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Biosynthesis of vitamin B_{12}

Discovery of the enzymes for oxidative ring contraction and insertion of the fourth methyl group

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In the vitamin B_{12} biosynthetic pathway the enzymes responsible for the conversion of precorrin-3 to precorrin-4 have been identified as the gene products of *cobG* and *cobJ* from *Pseudomonas denitrificans*. CobG catalyzes the oxidation of precorrin-3 to precorrin-3x (a hydroxy lactone) whereas CobJ is a SAM-dependent C-17 methyl transferase and is necessary for ring contraction. A mechanism for ring contraction is proposed.

Biosynthesis; Corrin; Precorrins-3, -3x, -4; ^{13}C-NMR, *Pseudomonas denitrificans*

1. INTRODUCTION

The pathway to corrins [1] in the aerobic organism *Pseudomonas denitrificans* involves the conversion of precorrin-3 (**1**), to hydrogenobyrinic acid (**6**) via the isolated intermediates corresponding to sequential oxidative ring contraction and insertion of C-methyl groups from *S*-adenosyl methionine (SAM) at C-17β ($\rightarrow$ Factor IV, **2**) [2], two further methylations at C-11, C-1 ($\rightarrow$ precorrin 6x, **3**) [3,4] reduction at C-18, 19 (precorrin 6y, **4**) [5], methylation at C-5, C-15 with decarboxylation of the C-12 acetate side chain (precorrin 8x, **5**) [6] and, finally [7] [1,5] sigmatropic shift of the C-11 methyl to C-12 to afford the cobalt-free corrin (**6**) (Scheme I).

Since most of the above steps have been defined through the use of mutants rather than single enzymes, the fascinating problem of the mechanism of the ring contraction step has remained enigmatic, and is further complicated by the discovery of a parallel, *anaerobic* pathway in *Propionibacterium shermanii*, which features early introduction of cobalt into the dipyrrocorphin, precorrin-3 [8].

2. MATERIALS AND METHODS

2.1. *Bacteria and plasmids*

E. coli strain TB1 was provided by Dr. Tom Baldwin, Texas A&M University. Plasmid pUC18 (*lac*PO) was purchased from Bethesda Research Laboratories, Bethesda, MD. *E. coli* strain BL21DE3 and plasmid pET21b (pT7) were purchased from Novagen, Madison, WI.

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2.2. *Expression of the* cobG *and* cobJ *genes*

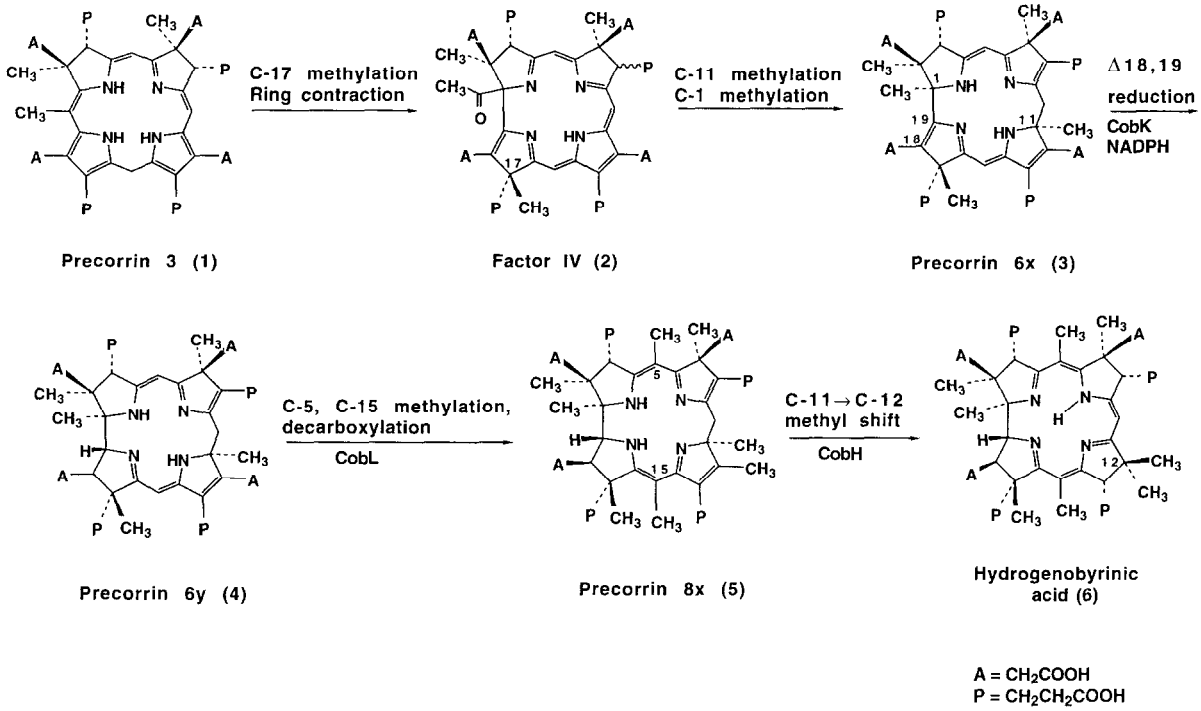
The *cobG* and *cobJ* genes were expressed in *E coli* using an expression cassette polymerase chain reaction technique [9] and *P denitrificans* genomic DNA as the template. The PCR primers were designed to provide convenient restriction enzyme sites and optimal translational signals for the expression of foreign genes in *E coli*. The sequence of the *cobJ* 5′ primer was CGCGC**GGATCC**AGGAGGAA-TTTAAA**ATG**ACCGGTACGCTCTATGTCGTCGGTACCGGA- providing a 5′ *Bam*HI restriction site (GGATCC), a strong ribosome binding site (AGGAGG), an optimal translational spacer element (AATTTAAA), the translational initiation signal (ATG) and codons for the next ten amino acids of the protein. The sequence of the *cobJ* 3′ primer was CGCGC**GTCGAC**TTACTGGCTCGCCCCTGCAT-AGAAGCGCGGTGT providing the codons for the last ten amino acid of the protein, a translational stop signal (TAA) and a 3′ *Sal*I restriction site (GTCGAC) The PCR product was digested with *Bam*HI and *Sal*I, ligated into *Bam*HI, *Sal*I-digested pET21b and transformed into BL21DE3 to give strain CR 377. The *cobG* gene was cloned into pUC18 in a similar manner, varying only the restriction sites (*Eco*RI and *Xba*I) and the amino acid codons of the primers, and transformed into TB1 to give strain CR370. SDS-PAGE analysis of whole cell lysates of strains CR370 and CR377 revealed the presence of new bands with $M_r \approx 45{,}000$ and 27,000, respectively, corresponding closely to the molecular weights of 46,990 Da and 27,105 Da for CobG and CobJ predicted from the DNA sequence.

2.3. *Synthesis of precorrin-3*

Precorrin-3 was synthesized in a multi-enzyme reaction as previously described [11,14].

2.4. *Synthesis of precorrin 3x and precorrin-4*

Precorrin-3x was synthesized in a 100 ml reaction containing 100 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, 2.0 mM $MgCl_2$, 0.4 mM NADH, 1.0 mM ATP, 3–5 mg of precorrin-3 and a cell lysate derived from strain CR370 (CobG) from which the small molecules were removed by passage through a Sephadex G-25 column. Precorrin-4 was synthesized in an identical reaction containing 0.25 mM SAM and a lysate derived from both CR370 (CobG) and CR377 (CobJ). After incubation aerobically for 4 h at 30°C, the products were isolated anaerobically by adsorption to DEAE-Sephadex and prepared for NMR analysis as previously described [11,14].



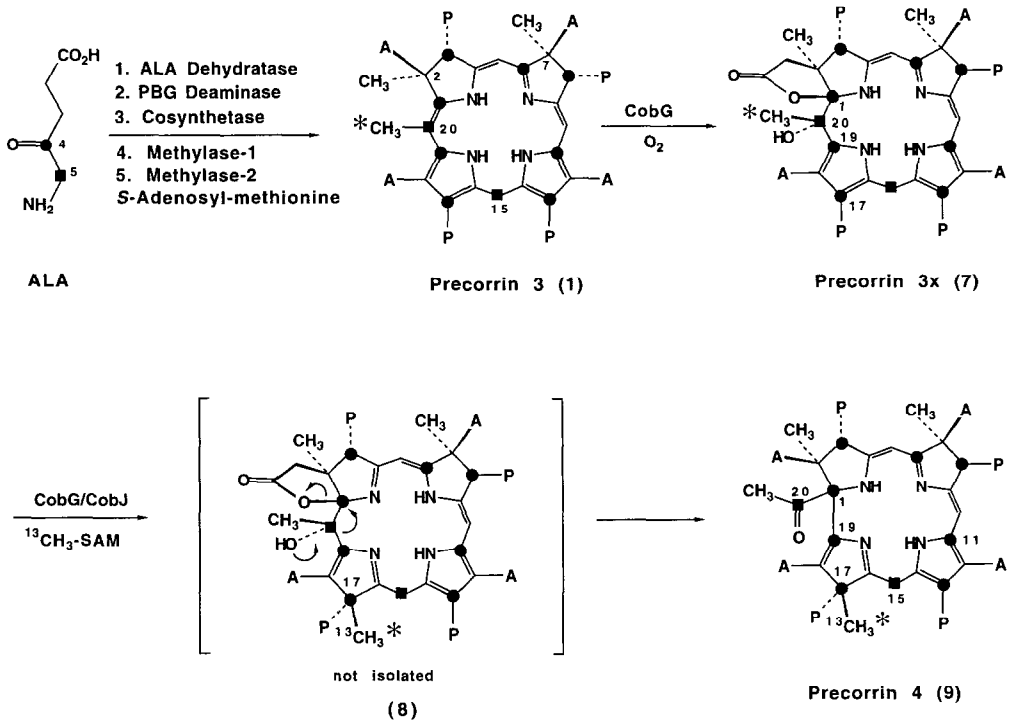
Scheme I

3. RESULTS AND DISCUSSION

Using ^{13}C-NMR spectroscopy as a probe for the activity of each of the overexpressed enzymes of the B_{12} pathway in *P. denitrificans* we have tested each of the individual enzymes in turn with precorrin-3 as substrate and find that CobG [10] serves as an O_2-dependent enzyme whose role is to install oxygen-derived functionality at C-20, thus preparing the macrocycle for ring contraction. Remarkably, the resultant spring-loaded mechanism **does not operate until after the fourth C-methylation has occurred at C-17**, an event which is mediated by CobJ [10] a SAM requiring enzyme, thereby defining both the ring contraction and C-17 methylation sequences.

The NMR assays for the activities of CobG and J were developed as follows. First, precorrin-3 was prepared in two ^{13}C-isotopomeric versions, A, from [^{13}C-4]-(●)-, and B, from [^{13}C-5]-(■)-5-aminolevulinic acid, using the multi-enzyme synthesis described earlier [11]. The reaction of isotopomer A (●) with CobG in presence of O_2 and NADH resulted in a spectrum almost identical with that of the substrate except for the disappearance of the signal (●) for C-1 at δ146 ppm and the appearance of a peak (●) at δ106 ppm corresponding to sp^3 geometry and a new environment at C-1. When isotopomer B (■) served as substrate, the pattern of NMR signals remained constant except for the resonance for C-20 (at δ103 ppm) which was replaced by a signal at δ78 ppm corresponding to oxygen insertion and resultant sp^3 hybridization at this center. In a separate experiment using precorrin-3 enriched at the C-20 methyl, the CH_3 signal (*) at C-20 (δ17.6 ppm) in precorrin-3 underwent a downfield shift to δ25 during incubation with CobG. The above spectral changes are in accord with the addition of oxygen at both positions 1 and 20 and together with the observation by infrared spectroscopy of a γ-lactone (1799 cm^{-1}) lead to the structural proposal (**7**) for the new product, precorrin-3x, whose formation can be rationalized by epoxidation at C-1, 20 by the O_2-dependent CobG enzyme followed by participation of the ring A carboxylate in a lactonisation-ring opening sequence. In the absence of O_2 no reaction of precorrin-3 with CobG was observed.

Next, addition of SAM and, in turn, each of the remaining putative methyl transferases in the *P. denitrificans* repertoire (CobF, J, M) to precorrin-3x (**7**) labeled from [4-^{13}C]ALA (●) resulted in a new signal for C-17 (δ66) corresponding to C-methylation at this center, **only when CobJ was present**. Confirmation that C-17 is indeed the site of the fourth methyl insertion came from double-labeled incubation of isotopomer A (●) and $^{13}CH_3$-SAM (*) in the presence of **both** CobG and J. In this experiment the new CH_3 signal (*) appearing at δ22.5 was coupled (J = 37 Hz) to the C-**17** resonance (●; δ66 ppm) (Scheme II). Most significantly, the ^{13}C-NMR spectrum of **9** also displays a new pair of coupled carbons C-1, C-19 (δ82, 142, J = 52 Hz) showing that



Scheme II

ring contraction occurs during incubation with CobJ. When the two-enzyme incubation was repeated using isotopomer B (■) the signal for C-20 in the product appeared at δ210 ppm indicating that ring contraction is accompanied by the genesis of a new methyl ketone function pendant from C-1, by a process which corresponds formally to the pinacol type rearrangement [12] illustrated in Scheme II (**7** → **8** → **9**). Analysis of the full set of spectral data (NMR; FAB-MS; FT-IR) leaves no doubt that the new isolate is precorrin-4 (**9**). Aerial oxidation of **9** to factor IV (**2**) [2] completed the proof of structure and absolute stereochemistry (except at position C-1) for precorrin-4 (**9**), the long-sought tetramethylated intermediate of corrin biosynthesis.

It is of considerable interest for the evolution of B_{12} synthesis that Nature should use both a 'modern' aerobic pathway (i.e. less than 2×10^9 years old) involving metal-free substrates for most of the way (Scheme I) as well as the ancient, anaerobic sequence (dating to ca. 3.8×10^9 years) in which cobalt is inserted very early [8], implying a corresponding dichotomy of mechanism of ring contraction in which O_2 is replaced in the metallo system by a 2 electron valency change. Thus the cobalt complex of precorrin-3x could be reached in the *Salmonella typhimurium* and *P. shermanii* series via internal redox of $Co^{III} \rightarrow Co^{I}$ which is formally equivalent to a 2-electron oxidation [13].

The discovery of the ring contractase system (CobG and CobJ) and the fourth methylating enzyme for C-17 (CobJ) reveals the way in which aerobic corrin biosynthesis uses an early oxidative ring contraction step concomitant with C-17 methylation on the β- (upper) face of the substrate, precorrin-3x, leaving only the enzymes mediating the fifth (C-11) and sixth (C-1) C-methylation steps to be identified, one of which must also involve deacetylation at C-1. From homology comparisons these are predicted to be CobM and CobF respectively and we have recently demonstrated C-methylation α to the pyrrolic N with a model substrate using CobM [14,15]. CobF then becomes the prime candidate for the C-1 methyl transferase catalyzing the insertion of the sixth methyl group, perhaps concerted with deacetylation at C-1 to reach precorrin-6x (Scheme I). The route from precorrin-6x to (cobalt-free) hydrogenobyrinic acid (Scheme I) has recently been described at the enzyme level [5,7], so that almost every intermediate in the aerobic pathway has now been identified by NMR spectroscopy and all of the functions necessary for corrin biosynthesis assigned in *P. denitrificans*. There now remains the experimentally challenging problem of analyzing the parallel, yet distinct, pathway in *S. typhimurium* [16] and *P. shermanii* for in these organisms the gene products are handling substrates in the form of cobalt-complexes whose valency changes are believed to be at the heart of the mechanism of ring contraction in these anaerobic bacteria.

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