JOURNAL OF BACTERIOLOGY, Feb. 1992, p. 1050–1052
0021-9193/92/031050-03$02.00/0

Vol. 174, No. 3

NOTES

Biosynthesis of Vitamin B_{12} in *Pseudomonas denitrificans*: the Biosynthetic Sequence from Precorrin-6y to Precorrin-8x Is Catalyzed by the *cobL* Gene Product

FRANCIS BLANCHE,[1]* ALAIN FAMECHON,[1] DENIS THIBAUT,[1] LAURENT DEBUSSCHE,[1] BEATRICE CAMERON,[2] AND JOEL CROUZET[2]

Département Analyse[1] and Unité de Biologie Moléculaire, Institut des Biotechnologies,[2] Centre de Recherche de Vitry-Alfortville, Rhône-Poulenc Rorer S.A., B.P. 14, 94403 Vitry-sur-Seine Cedex, France

Received 29 July 1991/Accepted 18 November 1991

A protein catalyzing methylation at C-5 and C-15 and decarboxylation of the acetic acid side chain at C-12 on precorrin-6y to yield precorrin-8x was purified to homogeneity from a recombinant strain of *Pseudomonas denitrificans*. It was sequenced at the N terminus and shown to be encoded by the *cobL* gene.

Previous studies on a recombinant strain of *Pseudomonas denitrificans* have led to the isolation (12) and structure elucidation (9) of precorrin-6x (see structures in Fig. 1), a precursor of the corrin hydrogenobyrinic acid (3, 6). The enzymatic reactions involved in the conversion of precorrin-6x to hydrogenobyrinic acid have been investigated. Two of these reactions are described in the accompanying reports (2, 11). The first one, catalyzed by the *cobK* gene product, is the NADPH-dependent reduction of precorrin-6x to a dihydro derivative, called precorrin-6y (2, 14). The other reaction is a rearrangement into hydrogenobyrinic acid of its isomeric ultimate precursor, named precorrin-8x (11). This reaction is catalyzed by the product of the *cobH* gene (11). The present note reports on the study of the enzymatic conversion of precorrin-6y to precorrin-8x. Based on the structural dissimilarities between these two compounds, this conversion is expected to involve three biosynthetic events (Fig. 2): *S*-adenosyl-L-methionine (SAM)-dependent methylation at C-5 and C-15 and decarboxylation of the acetic acid side chain at C-12.

Precorrin-6y-dependent methyltransferase activity was assayed by incubating protein fractions (0.1 to 0.2 U of activity) for 1 h at 30°C in 250 μl of Tris hydrochloride (pH 7.7)–1 mM EDTA (buffer A), containing precorrin-6y (1 nmol) (2) and [*methyl*-^{3}H]SAM (6.25 nmol; 100 nCi nmol^{-1}). The reaction was stopped by heating the incubation mixture at 80°C for 10 min, and after centrifugation (5,000 × *g* for 10 min), radioactive tetrapyrrolic acids were separated from SAM on a DEAE-Sephadex column, eluted with 1 N hydrochloric acid, and counted as previously described (1). One unit of activity was defined as the amount of enzyme necessary to transfer 1 nmol of methyl group per h under the conditions described above.

It was found that an amplified precorrin-6y-dependent methyltransferase activity (about 2.0 U mg of protein^{-1} versus 0.1 U mg^{-1}) was detected in cell extracts, prepared as previously described (1), from *P. denitrificans* SC510 Rif^r harboring plasmid pXL253 (4). This plasmid amplifies *P. denitrificans cobF* to *cobM* genes, eight cobalamin biosynthetic genes arranged in alphabetical order on the same strand, except *cobK*, which is on the complementary strand (2, 4). The purification of this C-5 and/or C-15 methyltransferase activity from extracts of SC510 Rif^r(pXL253) (10) was undertaken. Since precorrin-6y is the end product of precorrin-6x transformation in SAM-free incubation systems from *P. denitrificans* (2), we assumed that methylation at C-5 and/or C-15 occurs before decarboxylation. Proteins (109 mg) obtained from 1 g of wet cells were first fractionated on a Mono Q HR 10/10 column (Pharmacia) with a 135-ml linear 0 to 0.4 M gradient of potassium chloride in 0.1 M Tris hydrochloride (pH 7.7)–0.5 mM dithiothreitol–15% glycerol (wt/vol) (buffer B). Active fractions were pooled, concentrated to 1 ml with Centricon 10 microconcentrators (Amicon), diluted with 2 volumes of buffer B, and chromatographed on a Mono Q HR 5/5 column (Pharmacia) eluted

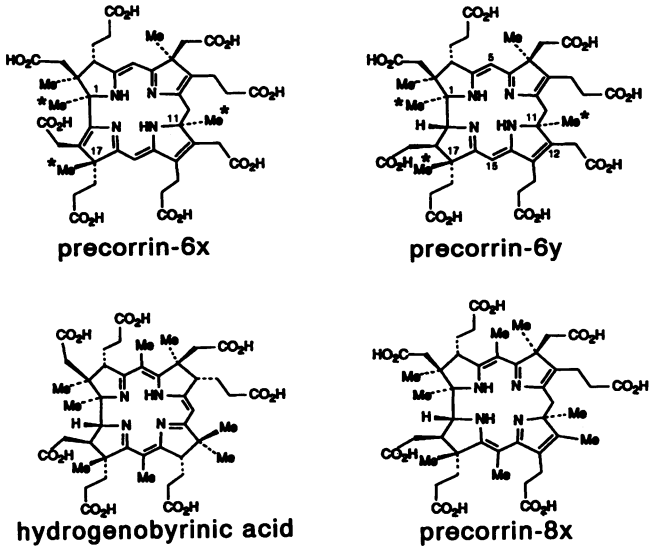

FIG. 1. Structures. *, Positions of labels in ^{14}C-labeled species. Me, methyl.

* Corresponding author.

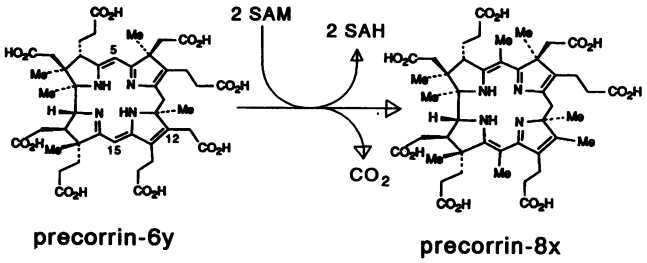

FIG. 2. Biosynthesis of precorrin-8x from precorrin-6y in *P. denitrificans*. Me, methyl; SAH, *S*-adenosyl-L-homocysteine.

with a 30-ml 0 to 0.4 M gradient of potassium chloride in buffer B (pH 7.5). The fraction containing activity was mixed with 1 volume of 1.7 M ammonium sulfate and chromatographed on a Phenyl-Superose HR 5/5 column (Pharmacia) eluted with a 0.85 to 0 M linear decreasing gradient of ammonium sulfate in buffer B. The active fraction was concentrated to 150 μl with a Centricon 10 microconcentrator and fractionated on a Bio-Sil SEC-250 column (600 by 7.8 mm) (Bio-Rad) at 0.4 ml min^{-1} in 20 mM potassium phosphate–50 mM sodium sulfate, pH 6.8. The protein was purified 170-fold with a 34% yield (Table 1). Using previously described procedures (1, 10), it was shown to be homogeneous (purity, ≥98%) and exhibited the following characteristics: a K_m for precorrin-6y of 2.2 ± 0.3 μM and a V_{max} of 750 ± 40 U mg^{-1} at 100 μM SAM, a molecular weight of 43,000 ± 1,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3), an apparent molecular weight of 320,000 ± 20,000 by gel permeation high-performance liquid chromatography (HPLC) (on a Bio-Sil SEC-250 column), a single N-terminal amino acid sequence (NH_2-ADVSNSE), and a single symmetrical peak when analyzed by reverse-phase HPLC (data not shown).

The *cobL* gene carried by pXL253 has been shown (4) to encode a protein of M_r 42,900 having the same amino-terminal sequence as the protein studied here; therefore, *cobL* is the structural gene of this protein. In agreement with this conclusion, precorrin-6y-dependent methyltransferase activity was found unamplified in cell extracts of SC510 Rif^r harboring pXL1503, a plasmid corresponding to pXL253 with the Ω-Sp^r fragment (7) cloned into the *Sst*I site internal to the *cobL* gene. The spectrum of CobL showed one maximum at 280 nm (ε, 6.9 × 10^4 M^{-1} cm^{-1} $subunit^{-1}$) and no absorbance in the visible region. To identify the product(s) of CobL activity, [1,11,17-*methyl*-^{14}C]precorrin-6y (25 nmol; 4.0 nCi $nmol^{-1}$; prepared from [1,11,17-*methyl*-^{14}C]precorrin-6x as described elsewhere (2); see positions of labels in Fig. 1) was incubated for 3 h at 30°C with SAM (1.0 μmol) and pure CobL (100 U) in 5 ml of buffer A. The incubation mixture was acidified to pH ≈ 1 with hydrochloric acid and centrifuged. Tetrapyrrolic acids were concentrated on a small column of LiChroprep RP-18 (Merck), eluted with water–*t*-butyl alcohol (4:1), and separated by reverse-phase HPLC as described elsewhere (11). One main product was obtained (13.5 nmol; 54% yield). It was shown by HPLC, UV-visible-light spectroscopy, and enzymatic conversion into hydrogenobyrinic acid by pure CobH (45% yield) (11) to be identical with authentic precorrin-8x prepared as described elsewhere (11). Degradation products of precorrin-8x accounted for the remainder of the radioactivity. This result established that CobL not only catalyzes methylation at homologous positions at C-5 and C-15, but also decarboxylates the acetic acid side chain at C-12. In SAM-free incubation mixtures, precorrin-6y was not transformed by pure CobL, whereas it was routinely converted to precorrin-8x in ≥50% yield in the control with SAM. This result indicated that at least one methylation precedes C-12 decarboxylation. The difficulty of establishing the order of methylation at C-5 and C-15 by pulse-labeling experiments (3, 8, 13) and the failure to isolate a monomethylated derivative of precorrin-6y during this study argue for a sequence with decarboxylation at C-12 occurring after methylations at both positions C-5 and C-15.

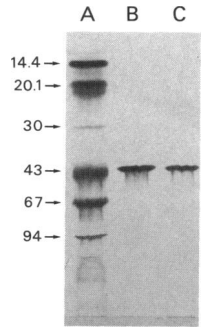

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified CobL. Lane A, molecular weight markers (×10^3) (50 to 100 ng per band); lanes B and C, pure CobL (100 and 50 ng, respectively). Gel stained with silver.

TABLE 1. Purification of CobL from *P. denitrificans* SC510 Rif^r(pXL253)[a]

Purification step	Vol (ml)	Amt of protein (mg)	Sp act (U mg of protein^{-1})	Recovery (%)	Purification (fold)
Crude extract	7.0	109	2.0		
Mono Q 10/10	10.0	4.5	36	74	18
Mono Q 5/5	3.5	2.1	67	65	33
Phenyl-Superose	5.7	0.48	170	37	85
Bio-Sil SEC-250	1.7	0.22	340	34	170

[a] Grown in 250-ml Erlenmeyer flasks containing 25 ml of PS4 medium (10).

Putative SAM-methyltransferase homologous sequences have been identified on the amino-terminal one-half of the CobL protein (4). Compared with other established (SAM: uroporphyrinogen III methyltransferase, SAM:precorrin-2 methyltransferase) or proposed (CobF, CobJ, CobM) methyltransferases of the corrin pathway (4, 5), CobL has an additional carboxy-terminal domain of M_r ≈ 20,000 (4) which probably carries C-12 decarboxylase activity. This suggests that *cobL* results from a fusion between ancestral genes encoding C-5/C-15 methylase and C-12 decarboxylase activities separately. Four of the eight C-methylations occurring during the biosynthesis of the corrin macrocycle have now been shown to be performed by two enzymes (at C-2 and C-7 by SAM:uroporphyrinogengen III methyltransferase and at C-5 and C-15 by CobL), whereas the methyltransfer at C-20 is catalyzed by SAM:precorrin-2 methyltransferase. The remaining three methylations at C-1, C-11, and C-17 are likely to be catalyzed by the CobF, CobJ, and CobM proteins (4). Therefore, only six methyltransferases are required to perform the eight methylation reactions in *P. denitrificans*.

D. Faucher and F. Cuine are acknowledged for sequencing of CobL.

REFERENCES

1. **Blanche, F., L. Debussche, D. Thibaut, J. Crouzet, and B. Cameron.** 1989. Purification of *S*-adenosyl-L-methionine:uroporphyrinogen III methyltransferase from *Pseudomonas denitrificans*. J. Bacteriol. **171**:4222–4231.
2. **Blanche, F., D. Thibaut, A. Famechon, L. Debussche, B. Cameron, and J. Crouzet.** 1992. Precorrin-6x reductase from *Pseudomonas denitrificans*: purification and characterization of the enzyme and identification of the structural gene. J. Bacteriol. **174**:1036–1042.
3. **Blanche, F., D. Thibaut, D. Frechet, M. Vuilhorgne, J. Crouzet, B. Cameron, G. Müller, K. Hlineny, U. Traub-Eberhard, and M. Zboron.** 1990. Hydrogenobyrinic acid: isolation, biosynthesis, and function. Angew. Chem. Int. Ed. Engl. **29**:884–886.
4. **Crouzet, J., B. Cameron, L. Cauchois, S. Rigault, M.-C. Rouyez, F. Blanche, D. Thibaut, and L. Debussche.** 1990. Genetic and sequence analysis of an 8.7-kilobase *Pseudomonas denitrificans* fragment carrying eight genes involved in transformation of precorrin-2 to cobyrinic acid. J. Bacteriol. **172**:5980–5990.
5. **Crouzet, J., L. Cauchois, F. Blanche, L. Debussche, D. Thibaut, M.-C. Rouyez, S. Rigault, J.-F. Mayaux, and B. Cameron.** 1990. Nucleotide sequence of a *Pseudomonas denitrificans* 5.4-kilobase DNA fragment containing five *cob* genes and identification of structural genes encoding *S*-adenosyl-L-methionine:uroporphyrinogen III methyltransferase and cobyrinic acid *a*,*c*-diamide synthase. J. Bacteriol. **172**:5968–5979.
6. **Podschun, T. E., and G. Müller.** 1985. Hydrogenobyrinic acid and vitamin B_{12}. Angew. Chem. Int. Ed. Engl. **24**:46–47.
7. **Prentki, P., and H. M. Krisch.** 1984. *In vitro* insertional mutagenesis with a selectable DNA fragment. Gene **29**:303–313.
8. **Scott, A. I., H. J. Williams, N. J. Stolowich, P. Karuso, M. D. Gonzalez, G. Müller, K. Hlineny, E. Savvidis, E. Schneider, U. Traub-Eberhard, and G. Wirth.** 1989. Temporal resolution of the methylation sequence of vitamin B_{12} biosynthesis. J. Am. Chem. Soc. **111**:1897–1900.
9. **Thibaut, D., F. Blanche, L. Debussche, F. J. Leeper, and A. R. Battersby.** 1990. Biosynthesis of vitamin B_{12}: structure of precorrin-6x octamethyl ester. Proc. Natl. Acad. Sci. USA **87**: 8800–8804.
10. **Thibaut, D., M. Couder, J. Crouzet, L. Debussche, B. Cameron, and F. Blanche.** 1990. Assay and purification of *S*-adenosyl-L-methionine:precorrin-2 methyltransferase from *Pseudomonas denitrificans*. J. Bacteriol. **172**:6245–6251.
11. **Thibaut, D., M. Couder, A. Famechon, L. Debussche, B. Cameron, J. Crouzet, and F. Blanche.** 1992. The final step in the biosynthesis of hydrogenobyrinic acid is catalyzed by the *cobH* gene product with precorrin-8x as the substrate. J. Bacteriol. **174**:1043–1049.
12. **Thibaut, D., L. Debussche, and F. Blanche.** 1990. Biosynthesis of vitamin B_{12}: isolation of precorrin-6x, a metal-free precursor of the corrin macrocycle retaining five *S*-adenosylmethionine-derived peripheral methyl groups. Proc. Natl. Acad. Sci. USA **87**:8795–8799.
13. **Uzar, H. C., A. R. Battersby, T. A. Carpenter, and F. J. Leeper.** 1987. Biosynthesis of porphyrins and related macrocycles. Part 28. Development of a pulse labelling method to determine the *C*-methylation sequence for vitamin B_{12}. J. Chem. Soc. Perkin Trans. I **1987**:1689–1696.
14. **Weaver, G. W., F. J. Leeper, A. R. Battersby, F. Blanche, D. Thibaut, and L. Debussche.** 1991. Biosynthesis of vitamin B_{12}: the site of reduction of precorrin-6x. J. Chem. Soc. Chem. Commun. **1991**:976–979.