desmethyl-cobyrinic acid *a*,*c*-diamide) (Fig. 1). Even though the cobalt-precorrin-6A intermediate has yet to be isolated, and there is some mystery as to why the presence of the two amidating enzymes, CbiA and CbiP, was necessary for C-1 methylation, the engineered system has provided the first solid evidence that CbiD is required. CbiD has a potential *S*-adenosyl-L-methionine binding site and so is probably the actual methyltransferase, but it could also work in conjunction with one of the other methyltransferases, e.g., CbiF. The structure of CbiD from *Archaeoglobus fulgidus* has been determined but provided no clues to its activity (unpublished information available from the Protein Data Bank website, http://www.rcsb.org/pdb/cgi/explore.cgi?pdbId=1SR8).

CbiT IS BOTH A C-15 METHYLTRANSFERASE AND A DECARBOXYLASE

In the aerobic pathway, methylation at C-5 and C-15 and decarboxylation of the C-12 acetate side chain of precorrin-6B are catalyzed by a single enzyme, CobL. In *S. enterica*, however, CobL is split into two separate enzymes, CbiE and CbiT. Because of the similarity of CbiE to other methyltransferases of the B_{12} pathway, it has long been assumed that CbiE is the methyltransferase that catalyzes the addition of the two methyl groups to cobalt-precorrin-6B and that CbiT then decarboxylates the C-12 acetate side chain to afford cobalt-precorrin 8. However, the structure of CbiT (6) and its similarity to methyltransferases not of the B_{12} pathway have led to the suggestion that it, too, might be a methyltransferase. The paper by Santander et al. (14) provides the first experimental evidence that CbiT alone can catalyze both methylation at C-15 and decarboxylation of the C-12 acetate side chain, even in the absence of C-1 methylation. The products synthesized from cobalt-precorrin-3 in the presence of CbiF, CbiG, CbiH, and CbiT were methylated at C-15 or were methylated at C-15 and decarboxylated, but they were never decarboxylated without C-15 methylation. This result suggests that CbiT catalyzes C-15 methylation first, followed by decarboxylation. In the absence of CbiT, neither methylation at C-15 nor decarboxylation was observed. If CbiT is the C-15 methyltransferase, then CbiE remains as the C-5 methyltransferase. Figure 1 depicts the CbiE-catalyzed methylation at C-5 as occurring before the action of CbiT, but this order needs to be confirmed.

TABLE 1. Functions of the *S. enterica cbi* gene products involved in the conversion of precorrin-2 to cobinamide

Enzyme	Function
CbiMNQO[a]	Cobalt transport
	Precorrin-2
CbiK	↓ Cobalt insertion
	Cobalt-precorrin-2
CbiL	↓ Methylation at C-20
	Cobalt-precorrin-3
CbiH	↓ Ring contraction
	↓ Lactone formation
	Cobalt-precorrin-4
CbiF	↓ C-11 methylation
	Cobalt-precorrin-5A
CbiG	↓ Lactone opening
	↓ Acetaldehyde extrusion
	Cobalt-precorrin-5B
CbiD	↓ C-1 methylation
	Cobalt-precorrin-6
CbiJ[a]	↓ C-18–C-19 reduction
	Cobalt-dihydro-precorrin-6
CbiE[a]	↓ C-5 methylation
	Cobalt-precorrin-7
CbiT	↓ C-15 methylation
	↓ Decarboxylation
	Cobalt-precorrin-8
CbiC	↓ Methyl rearrangement
	Cobyrinic acid
CbiA	↓ *a*,*c*-Amidation
	Cob(II)yrinic acid *a*,*c*-diamide
Unknown	↓ Cobalt reduction
	Cob(I)yrinic acid *a*,*c*-diamide
CobA	↓ Adenosylation
	Ado-cob(I)yrinic acid *a*,*c*-diamide
CbiP[a]	↓ *b*,*d*,*e*,*g*-Amidation
	Ado-cobyric acid
CbiB	↓ Aminopropanol attachment
	Ado-cobinamide

[a] Experimental confirmation not available.

FIG. 1. The anaerobic pathway from uroporphyrinogen III to adenosyl-cobinamide in *S. enterica*. The structures for the cobalt-precorrin-6A, -6B, -7, and -8 (labels shown in red) are tentative, based on our knowledge of the corresponding cobalt-free intermediates of the aerobic pathway.

CbiA IS THE *a* AND *c* SIDE-CHAIN AMIDASE

The paper by Roessner et al. (12) reported a genetically engineered strain of *E. coli* containing 10 *cbi* genes (all *cbi* genes except *cbiA*, *cbiB*, and *cbiP*) that accumulated 1-desmethyl-cobyrinic acid. The addition of the *cbiA* gene to this strain resulted in the accumulation of a bisamidated product (1-desmethyl-cobyrinic acid *a*,*c*-diamide) (Fig. 1), showing that CbiA is responsible for amidation of the two side chains. In addition, CbiA from *S. enterica* has been overexpressed, and its mechanism of action has been studied in detail (4), providing conclusive evidence that it is the *a*,*c*-amidase.

CONCLUDING REMARKS

Experimental evidence is now in hand for the functions of 9 (CbiA, -B, -D, -T, -F, -G, -H, -K, and -L) of the 13 Cbi enzymes. The confirmation of the predicted activities of CbiC, CbiE, CbiJ, and CbiP and the biosynthesis and confirmation of the structures of cobalt-precorrin-6A, -6B, -7, and -8 remain among the final challenges (along with the synthesis of dimethylbenzimidazole and attachment of the lower ligand) in determining the anaerobic pathway to cobalamin. CbiJ has been assigned the function of the reduction of cobalt-precorrin-6A to cobalt-precorrin-6B (Fig. 1) based on its similarity to CobK, which catalyzes the reduction of precorrin-6A to precorrin-6B in the aerobic pathway and, as described above, CbiE is the most likely candidate for the C-5 methyltransferase for the conversion of cobalt-precorrin-6B to cobalt-precorrin-7. CbiT probably catalyzes the conversion of cobalt-precorrin-7 to cobalt-precorrin-8, and catalysis of the conversion of cobalt-precorrin-8 to cobyrinic acid (Fig. 1) has been assigned to CbiC based on its similarity to CobH of the aerobic pathway. In addition, CobH can substitute for CbiC in a genetically engineered strain of *E. coli* (12) that synthesizes 1-desmethyl-cobyrinic acid (C. A. Roessner, unpublished results), confirming its function as the precorrin-8 methylmutase. CbiP has been assigned the function of amidation of the *b*, *d*, *e*, and *g* side chains based on its similarity to CbiA and CobQ, which performs the same function in the aerobic pathway. The functions of the Cbi enzymes are summarized in Table 1.

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