Volume 301, number 1, 73–78 FEBS 10919 April 1992

Expression of 9 *Salmonella typhimurium* enzymes for cobinamide synthesis

Identification of the 11-methyl and 20-methyl transferases of corrin biosynthesis

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Received 18 February 1992

Nine of the *cbi* genes from the 17.5 kb *cob* operon of *Salmonella typhimurium* previously shown by genetic studies to be involved in the biosynthesis of cobinamide from precorrin-2, have been subcloned and expressed in *Escherichia coli*. Seven of the gene products were found in the soluble fraction of cell lysates and have been purified. The gene products corresponding to *cbi E, F, H* and *L* were shown by SAM binding and by homology with other SAM-binding proteins to be candidates for the methyltransferases of vitamin B_{12} biosynthesis. The enzymatic functions of the gene products of *cbiL* and *cbiF* are associated with C-methylation at C-20 of precorrin-2 and C-11 of precorrin-3.

Precorrin methyltransferase; ^{13}C-NMR; Corrin biosynthesis; *Salmonella typhimurium*

1. INTRODUCTION

Recent advances in the molecular biology and biochemistry of vitamin B_{12} biosynthesis [1–13] have led to the isolation of several enzymes responsible for the synthesis of isolable intermediates between uroporphyrinogen III and hydrogenobyrinic acid. Thus, transformation of a vitamin B_{12} production strain of *Pseudomonas denitrificans* with a plasmid bearing eight genes (*cobF–M*) has resulted in the isolation of several enzymes for the synthesis of hydrogenobyrinic acid. The description of the genetics of vitamin B_{12} synthesis in *Salmonella typhimurium* [10,11] and of the nucleotide sequence [Dr. John Roth, University of Utah, personal communication] of plasmids bearing the genes of the 17.5 kb *cob* operon, including the CobI region (*cbiA-O* genes) involved in cobalamin synthesis, in this organism has opened the door to the expression and isolation of the enzymes homologous to those of *P. denitrificans*. Amino acid sequence comparisons of the gene products predicted from the nucleotide sequence of the *P. denitrificans* and *S. typhimurium* genes revealed homology between: *cobH* and *cbiC*; *cobI* and *cbiL*; *cobJ* and *cbiH*; *cobK* and *cbiJ*; *cobL* and *cbiE + T*; and the *cobM* and *cbiF* gene products. No homologs were found for the *cobF, cobG, cbiD*, or *cbiG* gene products.

The *P. denitrificans* enzymes responsible for reduction of precorrin 6x to 6y (*cobK*), the bis-methylation and decarboxylation of 6y to 8x (*cobL*), and the rearrangement of the latter to hydrogenobyrinic acid (*cobH*) have recently been described [7–9] (see Scheme 1). The remaining genes (*cobF, G, J, M*) most likely encode enzymes responsible for the synthesis of precorrin-6x from precorrin-3, a process which must involve several labile intermediates derived from SAM-dependent C-methylations at C-1, C11, C-17, and ring contraction/deacylation (from C-20 and its attached methyl). None of these intermediates has been isolated from lysates of the recombinant *P. denitrificans* strain and are presumably destroyed by the procedures used for the isolation of precorrin 6x (oxidation/esterification). Since the structures of these labile intermediates and the function of the enzymes required for their synthesis can only be determined by procedures which allow direct observation of the enzymatic products, such as NMR spectroscopy, we have recently developed a protocol combining genetically engineered enzymes for the synthesis of precorrins-2 and -3 with ^{13}C NMR spectroscopy to assign the structures of these key intermediates [12,13]. As a logical extension of these studies, we now present evidence that the *S. typhimurium cbiL* gene encodes precorrin-3 synthase and, further, that the *cbiF* gene encodes the SAM-dependent methyltransferase responsible for C-methylation at C-11 of precorrin-3.

Abbreviations: ALA, 5-aminolevulinic acid; PBG, porphobilinogen; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SAM, *S*-adenosylmethionine; NMR, nuclear magnetic resonance.

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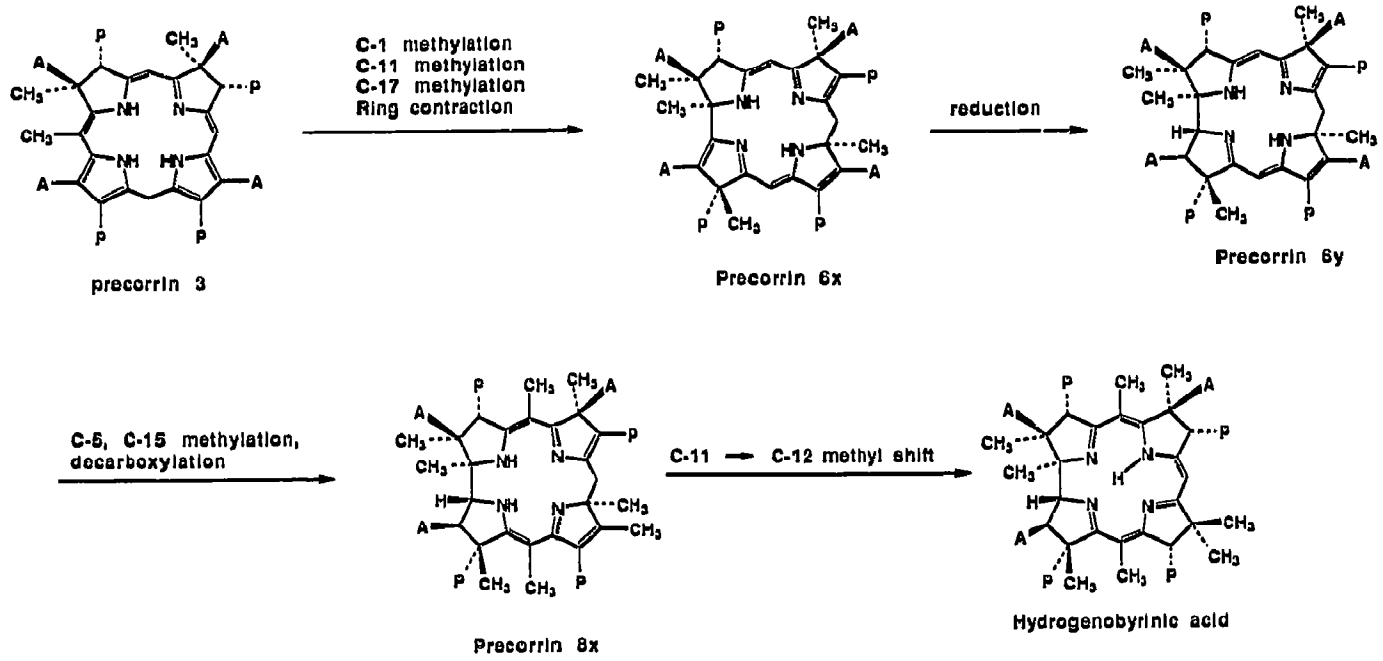

Scheme 1

2. MATERIALS AND METHODS

2.1. *Synthesis of labeled substrates*

[5-^{13}C]-, [4-^{13}C]- and [3-^{13}C]5-aminolevulinic acid (ALA) isotopomers were prepared as previously described [14,15]. [2,11-$^{13}C_2$]-, [3,5-$^{13}C_2$]- and [4,6-$^{13}C_2$]porphobilinogen (PBG) were enzymatically synthesized [16] from the appropriately labeled ALA using ALA dehydratase isolated from a recombinant strain of *E. coli* (strain CR261, C. Roessner, unpublished results). Precorrin-3, either ^{13}C-labeled or unlabeled, was synthesized using a mixture of enzymes isolated from recombinant strains of *E. coli* as previously described [13]. *S*-Adenosyl-[^{13}CH$_3$]L-methionine ([^{13}C]SAM) was prepared as previously described [17].

2.2. *Bacteria and plasmids*

E. coli strain TB1 was provided by Dr. Tom Baldwin, Texas A&M University. Plasmid pUC18 (*tac*PO) was purchased from Bethesda research laboratories, Bethesda MD. *E. coli* strain XA90 and plasmid pHN1+ (*tac*PO) were supplied by Dr. Gregory Verdine, Harvard University. Plasmids pJE1, pJE2, pZT365 and pZT366 bearing the *cbi* genes in plasmid pBR329 were generously provided by Dr. John Roth, University of Utah.

2.3. *Subcloning of the* cbi *genes*

A scheme for subcloning the *cbi* genes from plasmids pJE1, pJE2, pZT365 or pZT366 is shown in Fig. 1. The genes were initially subcloned into the expression vector pUC18 using standard recombinant DNA techniques [18]. TB1 cells transformed with plasmids bearing the proper insert were analyzed by SDS-PAGE [19] for expression of the gene as determined by the appearance of a protein band of the appropriate molecular weight and not seen in control TB1 cells bearing pUC18 only. If no expression was detected, the gene was subcloned into pHN1+, transformed into XA90 and again analyzed for expression. *Cbi* genes which displayed no expression by direct subcloning into either pUC18 or pHN1+ were supplied with optimal translational signals using an expression cassette polymerase chain technique [20] and inserted into either pUC18 or pHN1+. *P. denitrificans cob* genes were also expressed using an expression cassette polymerase chain reaction.

2.4. *Expression and purification of the* cbi *and* cob *gene products*

The gene products were purified from 4–8 l of either TB1 or XA90 cells bearing plasmids containing the genes. The TB1 cells were grown overnight with good aeration at 37°C in LB medium containing 50 μg/ml ampicillin. The XA90 cells were grown in the same medium to an OD_{550} = 0.5, induced by the addition of IPTG to 0.4 mM, and then further incubated overnight. The cells were collected by centrifugation, suspended in 1/50 volume PE buffer (100 mM KH_2PO_4, 2.0 mM EDTA, adjusted to pH 8.0 with NaOH) containing 50 μg/ml lysozyme, and allowed to stand at room temperature for 30 min. The cells were lysed by sonication and the lysate centrifuged for 10 min at 12,000 × g. The gene products were then purified by a combination of ammonium sulfate fractionation, gel filtration, anion exchange chromatography on DEAE-Sephacel, and, when necessary to obtain enzyme of sequencing purity, FPLC on a MonoQ anion-exchange column. Purified proteins were tested for SAM binding as previously described [2].

2.5. *Assay for methylation of precorrin-3*

Precorrin-3 (100 μg, derived by reduction of Factor III) and [^{14}C]SAM (0.2 μCi, 9.5 Ci/mmol, New England Nuclear) were added to 5.0 ml of a cell lysate that had been prepared anaerobically from a 50 ml culture of cells bearing plasmids expressing the *cbi* gene. The cells were collected by centrifugation, washed with 10 ml of degassed buffer (100 mM Tris-HCl, 1.0 mM EDTA, 100 mM KCl, 5.0 mM DTT), resuspended in 5.0 ml of degassed buffer containing 50 μg/ml lysozyme, incubated at 37°C for 30 min, and lysed by sonication. All of the above steps were carried out under argon. After incubation for 6 h at 30°C, the product was adsorbed onto 0.1 g of DEAE and washed thoroughly with water to remove unincorporated SAM. The product was then eluted with 1.5 ml of 0.1 N HCl and the radioactivity in 0.5 ml of the eluate determined.

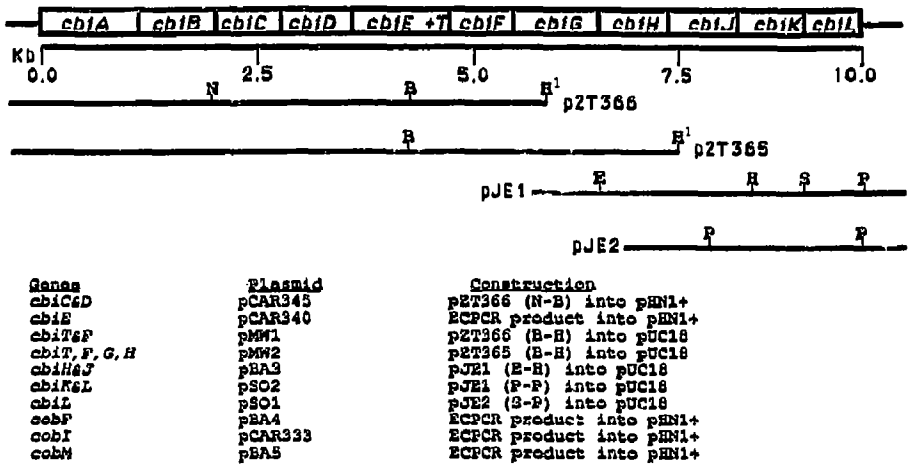

Fig. 1. Scheme for subcloning the *cbi* and *cob* genes. Restriction enzymes used: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nae*I; P, *Pst*I; S, *Sal*I; H, ^{1}*Hin*dIII site in BR329.

3. RESULTS AND DISCUSSION

Ten of the *cbi* genes found in the *S. typhimurium cob* operon were subcloned for expression from the 4 different plasmids obtained from Dr. J. Roth (Fig. 1). All of these genes except *cbiD* were expressed in *E. coli* as determined by SDS-PAGE, and seven of the gene products were purified from the soluble fraction of cell lysates of the appropriate strain (Fig. 2). Two of the gene products, those of *cbiG* and *cbiJ*, were found in the insoluble fraction (not shown). The amino-terminal sequence of each of the purified proteins was determined (Fig. 2) and used to confirm the location of the open reading frames predicted from the nucleotide sequence (J. Roth et al., in preparation). The gene products of *cbiE*, *cbiF*, *cbiH* and *cbiL* were shown to be SAM binding proteins, and based on their homology with other methyltransferases [8] are considered to be the most likely candidates for methyltransferase activity. The *P. denitrificans cobL* gene product has recently been reported [9] to have two functions, methylation (of C-5 and C-15) and decarboxylation of the ring C acetate. SDS-PAGE and NH_2-terminal sequence analysis revealed that two separate gene products in *S. typhimurium* (*cbiE* and *cbiT*) correspond to the *cobL* gene product with *cbiE* homologous to the methyltransferase region and *cbiT* homologous with the decarboxylase region.

From its homology with the *P. denitrificans cobI* gene product (31% identity, 71% conservation), the *cbiL* gene product was predicted to be the *S. typhimurium* precorrin-2 methyltransferase (M-2). When lysates of cells expressing *cbiL* were incubated with the multi-enzyme precorrin-2 synthesizing system and analyzed for the synthesis of precorrin-3, all of the lysates of cells expressing *cbiL* displayed M-2 activity indistinguishable from that of a lysate of cells expressing *cobI*, as demonstrated by the appearance, after esterification and extraction, of a blue product corresponding in R_f to Factor III octamethylester on TLC plates. Lysates of cells expressing the other putative methyltransferases did not display such M-2 activity. The blue product, isolated by preparative HPLC from large scale incubations with purified enzyme, was identical with Factor III octamethylester (HPLC retention time, UV/VIS spectrum, proton NMR, and FAB MS). Thus, the *cbiL* gene encodes M-2 in *S. typhimurium*. However, the specific

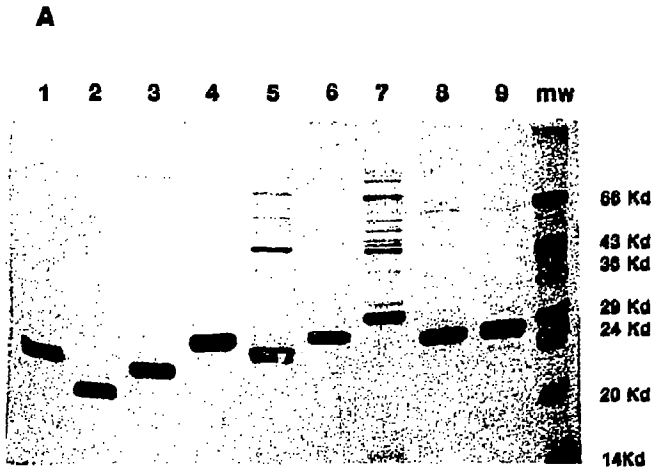

Fig. 2. A. SDS-PAGE analysis of the purified gene products of: 1, *cbiC*; 2, *cbiE*; 3, *cbiT*; 4, *cbiF*; 5, *cbiH*; 6, *cbiK*; 7, *cbiL*; 8, *cobF*; 9, *cobI*. Lane 10, molecular weight markers. B. Amino terminal sequence of the purified proteins.

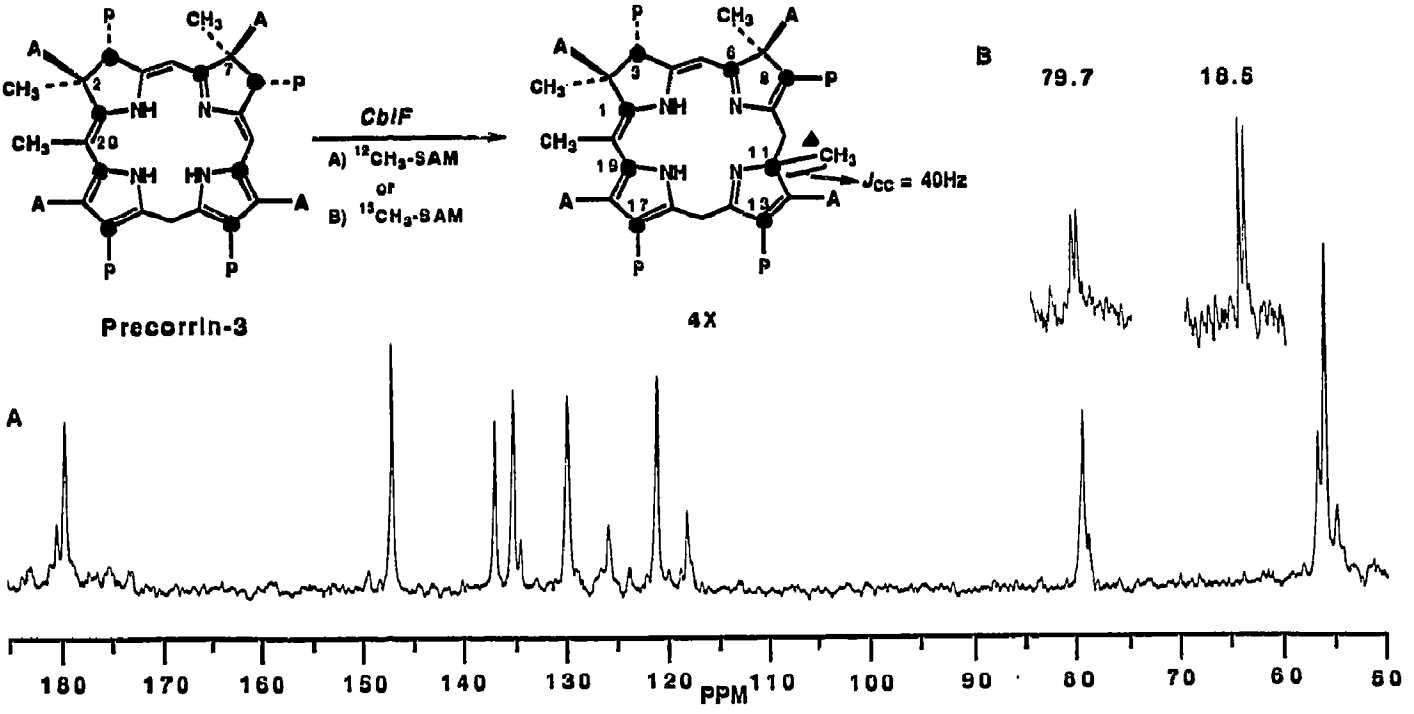

Fig. 3. ^{13}C NMR spectra of compound 4x derived from overnight incubations containing the *cbiF* gene product, precorrin-3 prepared from [3,5-$^{13}C_2$]PBG (3 mg), and [^{12}C]SAM (A) or [^{13}C]SAM (B). The samples, in 2 M KCl containing 20% D_2O, were maintained under argon while spectra were recorded using the parameters previously described [13].

activity of the M-2 expressed from *cbiL* in *E. coli* is approximately tenfold less than that expressed from *cobI* in *E. coli*.

Cell lysates of *E. coli* expressing the remaining suspected methyltransferases, including the *cobF* and *cobM* gene products of *P. denitrificans*, were tested for activity with precorrin-3 and labeled SAM as substrates. Only lysates expressing the *cbiF* gene product of *S. typhimurium* possessed the ability to incorporate ^{14}C in the radioassay which, when repeated with [^{13}C]SAM, displayed a single ^{13}C methyl signal at 18.5 ppm. The new product demonstrated a yellow absorbance spectrum (λ_{max} = 436 nm in 2.0 M KCl) typical of an interrupted chromophore. The product rapidly turned red and then brown when exposed to air, and attempts to stabilize it by methyl ester formation, a standard procedure with precorrins, resulted in decomposition of the product. To determine the site of C-methylation by the *cbiF* gene product, precorrin-3, derived from [3,5-$^{13}C_2$]-labeled PBG, was incubated anaerobically overnight in the presence of the enzyme, either purified or in cell-free lysates, and [^{13}C]SAM. The product was concentrated and isolated by adsorption to and elution from DEAE-Sephadex, extreme care being exercised to maintain anaerobicity throughout the isolation and NMR analysis. The resulting ^{13}C NMR spectrum displayed two new

Table 1

^{13}C-NMR chemical shifts[a] of 4x derived from various ^{13}C-isotopomers of PBG

from [3,5-$^{13}C_2$]PBG		*from [4,6-$^{13}C_2$]PBG*		*from [2,11-$^{13}C_2$]PBG*		*from [^{13}C-methyl]SAM*	
55.5	C3	20.9		27.5 (J = 48, 51 Hz)	C15	25.6	C7′
79.7	C11	21.3	C8′, C13′, C17′[b]	32.8 (J = 49 Hz)	C10	19.7	C2′
120.7	C17	21.6		81.7 (J = 71 Hz)	C5	18.5	C11′
129.4	C19	26.3	C3′	108.5	C20	17.7	C20′
134.8[b]	C8	45.0[b]	C7	122.5 (J = 51 Hz)	C16		
136.5[b]	C13	57.9[b]	C2	133.5 (J = 49 Hz)	C9		
146.3	C1	114.7	C18	167.8 (J = 71 Hz)	C4		
178.6	C6	163.8	C12	178.3 (J = 48 Hz)	C14		

[a] Samples in 2.0 M KCl containing 20% D_2O and referenced to dioxane, δ 66.5.
[b] Not unambiguously assigned.

doublets, one in the methyl region at 18.5 ppm and one in the sp^3 region at 79.7 ppm (Fig. 3B). Both doublets had equivalent coupling constants (40 Hz) demonstrating the insertion of one [^{13}C]SAM-derived methyl group on a ^{13}C-enriched PBG-derived carbon, which could be confirmed by the appropriate single-label experiments; i.e. NMR analysis of the product derived from [3,5-$^{13}C_2$]PBG and [^{12}C]SAM (Fig. 3A) or derived from [^{12}C]PBG and [^{13}C]SAM (not shown) revealed a clear singlet for each carbon at δ 79.7 and 18.5 ppm, respectively. The ^{13}C-NMR spectrum (Fig. 3A) also revealed six sp^2 carbons but only one other sp^3 carbon located at 55.5 ppm corresponding to C-3 and indicative of a double bond migration into ring B, i.e. C-8 is now sp^2 hybridized.

The complete chromophore of compound 4x was established by ^{13}C NMR analysis of a sample derived from [2,11-$^{13}C_2$]PBG. Unexpectedly, two sp^2 (meso; δ 81.7, 108.5 ppm) and two sp^3 (δ 27.5, 32.8 ppm) carbons were observed. Based on the chemical shifts and coupling patterns of these carbons, we can unambiguously assign the single resonance at 108.5 ppm to C-20 while the doublet at 81.7 ppm can be assigned to C-5. Accordingly, compound 4x must contain two meso methylenes, at C-10 and C-15 (δ 32.8 and 27.5 ppm, respectively) as shown in the proposed structure (Fig. 3). The NMR spectrum of compound 4x derived from [4,6-$^{13}C_2$]PBG was obtained to complete the chemical shift assignments of the ring carbons (Table I).

The above NMR analysis of compound 4x confirms that the *cbiF* gene product catalyzes the SAM-dependent methylation of precorrin-3, inserting one methyl group on a ^{13}C-enriched carbon derived from [3,5-$^{13}C_2$]PBG. Based on the methylation pattern of the precorrins and cobyrinic acid, the possible methylation sites are C-1, C-11, C-16 and C-17. The observed chemical shift (δ 79.6) of the C-methylated carbon is diagnostic for methylation at an α-pyrrolic center (C-1, C-11, or C-16) but not for β-methylation, thus eliminating C-17 as a potential site. Further distinction between C-1, C-11 and C-16 can be made by analysis of the chemical shifts and coupling patterns in the spectrum derived from [2,11-$^{13}C_2$]PBG. Had methylation occurred at C-1, the signal for the adjacent C-20 would have reflected a necessary sp^3 geometry. C-16 methylation can be ruled out from the diagnostic sp^2 signals assigned to C-14 and C-16 (Table I). The necessary conclusion that the new methyl group has been inserted at C-11 is at first sight highly unexpected since pulse labeling experiments have suggested that C-17 is the site of the first C-methylation of precorrin-3 (followed by methylation at C-12 [21,22]), and since all of the corrinoid vitamin B_{12} precursors characterized prior to the isolation of precorrin 6x have displayed methylation at C-12, not at C-11. Precorrin 6x, however, is methylated at C-11 [6] and it has been shown this methyl group eventually migrates to C-12 in the formation of hydrogenobyrinic acid, thus re-enforcing the conclusion that the *cbiF* gene product is indeed the C-11 methyltransferase and that compound 4x has the structure shown in Fig. 3. Whether compound 4x is the next intermediate following precorrin-3 or has resulted from non-sequential methylation has yet to be determined. However, it now appears that C-11 methylation is indeed the function of the *cbiF* gene product regardless of whether the substrate is precorrin-3 or a subsequent intermediate. Based on homology with *cbiF*, the *P. denitrificans cobM* gene product presumably performs the same function, although a lysate derived from recombinant *E. coli* cells expressing the *cobM* gene product in this organism did not methylate precorrin-3. Additionally, precorrin-2 was not a substrate for methylation by *cbiF*.

Previously, *E. coli* uroporphyrinogen III methyltransferase (the *cysG* gene product, M1) was shown to over-methylate precorrin-2 at C-12 resulting in the formation of a trimethylpyrrocorphin [12]. A similar chromophore, which resulted from a single methylation, was observed when precorrin-3 was incubated with purified *cysG* gene product. The site of this C-methylation has tentatively been assigned to C-12 (unpublished results). Compound 4x, however, was not a substrate for methylation by the *cysG* gene product, presumably due to stereo-electronic changes induced by methylation at C-11.

At this stage of our knowledge of the intermediates of cobinamide synthesis, we cannot designate compound 4x as the next intermediate, precorrin 4x, since we have as yet to show it can be converted to cobyrinic acid. It is interesting to note that precorrin 6x was not converted to cobyrinic acid in a cell-free system either [5] but rather to the cobalt-free corrinoid, hydrogenobyrinic acid, even when the incubation mixture included cobalt. The failure of precorrin 6x to serve as an intermediate for cobyrinic acid by a cobyrinic acid synthesizing system suggests that, in vivo, cobalt insertion is required prior to the formation of precorrin 6x. Indeed, evidence that cobalt insertion occurs as early as the precorrin-3 stage has been obtained using *P. shermanii* extracts [23]. Since compound 4x and precorrin 6x were both synthesized in the absence of cobalt (and in the absence of a cobalt inserting system), the enzymes involved may have never seen their proper substrates, viz. cobalto-precorrin-3-4-5, etc, resulting in the synthesis and accumulation of both 4x and of precorrin 6x as metal-free derailment products.

It now appears that some organisms use different pathways to corrins. Thus, the photosynthetic anaerobic bacteria *Rhodopseudomonas spheroides* and *Chromatium vinosium* product cobalt-free corrinoids [24] as does the *Pseudomonas denitrificans* strain that has been genetically altered by introduction of a plasmid bearing eight genes for cobinamide synthesis [5]. On the other hand, hydrogenobyrinic acid is not a precursor of cobyrinic acid in *P. shermanii* [25] and even in the genetically

altered *P. denitrificans* strain, cobalt can be inserted into the product, hydrogenobyrinic acid, only by chemical means. Thus it would appear that, as suggested above, the true substrates for the enzymes of cobyrinic acid synthesis in anaerobes are the cobalt complexes of precorrin-2-3-4, etc., and the specificities of the various methyltransferases (M-2, M-3, etc.) may be modulated by the presence (or absence) of cobalt. The appropriate experiments are in hand to test this thought which also rationalizes the apparent discrepancy between the sequence of C-methylation (C-20>C-17>C-12>C-1>C-5>C-15) derived from pulse label experiments and the regiospecificity of the enzyme responsible for C-methylation of precorrin-3 at C-11 as disclosed in this report. With the remaining biosynthetic enzymes now in hand, the intermediates between precorrin-3 and cobyrinic acid can be identified, although their predictable instability towards oxygen presents a formidable challenge in solving the necessary problems of isolation and structure.

Acknowledgements: We thank Dr. John Roth (University of Utah) for supplying plasmids pZT365, pZT366, pJE1, and pJE2 and the nucleotide sequence of the *cbi* genes prior to publication, Dr. Gregory Verdine (Harvard University) for pHN1+ and XA90, and NIH for financial support.

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