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Biosynthesis of the Corrin Macrocycle of Coenzyme B_{12} in *Pseudomonas denitrificans*

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Studies with cell-free protein preparations from a series of recombinant strains of *Pseudomonas denitrificans* demonstrated that precorrin-3 is converted into a further trimethylated intermediate, named precorrin-3B, along the pathway to coenzyme B_{12}. It was then shown that the part of the pathway from precorrin-3 (called precorrin-3A hereafter) to precorrin-6x involves three intermediates, precorrin-3B, precorrin-4, and precorrin-5. Precorrin-3B was isolated in its native (reduced) as well as its oxidized (factor-IIIB) states, and precorrin-4 was isolated in its oxidized form only (factor-IV). Both factors were in vitro precursors of precorrin-6x. The synthesis of precorrin-6x from precorrin-3A was shown to be catalyzed by four enzymes, CobG, CobJ, CobM, and CobF, intervening in this order. They were purified to homogeneity. CobG, which converts precorrin-3A to precorrin-3B, was found to be an iron-sulfur protein responsible for the oxidation known to occur between precorrin-3A and precorrin-6x, and CobJ, CobM, and CobF are the C-17, C-11, and C-1 methylases, respectively. The acetate fragment is extruded after precorrin-4 formation. This study combined with our recent structural studies on factor-IV (D. Thibaut, L. Debussche, D. Fréchet, F. Herman, M. Vuilhorgne, and F. Blanche, J. Chem. Soc. Chem. Commun. 1993:513–515, 1993) and precorrin-3B (L. Debussche, D. Thibaut, M. Danzer, F. Debu, D. Fréchet, F. Herman, F. Blanche, and M. Vuilhorgne, J. Chem. Soc. Chem. Commun. 1993:1100–1103, 1993) provides a first step-by-step picture of the sequence of the enzymatic reactions leading to the corrin ring in *P. denitrificans*.

Until the end of the 1980s, most of the knowledge on coenzyme B_{12} (vitamin B_{12}) biosynthesis came from experiments with *Propionibacterium shermanii*. Since the cloning of coenzyme B_{12} biosynthetic genes (*cob* genes) of *Pseudomonas denitrificans* (12) and the purification of CobA (6), the C-methyltransferase which deflects uroporphyrinogen III into the pathway to coenzyme B_{12} (see Fig. 1), rapid progress has been made in clarifying the biosynthesis of this complex cofactor at both the biochemical and genetic levels. Vitamin B_{12} biosynthetic genes from *Salmonella typhimurium* were also recently sequenced (33a), and a likely function was attributed to several of them by comparison with the *cob* genes previously characterized in *P. denitrificans*.

In *P. denitrificans*, the pathway downstream from hydrogenobyrinic acid (Fig. 1), the first intermediate displaying the complete corrin ring system (11, 16), was fully elucidated recently (15 and references cited therein), and cobalt was proven to be introduced in hydrogenobyrinic acid *a,c*-diamide by a complex soluble enzyme named cobaltochelatase (16). In contrast, in the anaerobe *P. shermanii*, cobalt is known to be inserted well before the corrin macrocycle is completed (28, 29). Upstream from hydrogenobyrinic acid, the biosynthesis of the corrin ring has not yet yielded all of its secrets despite the discovery of a very informative intermediate, precorrin-6x (38, 41), and the step-by-step elucidation of the sequence from precorrin-6x to hydrogenobyrinic acid (7, 10, 40, 43, 44). Particularly, the part of the pathway between precorrin-3 and precorrin-6x is still largely unexplored, and the intervening intermediates, which are assumed to be very labile (33), remain to be discovered.

The conversion of precorrin-3 to precorrin-6x involves three *S*-adenosyl-L-methionine (SAM)-dependent C-methylations at C-17, C-11, and C-1 (in this order) (11, 33, 37, 38, 41, 45), ring contraction (formation of a bond between C-1 and C-19), loss of C-20 with its attached methyl group as a two-carbon fragment (presumably acetic acid) (2, 27), and oxidation of the macrocycle (38, 41). With respect to the ring contraction-deacylation process, various hypothetical sequences, which were all based on a very attractive biomimetic chemical model (the dihydrocorphinol-corrin rearrangement) introduced by Eschenmoser (20), were recently proposed (34, 36–38). All suggested a methylation at C-17 as the first committed step.

We reported the genetic and sequence analysis of an 8.7-kb *Eco*RI *P. denitrificans* fragment carrying eight *cob* genes, named *cobF* to *cobM*, involved in transforming precorrin-2 into hydrogenobyrinic acid (14, 16). The reactions catalyzed by the products of four of these eight *cob* genes are known. The CobI protein effects the SAM-dependent methylation at C-20 on precorrin-2, yielding precorrin-3 (39), and CobK, CobL, and CobH catalyze the conversion of precorrin-6x into hydrogenobyrinic acid (7, 10, 40). Consequently, the last four genes, *cobF*, *cobG*, *cobJ*, and *cobM*, are certainly involved in the remaining portion from precorrin-3 to precorrin-6x. Biochemical and genetic data indicate that *cobF*, *cobJ*, and *cobM* code for methyltransferases (14).

In this report, we outline experiments aimed at elucidating the pathway from precorrin-3 to precorrin-6x and the reactions catalyzed by the CobF, CobG, CobJ, and CobM enzymes.

MATERIALS AND METHODS

Bacteriological methods. The recombinant DNA techniques used in this study were carried out as reported previously (40). *Escherichia coli* MC1060 or TG1 was used for plasmid construction (Table 1). Plasmid pXL229 was constructed by

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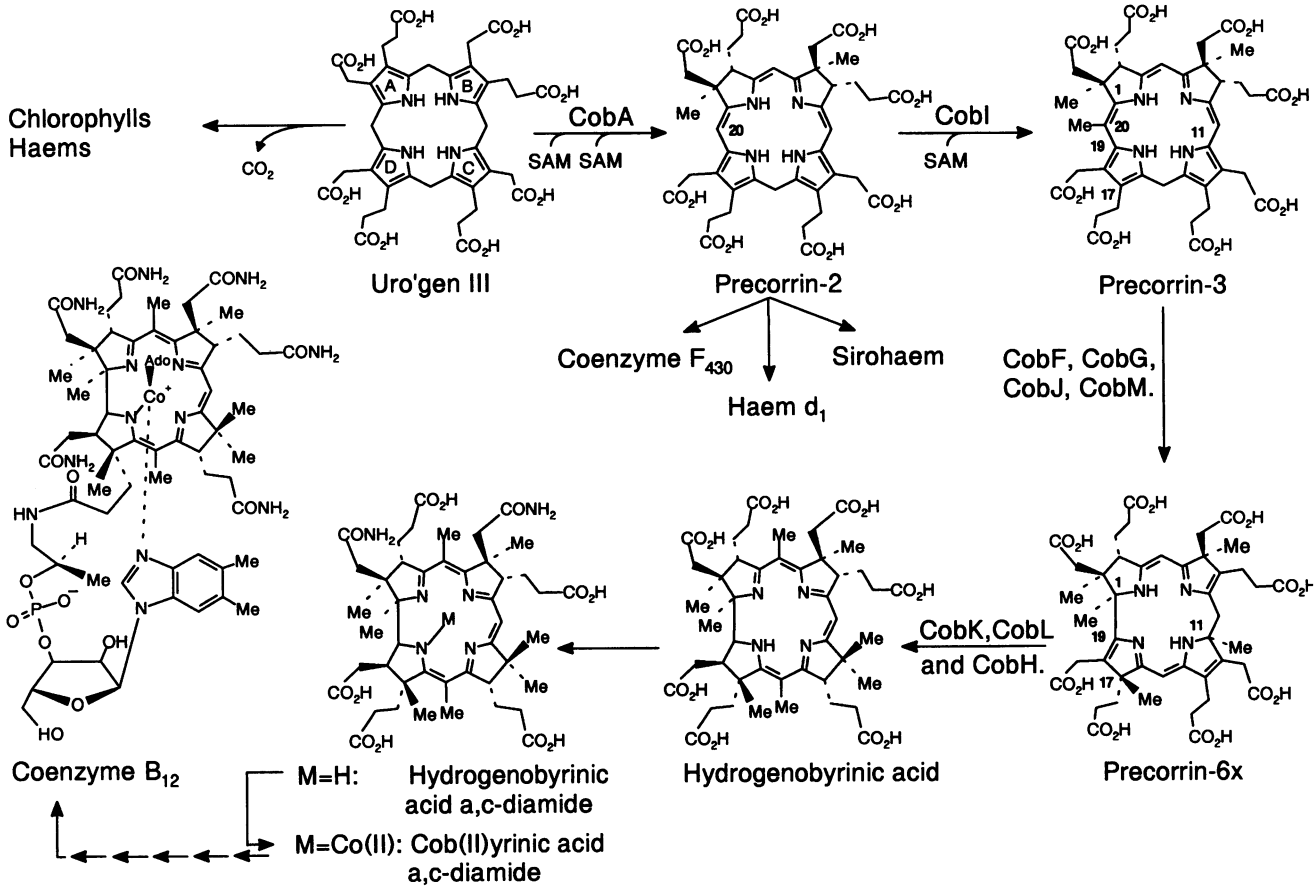

FIG. 1. Pathway from uroporphyrinogen III (ur'ogen III) to coenzyme B_{12} and other porphyrinoid cofactors. Ado, 5'-deoxy-5'-adenosyl.

cloning the *Eco*RI-*Bam*HI fragment from pXL152 into pKT230. pXL325 resulted from the cloning of the 4.1-kb *Eco*RI-*Sst*I fragment from pXL330 into pKT230. Plasmid pXL438 was obtained as follows: pXL330 was digested with *Bst*EII, treated with the Klenow fragment of *E. coli* DNA polymerase I, and then ligated with phosphorylated *Xba*I linkers; the plasmid was digested with *Xba*I and *Bgl*II, and the 5.7-kb fragment containing the *cobG* to *cobK* genes was cloned into pFR210 digested with *Bam*HI and *Xba*I. Plasmid pXL440 was constructed by cloning the 5.6-kb *Sal*I fragment from pXL330 into pFR210 digested with *Sal*I. pXL452 corresponds to the cloning of the 2.7-kb *Sst*I fragment from pXL330 into *Sst*I-linearized pXL435. An *Nde*I restriction site was introduced at the initiation codon of *cobF* by site-directed mutagenesis. The *Nde*I-*Hin*dIII cassette containing the *cobF* gene was cloned into *Nde*I-*Hin*dIII-digested pXL1841 to give plasmid pXL1546. In this plasmid, the *cobF* gene is downstream from the *E. coli* tryptophan promoter and the ribosome binding site of the *c*II gene of phage λ and upstream from the terminator region of the *E. coli rrnB* operon. pXL1546 is an RSF1010-derived plasmid and can be mobilized into *P. denitrificans* as described previously (12). The construction of pXL1663 was done in two steps. First, pXL330 was digested with *Xho*I and *Bgl*II, and the extremities were filled in with the Klenow fragment of *E. coli* DNA polymerase I and ligated with the 2-kb *Sma*I Ω fragment from pHP45Ω to give plasmid pXL1636. Because of the deletion of the 6.1-kb central region of the 8.7-kb fragment, pXL1636 has only kept the 5' end of *cobF* and the 3' end of *cobM*. Second, the *Eco*RI 4.6-kb fragment of pXL1636 was cloned into *Eco*RI-linearized pRK404 to give pXL1663. This plasmid was introduced into the Rif^r strain SC510, and marker exchange mutagenesis was performed as described elsewhere (6). This mutagenesis yielded a strain named G3575 displaying a deletion in the *cobF* to *cobM* genes. The genotype of G3575 was checked by Southern blot analysis. G3575 was found to no longer produce cobalamin when cultured in PS4 medium.

General methods and starting materials. Starting materials and protein extracts were prepared as described elsewhere (41). [*methyl*-^{13}C]SAM (>85 atom% ^{13}C) was a generous gift (G. Müller, Institut für Organische Chemie, Biochemie und Isotopenforschung, Stuttgart, Germany). Precorrin-3A accumulates in incubation mixtures containing crude protein preparations from the Rif^r strain SC510(pXL190), purified CobI, δ-aminolevulinic acid (ALA), and SAM under an argon atmosphere (39). Unlabeled precorrin-3A and ^{14}C-labeled precorrin-3A (Fig. 2, compound 3b; derived from [4-^{14}C]ALA) were isolated from such mixtures as reported previously (46). Methods for protein characterization (assay, determination of molecular weights by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE], and N-terminal sequencing) were carried out as described elsewhere (6, 39). CobG concentrations were also measured by quantitative amino acid analysis (6).

Incubation conditions and assays. Unless otherwise mentioned, assay incubations were done for 6 h at 30°C in a 1-ml final volume of 0.1 M Tris hydrochloride (pH 7.7)–1 mM EDTA (buffer A) containing 1 mM NADH, 200 μM SAM, 10

TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Marker and replicon	Relevant genotype, phenotype, properties, or fragment cloned	Reference or source
E. coli			
MC1060		Δ(*lacLOPZYA*)*X74 galU galK strA2 hsdR*	13
TG1		Δ(*lac-pro*) *thi supE hsdD5*/F′ *proAB lacI*^{q}*ZΔM15*	22
P. denitrificans			
SC510 (Rif^{r})		Cobalamin-overproducing strain resistant to rifampin at up to 100 mg liter^{-1}	6
G3575		Derived from the Rif^{r} strain SC510 Δ(*cobF* to *cobM*), obtained through double homologous recombination with pXL1663, producing no cobalamin	This study
Plasmids			
pFR210	Km^{r} RSF1010	Multicloning site	32
pKT230	Km^{r} RSF1010	Carries the Mob locus of RSF1010	1
pHP45Ω	Amp^{r} Sp^{r} ColE1	Sp^{r} cassette	31
pRK404	Tet^{r} RK2	Carries the Mob locus of RK2, multicloning site	19
pRK2073	Sp^{r} ColE1	Carries the *tra* genes of RK2	25
pXL152	Km^{r} RSF1010	12.8-kb *P. denitrificans Sau*3AI fragment cloned into *Bam*HI site of pXL59	12
pXL190	Km^{r} RSF1010	4.2-kb *P. denitrificans* fragment, *cobE*, *cobA*, *cobB*, and *cobC*	12
pXL229	Km^{r} RSF1010	3.3-kb *Eco*RI-*Bam*HI fragment from pXL152, *cobH* to *cobK*	This study
pXL253	Km^{r} RSF1010	8.7-kb *Eco*RI fragment from pXL151, *cobF* to *cobM*	14
pXL325	Km^{r} RSF1010	4.1-kb *Eco*RI-*Sst*I from pXL330, *cobF* to *cobH*	This study
pXL330	Amp^{r} ColE1	8.7-kb *Eco*RI fragment from pXL151 cloned into pUC13, *cobF* to *cobM*	10
pXL435	Km^{r} RSF1010	Carries the Mob locus of RSF1010, multicloning site	12
pXL438	Km^{r} RSF1010	5.7-kb *Bgl*II-*Bst*EII fragment from pXL330, *cobG* to *cobK*	This study
pXL440	Km^{r} RSF1010	5.6-kb *Sal*I fragment from pXL330, *cobF* to *cobJ*	This study
pXL452	Km^{r} RSF1010	2.7-kb, *Sst*I-*Eco*RI fragment from pXL330, *cobM*	12
pXL1546	Km^{r} RSF1010	*cobF* gene under the control of the *E. coli* tryptophan promoter	This study
pXL1636	Amp^{r} Sp^{r} ColE1	6.1-kb deletion in the 8.7-kb *Eco*RI fragment removing *cobF* to *cobM*	This study
pXL1663	Tet^{r} Sp^{r} RK2	4.6-kb fragment from pXL1636	This study
pXL1841	Km^{r} RSF1010	*Methanobacterium ivanovii corA* gene under the control of the *E. coli* tryptophan promoter	9

μM tetrapyrrolic substrate, and enzyme preparation (5 mg of crude protein extracts and/or about 10 U of each purified enzyme added). Before use, buffer A was carefully deoxygenated by bubbling argon through the solution. Incorporation of ice-cold components in the incubation medium was carried out in a glove box under an argon atmosphere, and assays were initiated by shifting the temperature to 30°C. In each assay, control blanks with either the protein or the tetrapyrrolic substrate absent were run in parallel.

In assay 1, the tetrapyrrolic substrate was unlabeled (trimethylisobacteriochlorin [TMIBC]; Fig. 2, compound 4d). To trace methylation of the tetrapyrrolic substrate, [*methyl*-^{3}H]SAM (1 μCi μmol^{-1}) was used. After incubation, the pigments were chromatographically separated from SAM and counted as described previously (6).

In assay 2, ^{14}C-labeled TMIBC (Fig. 2, compound 4b; 6 μCi μmol^{-1}) produced from [4-^{14}C]ALA was used as the tetrapyrrolic substrate. The incubation was stopped by adding 1 ml of 2 M hydrochloric acid, and after centrifugation of precipitated material, the radioactive tetrapyrrolic pigments contained in the supernatant were separated and quantitated by high-performance liquid chromatography (HPLC; ca. 2 ml injected) on a Nucleosil C-18 5-μm column (4.6 by 250 mm; Macherey-Nagel, Düren, Germany) run on a Gilson 305 gradient HPLC system and eluted at a flow rate of 1 ml min^{-1} with a linear gradient of 1 to 50% acetonitrile in 0.1 M potassium phosphate (pH 5.8). On-line quantitative detection of ^{14}C radioactivity during HPLC was performed as described elsewhere (40). Amounts of TMIBC, precorrin-6x, and factors were calculated from amounts of ^{14}C radioactivity detected in the corresponding peaks.

Trapping of the extruded acetic acid fragment. Incubations were run with [2,7,20-*methyl*-^{14}C]TMIBC (Fig. 2, compound 4c; prepared from unlabeled ALA and [*methyl*-^{14}C]SAM [2 μCi μmol^{-1}]) as the substrate. Incubation media were treated as described for assay 2, except that the radioactive peak found just after the exclusion peak of the HPLC column was collected (ca. 2 ml), neutralized by adding 400 μl of 1 N potassium hydroxide, and injected on a MonoQ HR 10/10 (Pharmacia) column previously washed with 10 ml of 2 N potassium hydroxyde and then equilibrated in water at a flow rate of 2 ml min^{-1}. After injection, the column was washed with water (50 ml) and elution was done with 10 mM potassium chloride. On-line quantitative detection of ^{14}C radioactivity during chromatography was performed as described above.

HPLC resolution of factor-IV epimers A and B, and evidence for biochemical interconversion. Factor-IV epimers A and B were separated by HPLC under isocratic conditions on a Nucleosil C-18 5-μm column (4.6 by 250 mm) eluted at a flow rate of 1 ml min^{-1} with 25% acetonitrile in 0.1% aqueous trifluoroacetic acid. Collected fractions containing factor-IV epimer A or epimer B were lyophilized and incubated at 30°C for 1 h at a final concentration of 5 μM in buffer A with a protein extract from G3575 (5 mg of protein) and 1 mM NADH in a 1-ml final volume. Incubations were stopped by adding 1 volume of 2 M hydrochloric acid, and after centrifugation of precipitated material, the supernatant (ca. 2 ml injected) was analyzed in the HPLC isocratic system described above. Control incubations without either NADH or protein were done in parallel.

Purification of precorrin-3B and resolution of epimers α and β. Precorrin-3B was purified as described for precorrin-8x (40) with the following modifications: all steps were carried out under an argon atmosphere, solvents were carefully deoxygenated by bubbling argon for several hours before use, and after the first chromatographic step on LiChroprep C-18 (Merck,

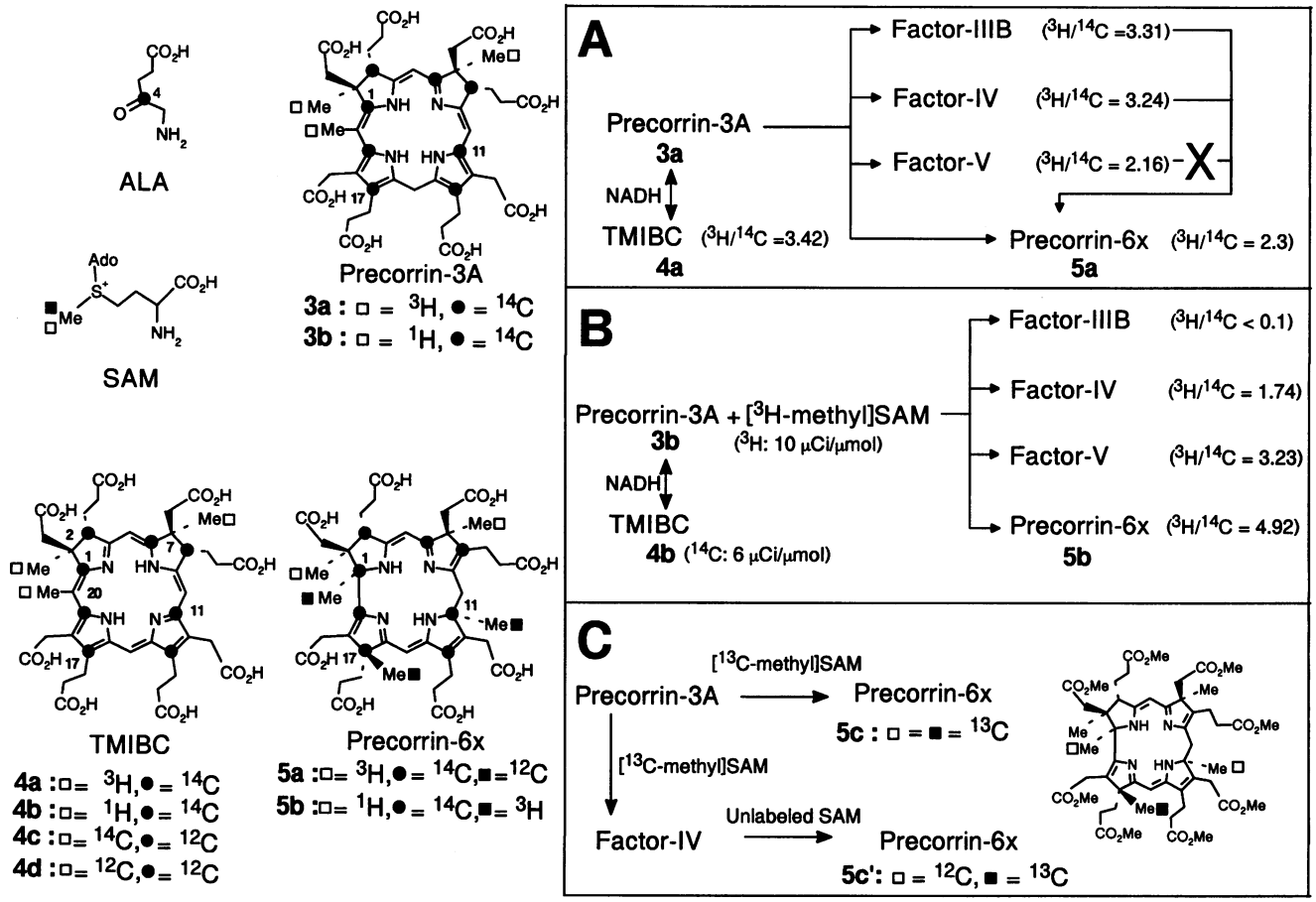

FIG. 2. Labeling experiments. (A) Preparation of ^{3}H,^{14}C-labeled factors from ^{3}H,^{14}C-labeled TMIBC, conversion into precorrin-6x, and loss of the C-20 methyl group; (B) extent of methylation of factors; (C) preparation of [*methyl*-^{13}C]precorrin-6x samples for NMR studies.

Darmstadt, Germany), precorrin-3B was directly purified in the desalting HPLC system (40). Epimers α and β were separated with the isocratic HPLC system used for resolution of factor-IV epimers (see above), except that the acetonitrile concentration was lowered to 17.5%.

Purification of CobF, CobG, CobJ, and CobM. CobF, CobG, CobJ, and CobM were separately purified from strains SC510(pXL1546), G3575(pXL325), G3575(pXL229), and G3575(pXL452), respectively. Fractions containing CobF were identified by SDS-PAGE analysis. Assays of CobG, CobJ, and CobM were carried out by using the conditions of assay 1, except that chromatographic fractions were incubated in the presence of 5 mg of the adequate complementation protein extract, i.e., protein extracts from G3575(pXL229) for CobG, from G3575(pXL325) for CobJ, and from G3575(pXL440) for CobM. Control blanks with complementation protein extracts alone were run in parallel. One unit of activity was defined as the amount of enzyme necessary to incorporate 1 nmol of methyl groups from SAM into pigments under the conditions of assays 1 and 2 described above. For each of the four protein purifications, proteins obtained from 5 g of wet cells of the relevant strain were first fractionated on a MonoQ HR 10/10 column (Pharmacia) with a linear 0 to 0.3 M gradient of potassium chloride in 50 mM Tris hydrochloride (pH 7.7)–1 mM dithiothreitol–15% (wt/vol) glycerol. The final purification of CobF was achieved by a gel permeation chromatographic step on a Bio-Sil SEC-250 column (Bio-Rad) identical to the final step used to purify CobL (7). For CobG, CobJ, and CobM purifications, active fractions from the MonoQ step were pooled, concentrated to 3 ml with Centriprep 10 concentrators (Amicon), mixed with 1 ml of 3.4 M ammonium sulfate, and chromatographed on a Phenyl-Superose HR 10/10 column (Pharmacia) eluted with a 0.85 to 0 M decreasing gradient of ammonium sulfate in buffer A. CobJ and CobM were further purified on a MonoQ HR 5/5 column eluted with a linear 0 to 0.3 M gradient of potassium chloride in buffer C, after desalting on PD10 columns (Pharmacia) equilibrated with 25 mM {1,3-bis[tris(hydroxymethyl)methylamino] propane} hydrochloride (pH 6.8)–1 mM dithiothreitol–15% (wt/vol) glycerol (buffer C). Final purification of CobM was achieved by gel permeation as described for CobF isolation.

Metal and sulfide analyses. CobG preparations were digested in concentrated nitric acid. Assays of Fe, Cu, Ni, Co, Mg, Mn, Ca, Zn, Cr, Mo, As, V, Se, Sr, Sn, and W in the resulting digestions were done by plasma emission spectroscopy with an Iris spectrometer (Thermo Jarrel Ash Corp., Franklin, Mass.). Fe concentrations were also determined by atomic absorption spectroscopy on a Perkin-Elmer 5100 graphite furnace spectrophotometer by using the method of standard additions. Acid-labile sulfide was quantitated by using the methylene blue formation assay described by Beinert (3).

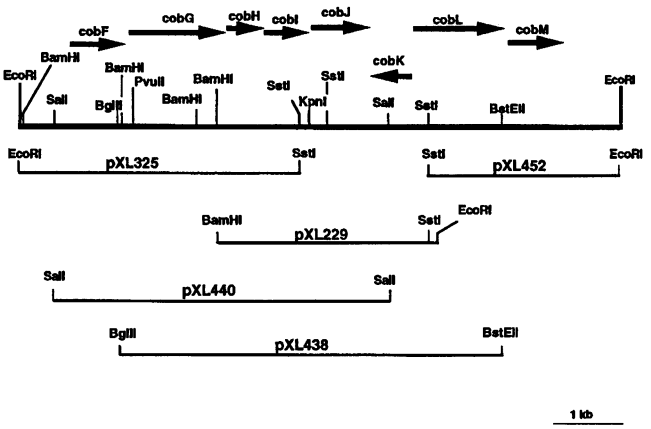

FIG. 3. Restriction map of the 8.7-kb *Eco*RI *P. denitrificans* DNA fragment carrying the *cobF* to *cobM* genes. The inserts of plasmids introduced into strain G3575 are shown.

RESULTS

Role of CobF, CobG, CobJ, and CobM and first evidence for a further trimethylated intermediate. The typical in vitro incubation system used to synthesize precorrin-6x from TMIBC contains SAM, NADH, and a protein extract from the Rif^r strain SC510(pXL253) (41). This extract catalyzes the NADH-dependent reduction of TMIBC into precorrin-3 and the subsequent conversion of precorrin-3 to precorrin-6x. Plasmid pXL253 carries the 8.7-kb *Eco*RI DNA insert containing eight *cob* genes, *cobF* to *cobM* (14). Available evidence (7, 10, 40) indicates that *cobF*, *cobG*, *cobJ*, and *cobM* certainly specify enzymes catalyzing reactions between precorrin-3 and precorrin-6x. To explore the relevant sequence of reactions, engineered SC510 strains amplifying *cobF*, *cobG*, *cobJ*, and *cobM* genes separately were constructed. A strain named G3575 was first constructed from the Rif^r strain SC510, in which genomic copies of *cobF* to *cobM* had been deleted. Several recombinant strains (Table 1) were then obtained by introducing plasmids carrying selected fragments of the 8.7-kb *Eco*RI DNA insert into strain G3575 (Fig. 3).

A first series of experiments was designed to detect which of these strains were still able to consume TMIBC (assay 2), to methylate it (assay 1), or to convert it into precorrin-6x (assay 2). The most salient observations done during this study (Table 2) were the following: (i) if one of the four genes mentioned above was missing, the ability to convert TMIBC into precorrin-6x was lost; (ii) only *cobG* was required to consume TMIBC; (iii) consumption of TMIBC was accompanied by methylation only if *cobJ* was present; and (iv) results obtained in assay 1 allow a classification of protein extracts in four classes which reflect the four expected biosynthetic methylation levels (Table 2, column 4) intervening between precorrin-3 and precorrin-6x. Importantly, these results indicate that the first intervening enzyme is CobG and that CobG catalyzes a reaction that is not a methylation. Therefore, the pathway to corrins involves a further trimethylated intermediate, and consequently, precorrin-3 must be renamed precorrin-3A according to the current nomenclature (45), while the new trimethylated intermediate is named precorrin-3B. The CobG-mediated reaction is followed by the three methyl transfers catalyzed by CobJ, CobM, and CobF, in this order. According to the established biosynthetic methylation sequence (11, 37, 45), these last three enzymes must introduce a methyl group at C-17, C-11, and C-1, respectively, from SAM, giving rise to precorrin-4, precorrin-5, and precorrin-6x. These conclusions, which were drawn from experiments with crude protein extracts, must be regarded as indications only and not rigorous proofs. They were, however, fully confirmed during subsequent studies with purified enzymes and intermediates (see below).

Factor-IIIB, factor-IV, and factor-V: detection, isolation, and labeling experiments. HPLC analyses (assay 2) revealed three new radioactive peaks in incubations that consumed TMIBC but produced no precorrin-6x, reflecting accumulation of unknown precorrin species. As demonstrated below, these products were not true precorrins but close derivatives which were named factors (18). One of these new peaks appeared in incubations with extract from G3575(pXL325), a strain expressing CobF, CobG, and CobH. In accordance with results outlined in the preceding paragraph, the corresponding factor was called factor-IIIB, this attached number indicating that it was related to the putative precorrin-3B. As shown in Table 2, factor-IIIB was no longer detected when both CobG and CobJ were present in the incubation mixture. Instead, another factor called factor-IV appeared, provided that CobM was not present. Finally, a third factor called factor-V accumulated, when CobG, CobJ, and CobM, but not CobF, were present. In parallel experiments done in the absence of SAM, factor-IV, factor-V, and precorrin-6x were no longer detected, CobG-dependent consumption of TMIBC was not hampered, and incubation mixtures accumulated factor-IIIB, provided that CobG was present. The amount of factor-IIIB thus produced was comparable to that amount of factor-IV, factor-V, or precorrin-6x formed in the corresponding experiments with SAM (data not shown).

By using the incubation conditions described in Materials and Methods, ^3H,^{14}C-labeled factor-IIIB, factor-IV, and factor-V were synthesized on a preparative scale (100-ml incubation volume) with appropriate protein extracts (see above; Table 2) from ^3H,^{14}C-labeled precorrin-3A (Fig. 2, compound 3a), which in turn had been generated via its aromatized form, TMIBC (compound 4a), from [4-^{14}C]ALA and [*methyl*-^3H]SAM. Factor-IIIB, factor-IV, and factor-V were purified by HPLC to a constant specific activity and a $^3H/^{14}C$ ratio as described for precorrin-8x (40). Conversion of these factors into precorrin-6x was carried out on a 20-ml scale by using protein extracts from the Rif^r strain SC510(pXL253) in the incubation conditions mentioned above.

Factor-IIIB and factor-IV were converted at a 20 to 40% yield into precorrin-6x (Fig. 2A). $^3H/^{14}C$ ratios and specific activities of precorrin-6x formed from both factors were the same as those of the precorrin-6x formed directly from ^3H,^{14}C-labeled TMIBC. Identity and amounts of precorrin-6x were established by HPLC and UV-visible spectrometry (38, 41). Factor-IIIB and factor-IV are therefore in vitro precursors of precorrin-6x. On the other hand, no transformation of factor-V into precorrin-6x was observed. Examination of the $^3H/^{14}C$ ratios reported in Fig. 2A clearly showed that both factor-IIIB and factor-IV still carried the C-20 methyl group because they had kept the same $^3H/^{14}C$ ratio (factor-IIIB, 3.31; factor-IV, 3.24) as that of TMIBC (compound 4a, 3.42). On the other hand, $^3H/^{14}C$ ratios of factor-V (2.16) and precorrin-6x (2.3) showed that they had lost the C-20 methyl group. Additional incubations with [2,7,20-*methyl*-^{14}C]TMIBC (compound 4c) as the substrate were done to trace the radioactive two-carbon fragment extruded during precorrin-6x formation, keeping in mind that the fragment extruded during cobyrinic acid biosynthesis in *P. shermanii* has been identified as acetic acid (2, 27). HPLC analyses of incubations affording either factor-V or precorrin-6x revealed the presence of one new radioactive peak found just after the exclusion peak of the HPLC C-18

TABLE 2. Transformation of TMIBC by protein extracts from recombinant strains of *P. denitrificans*

Strain(s) used	*cob* genes carried by the relevant plasmid[a]	ASSAY 1		ASSAY 2		
		methyl groups incorporated into pigments from SAM (nmol)[b]	methylation level of the product[c]	Disappearance of TMIBC (nmol)[b]	Production of precorrin-6x (nmol)[b]	New compound accumulated (nmol / species)[d]
G3575(pXL435)		-	3	-	-	-
SC510 Rif^r(pXL253)	**F G** H I **J** K L **M**	9 - 17	6	3 - 5	2.5 - 5	-
G3575(pXL325)	**F G** H	-	3	2 - 4	-	3 / factor-IIIB
G3575(pXL229)	H I **J** K	-	3	-	-	-
G3575(pXL440)	**F G** H I **J**	3 - 5	4	3 - 4	-	3.5 / factor-IV
G3575(pXL438)	**G** H I **J** K	2.5 - 3.5	4	2 - 3.5	-	2.5 / factor-IV
G3575(pXL452)	**M**	-	3	-	-	-
G3575(pXL440) + G3575(pXL452)[e]	**F G** H I **J** **M**	8- 14	6	3 - 6	2 - 5	-
G3575(pXL438) + G3575(pXL452)[e]	**G** H I **J** K **M**	5 - 9	5	2 - 5	-	3 / factor-V
G3575(pXL325) + G3575(pXL229)[e]	**F G** H H I **J** K	2 - 4	4	2 - 4	-	3 / factor-IV
G3575(pXL229) + G3575(pXL452)[e]	H I **J** K **M**	-	3	-	-	-
G3575(pXL325) + G3575(pXL452)[e]	**F G** H **M**	-	3	2 - 4	-	3 / factor-IIIB

[a] *cob* genes carried by the relevant plasmid are only indicated by the corresponding letters; the presence of the four genes of interest, *cobF*, *cobG*, *cobJ*, and *cobM*, is emphasized by using bold characters.

[b] Detection limit, 0.3 nmol. —, not detected. Extreme values were obtained from five replicates.

[c] Three methylations are required to convert precorrin-3 into precorrin-6x; from results of assay 1, protein extracts were classified as catalyzing either one, two, or the three methylation steps leading to tetra-, penta-, or hexamethylated species (45), respectively; when no methylation was detected, the biosynthetic methylation level remained unchanged (i.e., 3).

[d] Detection limit, 0.5 nmol; —, not detected. The results shown are averages of five replicates.

[e] Incubations with 5 mg of each protein extract.

column, which was absent in experiments accumulating factor-IIIB or factor-IV. The amount of radioactivity in this peak equaled half the amount found in factor-V or precorrin-6x. It was retained on a MonoQ HR 10/10 column under OH^- form equilibrated in water and quantitatively eluted by 10 mM potassium chloride (ca. 60 ml). Authentic [*methyl*-^{14}C]acetate used as a reference behaved identically both on the HPLC C-18 and MonoQ columns. On the grounds of earlier knowledge, it is thus highly likely that the radioactive material detected in these experiments is acetic acid, although the evidence is not absolutely firm. On the other hand, these results argue for acetate extrusion before the CobF-catalyzed step (which consists of at least methylation at C-1). Therefore, acetate extrusion would be done by CobM. However, we cannot exclude the possibility that the acetate fragment detected in our experiments is generated by chemical degrada-

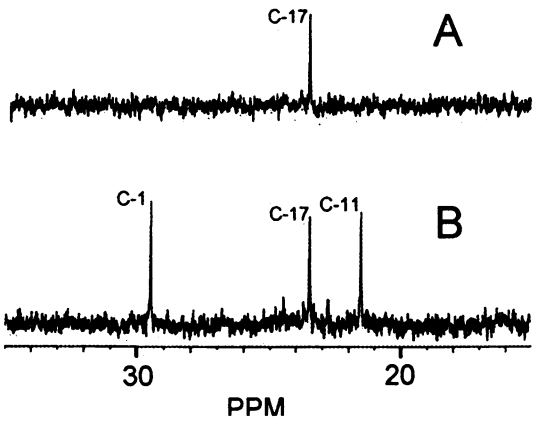

FIG. 4. Proton noise-decoupled ^{13}C NMR spectra of [17-*methyl*-^{13}C]precorrin-6x octamethylester (compound 5c'; A) and [1,11,17-*methyl*-^{13}C]precorrin-6x octamethylester (compound 5c; B).

tion of precorrin-5, e.g., during purification. This might explain why factor-V (which would be a degradation derivative of precorrin-5 in this case) was not converted into precorrin-6x.

In the experiment illustrated in Fig. 2B, precorrin-6x and factors were produced from ^{14}C-labeled TMIBC (compound 4b, 6 μCi μmol^{-1}) in the presence of [*methyl*-^{3}H]SAM (10 μCi μmol^{-1}). The measured $^{3}H/^{14}C$ ratio (i.e., 4.92) of the precorrin-6x produced (compound 5b) was in total agreement with the expected ratio (i.e., 5.0; three ^{3}H-labeled methyl groups from SAM incorporated into one molecule of ^{14}C-labeled precorrin-6x). In the same way, the measured $^{3}H/^{14}C$ ratios for factor-V (3.23), factor-IV (1.74), and factor-IIIB (<0.1) established that, during their formation from TMIBC, factor-V had incorporated two methyl groups, factor-IV had incorporated one, and factor-IIIB had incorporated none.

Factor-IV was synthesized from unlabeled TMIBC and [*methyl*-^{13}C]SAM and converted into precorrin-6x with unlabeled SAM. After esterification (40), the resulting precorrin-6x octamethyl ester (compound 5c' in Fig. 2C) was examined by proton noise-decoupled ^{13}C nuclear magnetic resonance (NMR) in $C_6{}^2H_6$ (Fig. 4). The spectrum showed only one signal corresponding to the methyl at C-17 against three with a sample of [1,11,17-*methyl*-^{13}C]precorrin-6x octamethyl ester (compound 5c) used as a reference (38), confirming that CobJ is the C-17 methylase.

Role of NADH in the reincorporation of factors by crude protein extracts. In absence of NADH, TMIBC, factor-IIIB, and factor-IV were not transformed as evidenced by assays 1 and 2, whereas precorrin-3A was converted in ≅60% yield into precorrin-6x under the same conditions. This result suggested that factor-IIIB and factor-IV are not true precorrins but oxidized derivatives generated by oxidation during the extraction-purification process. An NADH-dependent reducing system, analogous or identical to the known isobacteriochlorin-reducing system (10, 41) that allows TMIBC to reenter the pathway as precorrin-3A, was assumed to reintroduce these oxidation products into the pathway. Bearing in mind that precorrin intermediates are less-colored compounds than their oxidized counterparts, spectroscopic evidence of the reduction was obtained by the following experiment. TMIBC, factor-IIIB, or factor-IV was incubated with protein extracts from strain G3575. The color due to the tetrapyrrolic substrate disappeared within 1 h in incubations with NADH but remained unchanged in incubations without NADH. After protein precipitation with hydrochloric acid, the oxidized, colored substrate was quantitatively recovered in the NADH-containing incubations, as estimated by subsequent HPLC analysis. This experiment also showed that the reduction of TMIBC was not effected by CobG or any other Cob enzyme encoded by the 8.7-kb *Eco*RI DNA fragment, since strain G3575 does not express any of these proteins.

Heterogeneity of factor-IV. When analyzed by HPLC under isocratic conditions, factor-IV could be resolved in a mixture of two major compounds named A and B (A, ca. three parts; B, ca. two parts). The labeling studies described above were repeated with each compound, both giving the same results as the mixture. In particular, the rates of conversion into precorrin-6x were identical. Factor-IV (compound A) was a blue pigment and showed λ_{max}/nm values (H_2O–0.1% trifluoroacetic acid) of 369 (ε_{rel} = 1.00), 467 (0.11), 498 (0.11), 590 (0.34), and 629 (0.42). Compound B exhibited the same UV-visible-light spectrum, except for a slight 3-nm longwave shift of the secondary maximum to 632 nm. Both compounds showed by fast-atom bombardment–mass spectrometry a molecular weight equivalent to an *m/z* of 906, corresponding to the molecular formula $C_{44}H_{50}N_4O_{17}$, and the methyl ester derivatives (40) showed an *m/z* of 1,018. This clearly indicated that both compounds were octa acids and probably epimeric species. Purified isomers A and B were not converted to each other when they were reextracted and repurified by HPLC. However, each purified isomer gave the original mixture back (A/B ratio = 3:2 after HPLC) upon incubations with both NADH and a protein extract from strain G3575. From these observations, we inferred that NADH-dependent reduction of isomers A and B by G3575 protein extract led to the same product, precorrin-4, and that chemical oxidation of precorrin-4 occurring during isolation afforded isomers A and B with the observed ratio. This lent support indirectly to the view that A and B were epimers, the asymmetric center arising from double-bond migration during the oxidative purification procedure. Our structural studies on factor-IV have recently established that A and B are indeed epimers, probably at C-8 (42).

Purification of CobF, CobG, CobJ, and CobM. CobG was purified from strain G3575(pXL325) on the basis of its ability to complement extracts from G3575(pXL229) for the synthesis of factor-IV from TMIBC with assay 1 (Table 2). Conversely, CobJ was purified from G3575(pXL229) on the basis of its ability to complement extracts from G3575(pXL325). A similar approach was used to purify CobM, since protein extract from G3575(pXL452) did not show any activity in assay 1 but enhanced the response of protein extract from G3575 (pXL440) in the same assay. CobF was overexpressed in the Rif^r strain SC510(pXL1546) and thus easily purified to homogeneity on a molecular weight basis. In assay 2 with factor-IV as the substrate, pure CobF complemented protein extracts from G3575(pXL452) for the conversion of factor-IV to precorrin-6x. Each protein was obtained in a homogeneous state (>95%) as judged by SDS-PAGE (no contaminating band revealed by silver staining) and clean NH_2 terminus sequencing (no other sequence detected at a level of 5%). Table 3 provides purification data for CobG, CobJ, and CobM. As estimated by SDS-PAGE analysis, CobF was purified sixfold with an overall yield of about 90%. Molecular weights for the denaturated proteins were determined by SDS-PAGE: CobG, 46,000 ± 2,000; CobJ, 27,000 ± 1,000; CobM, 31,000 ± 2,000; CobF, 31,000 ± 2,000. These molecular weights and the four N-terminal sequences (15 amino acids; data not shown) were in total agreement with those predicted from the DNA sequences of the corresponding genes (14), except that the amino-terminal methionines appear to be processed off in the four mature proteins.

TABLE 3. Purification of CobG, CobJ, and CobM

Protein	Purification step	Vol (ml)	Amt of protein (mg)	Sp act[a] (U mg of protein^{-1})	Recovery (%)	Purification (fold)
CobG from G3575(pXL325)	Crude extract	20	370	0.7		
	MonoQ HR 10/10	18	24	7.8	72	11
	Phenyl-Superose	3	3.6	48	66	68
CobJ from G3575(pXL229)	Crude extract	22	450	4		
	MonoQ HR 10/10	18	34	38	67	9.5
	Phenyl-Superose	3	2.8	340	53	85
	MonoQ HR 5/5	2	0.33	2,200	40	550
CobM from G3575(pXL452)	Crude extract	20	380	5.4		
	MonoQ HR 10/10	18	24	70	82	13
	Phenyl-Superose	3	1.2	750	44	140
	MonoQ HR 5/5	0.8	0.17	3,700	31	690
	Bio-Sil SEC-250	0.7	0.06	6,500	19	1,200

[a] Specific activities were determined with 0.5 to 1 U of enzyme.

Conversion of precorrin-3A with pure enzymes. Pure CobG catalyzed the conversion of precorrin-3A to precorrin-3B. The yield was about 2 nmol of precorrin-3B (identified as factor-IIIB) per 100 μg (2.1 nmol) of protein, thus indicating that CobG transformed about one equivalent of precorrin-3A. The addition of CobJ (2.5 to 5 μg of CobJ per 100 μg of CobG) and SAM resulted in a quantitative shift to precorrin-4. Incubation mixtures containing the four purified enzymes synthesized low amounts of precorrin-6x (<0.2 nmol) in a poorly reproducible manner but always accumulated precorrin-4 (>1.5 nmol), despite the fact that CobM was added in excess (up to 5 μg). The addition of an oxygen-consuming system consisting of glucose-glucose oxidase-catalase (8) prevented oxidation of precorrin-3A during incubation but did not significantly increase CobG activity. However, this system resulted in reproducibly higher yields of synthesized precorrin-6x (ca. 1 nmol), provided that none of the three components of the oxygen-consuming system was omitted. This study proved that the in vitro conversion of precorrin-3A to precorrin-6x involves only the four enzymes specified by *cobG*, *cobJ*, *cobM*, and *cobF*. In the above conditions, TMIBC was not transformed at all.

Isolation and characterization of precorrin-3B. Preparative-scale incubations used previously for preparing factor-IIIB from ^{14}C-labeled TMIBC (Fig. 2, compound 4b) were used to search out precorrin-3B. By using the HPLC procedure especially designed for this purpose (see Materials and Methods), we isolated a new radioactive compound which was quantitatively converted into precorrin-6x by protein extracts from the Rif^r strain SC510(pXL253). When incubated at 1 μM with only SAM (200 μM) and purified CobJ (5 μg ml^{-1}), this compound was converted with an 85% yield in 1 h into precorrin-4 (identified as factor-IV by HPLC and UV-visible-light spectroscopy), providing evidence that the newly isolated compound was precorrin-3B. In contact with air, precorrin-3B changed into factor-IIIB within a few minutes.

Precorrin-3B was resolved into two compounds named α and β by HPLC under isocratic elution conditions. Both compounds were converted into precorrin-4 with the same yields and steady-state rates. Ratios of epimer A/epimer B found in factor-IV formed from either α or β were identical (i.e., 3:2).

α was a pale-yellow compound and showed a λ_{max}/nm (H_2O–0.1% trifluoroacetic acid) of 376 (ε, 17,000 ± 2,000 M^{-1} cm^{-1}). β exhibited the same UV-visible-light spectrum but was 3 nm longwave shifted, suggesting that α and β are epimers. α and β exhibited the same molecular weight equivalent to an *m/z* of 894 by fast-atom bombardment–mass spectrometry, corresponding to the molecular formula $C_{43}H_{50}N_4O_{17}$. Therefore, precorrin-3B has one more oxygen atom than precorrin-3A and is at the same level of oxidation as precorrin-6x plus acetic acid, the extruded two-carbon fragment. This demonstrates that CobG carries out the oxidation which is known to occur during the conversion of precorrin-3A into precorrin-6x (38, 41). Subsequent structural studies confirmed that α and β are epimeric, probably at C-3 (17).

CobG is an iron-sulfur (Fe-S) protein. CobG was a brown-green protein. Its UV-visible-light spectrum exhibited a maximum A_{400} reminiscent of an iron-sulfur (Fe-S) protein. One mole of CobG was found to contain 4.6 ± 0.5 mol of Fe, less than 0.15 mol of Cu, and less than 0.05 mol of Ni, Co, Mg, Mn, Ca, Zn, Cr, Mo, As, V, Se, Sr, Sn, and W. Freshly prepared protein samples contained 4.1 ± 0.6 mol of inorganic sulfur per mol of CobG. These data along with the absence of absorbance maxima between 500 and 650 nm suggest that Fe atoms are present as nonheme iron in Fe-S clusters. Upon handling or storing CobG preparations, the sulfur content, the A_{400}, and the enzymatic activity dropped simultaneously. Whatever the preparation studied, the A_{400} coefficient per mol of inorganic sulfur was in the range of 3,900 to 4,500 cm^{-1} M^{-1}. The preparations of purified CobG showing the highest activity exhibited a molar absorption coefficient of 18,000 cm^{-1} M^{-1} at 400 nm and an A_{400}/A_{280} ratio of 0.36. These data indicate that CobG is an Fe-S protein containing most probably four iron and four sulfur atoms in one [4Fe-4S] or two [2Fe-2S] clusters. The C-terminal domain of CobG displays weak similarity (20 to 25%; data not shown) to the domains postulated to provide the ligands of the [4Fe-4S] cluster in *S. typhimurium* and *E. coli* sulfite reductase hemoprotein CysI (EC 1.8.1.2), spinach ferredoxin-nitrite reductase (EC 1.7.7.1), and cyanobacterial ferredoxin-sulfite reductase (EC 1.8.7.1) (23, 30).

Fourier-transformed infrared spectroscopy and extensive ^{13}C-labeling experiments in combination with NMR studies have rigorously established the structure of precorrin-3B (Fig. 5) (17), thus confirming that the CobG-catalyzed reaction is oxidative. In vitro conversion of precorrin-3A to precorrin-3B has been carried out during this study with purified CobG preparations without any added cofactor. Therefore, CobG was the actual electron acceptor for the reaction, and the enzyme could not be oxidized back to its original state for a further catalytic turn. This explains why pure CobG alone

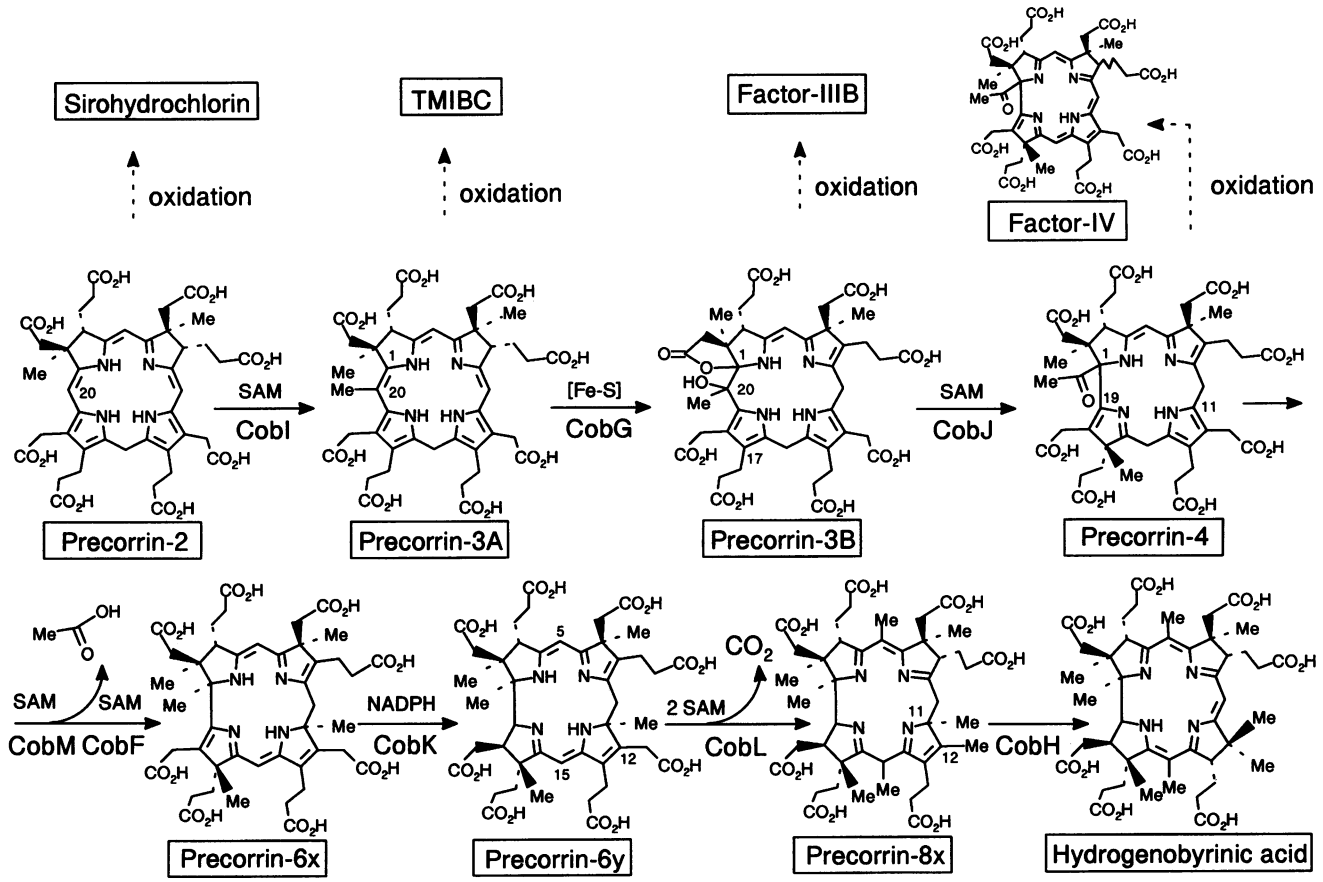

FIG. 5. Biosynthesis of the corrin hydrogenobyrinic acid in *P. denitrificans*. Enzymes, cofactors, intermediates, and oxidized derivatives are shown.

could not transform more than one equivalent of precorrin-3A. In this regard, it is noteworthy that CobG activity either alone or in crude extracts was not significantly increased in the presence of common redox cofactors such as NAD^+ (1 mM), $NADP^+$ (1 mM), flavin adenine dinucleotide (50 μM), and flavin mononucleotide (50 μM). Therefore, the ultimate electron acceptor remains to be discovered.

It is well established that Fe-S clusters are involved not only in redox processes (as electron carriers or as active centers of hydrogenases [4]) but also as active centers of a group of enzymes that are not involved in oxidation-reduction, i.e., some (de)hydrases of the aconitase type (4). Therefore, if it is plausible to assume that the Fe-S cluster of CobG participates in redox reactions during precorrin-3B formation, one cannot rule out the possibility that it plays a role in a hypothetical hydration of precorrin-3A.

DISCUSSION

This report reveals the existence of a quite unexpected further trimethylated precursor of coenzyme B_{12}, precorrin-3B. It also provides the first biochemical data on CobF, CobG, CobJ, and CobM, the four enzymes that catalyze the conversion of precorrin-3A (previously named precorrin-3) into precorrin-6x, and sheds light on the individual step each of these enzymes catalyzes. When combining these results with the structural information gained recently on factor-IV and precorrin-3B (17, 42) and the conclusions of previously reported studies on the conversion of precorrin-6x (7, 10, 40), we can draw for the first time a step-by-step sequence of the enzymatic reactions involved in the biosynthesis of the corrin ring (Fig. 5). After precorrin-2, the last precursor common to siroheme, coenzyme F_{430}, heme d_1, and coenzyme B_{12} (21, 26) (Fig. 1), the pathway specifically dedicated to corrinoids is initiated by the introduction of the C-20 methyl group which is expelled later along with the C-20 bridge carbon in the form of acetic acid, as a result of the ring contraction process (2, 27). The C-20 methylation reaction is catalyzed by CobI and yields precorrin-3A (39, 46). This one is converted to precorrin-3B by an Fe-S enzyme, CobG, which catalyzes a complex oxidative reaction involving C-20 hydroxylation and γ-lactone formation from ring-*A* acetate to C-1. It is possible that precorrin-3B is generated first as a δ-lactone at C-20 with which the γ-lactonic intermediate could be in rapid exchange (17). The next step, the ring contraction itself, is catalyzed by CobJ, a typical methyltransferase. The entire ring contraction process seems to be initiated by the methylation at C-17. It leads to precorrin-4 with an acetyl group at C-1 (42) and not at C-19 as previously assumed (2, 20, 27). Precorrin-4, which has been isolated in its oxidized form only, must have the structure given in Fig. 5 or one of its double-bond tautomers. Precorrin-4 is methylated at C-11 by CobM, and after removal of the acetyl group, the CobF-dependent methylation at C-1 gives precorrin-6x. Our study did not unambiguously establish which one of the two enzymes (CobM or CobF) removes the acetyl group, although factor-V has lost this group. Nevertheless, it is

noteworthy that, as for the ring contraction process, the deacylation reaction is effected by a typical methyltransferase, CobM or CobF. The CobK-catalyzed NADPH-dependent reduction of precorrin-6x affords a further hexamethylated intermediate (10, 44), precorrin-6y. Precorrin-6y is, in turn, methylated at C-5 and C-15 and decarboxylated at C-12 by a bifunctional protein which is the product of the *cobL* gene, to give precorrin-8x (7, 43). This one is finally rearranged by a (probably intramolecular) C-11 to C-12 methyl migration catalyzed by CobH (40) to hydrogenobyrinic acid, the simplest intermediate having the corrin ring system.

It has been proposed that vitamin B_{12} is an evolutionarily ancient cofactor (5, 20). The unexpected features of the ring contraction process revealed during this study open the way to further explore the possibility of prebiotic emergence of the corrinoid template suggested by Eschenmoser (20). In this respect, it is noteworthy that precorrin-3B formation involves an Fe-S cluster, a structure belonging to the most ancient biologically active metal cofactors (4), and it is also very interesting that the ring contraction is catalyzed by CobJ, a typical methyltransferase, thus suggesting that ring contraction is triggered by C-17 methylation.

It has been shown that carbonyl oxygen label exchange occurs at the acetamido group of ring-*A* during coenzyme B_{12} biosynthesis in *P. shermanii* (24, 35), thus providing the first experimental support for Eschenmoser's hypothesis which assumes that a lactone involving a peripheral acetate chain may play a key role in the ring contraction process (20). If such a label exchange also occurs during ring contraction in *P. denitrificans*, a study of the origin and fate of oxygen atoms bound to C-1 and C-20 in precorrin-3B will be extremely useful for elucidating the mechanism of this fascinating reaction.

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