JOURNAL OF BACTERIOLOGY, Nov. 1992, p. 7452–7454
0021-9193/92/227452-03$02.00/0

Vol. 174, No. 22

NOTES

Purification and Characterization of Cob(II)yrinic Acid *a,c*-Diamide Reductase from *Pseudomonas denitrificans*

FRANCIS BLANCHE,* LAURENT MATON, LAURENT DEBUSSCHE, AND DENIS THIBAUT

Département Analyse, Centre de Recherche de Vitry-Alfortville, Rhône-Poulenc Rorer S.A., B.P. 14, 94403 Vitry-sur-Seine Cedex, France

Received 15 May 1992/Accepted 5 September 1992

An NADH-dependent flavoenzyme exhibiting cob(II)yrinic acid *a,c*-diamide reductase activity was purified 6,300-fold to homogeneity from *Pseudomonas denitrificans* and sequenced at its N terminus. This enzyme of the cobalamin biosynthetic pathway reduced to the Co(I) state all of the Co(II)-corrinoids isolated from this microorganism.

In bacteria, the enzymatic conversion of aquacobalamin to its coenzyme form, 5'-deoxy-5'-adenosyl (Ado)-cobalamin (Fig. 1), is a three-step process involving reduction of Co(III) in aquacobalamin to Co(II) by aquacobalamin reductase (EC 1.6.99.8) (Fig. 2, reaction 1), reduction of Co(II) to Co(I) by cob(II)alamin reductase (EC 1.6.99.9) (reaction 2), and ATP-dependent Coβ-adenosylation of Co(I) by cob(I)alamin adenosyltransferase (EC 2.5.1.17) (reaction 3) (16, 19).

During coenzyme B_{12} biosynthesis in *Pseudomonas denitrificans*, addition of the adenosyl group as the axial upper (Coβ) ligand of cobalt occurs on cob(I)yrinic acid *a,c*-diamide (Fig. 1) (1, 11); the enzyme catalyzing this reaction has been purified to homogeneity in our laboratory (11). In addition, our recent studies (12) have shown that hydrogenobyrinic acid *a,c*-diamide (Fig. 1) is the substrate of cobaltochelatase and that the product of this enzyme's activity is cob(II)yrinic acid *a,c*-diamide (Fig. 1). Taken together, these results imply the existence of a cob(II)yrinic acid *a,c*-diamide reductase activity that is required to reduce cob(II)yrinic acid *a,c*-diamide to the Co(I) state, allowing adenosylation.

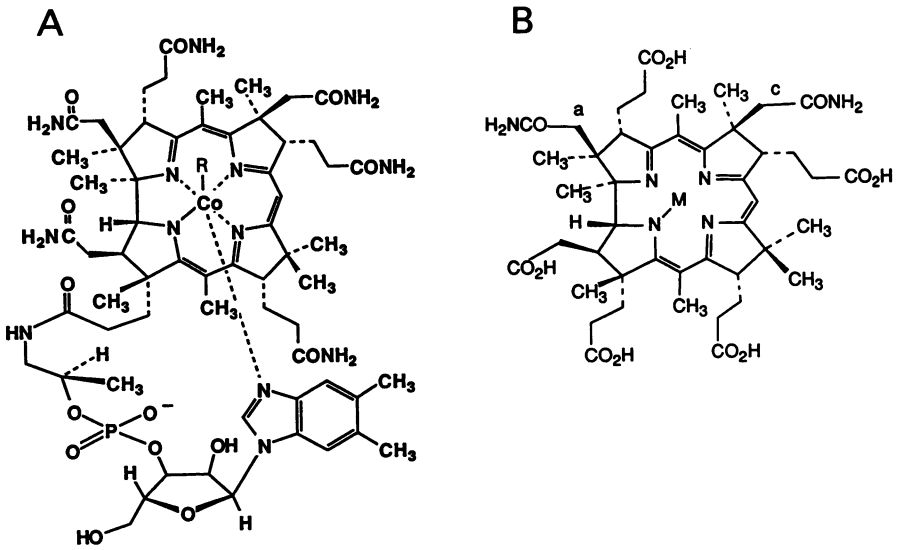

FIG. 1. Chemical structures. (A) Aquacobalamin (R = H_2O), coenzyme B_{12} or Ado-cobalamin (R = Ado). (B) Hydrogenobyrinic acid *a,c*-diamide (M = H), cob(II)yrinic acid *a,c*-diamide [M = Co(II)]; cob(I)yrinic acid *a,c*-diamide [M = Co(I)].

In this report we describe for the first time, as far as we know, the purification and characterization of this cob(I-I)yrinic acid *a,c*-diamide reductase activity. Two assays were used to detect and quantify this activity. In assay 1 we trapped, with iodoacetic acid, the extremely nucleophilic and air-sensitive Co(I)-corrinoid, the product of reductase activity, to form a stable carboxymethyl derivative (15, 17). In assay 2, the Co(I)-corrinoid produced was adenosylated in situ by adding an excess of cob(I)alamin adenosyltransferase. The presence in both assays of an oxygen-consuming system consisting of glucose-glucose oxidase-catalase was

* Corresponding author.

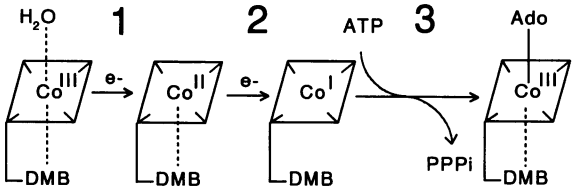

FIG. 2. Enzymatic conversion of aquacobalamin to Ado-cobalamin. The boxlike structure represents the fully amidated corrin ring shown in Fig. 1 for cobalamin. The bracket below the ring represents the nucleotide loop. DMB, dimethylbenzimidazole.

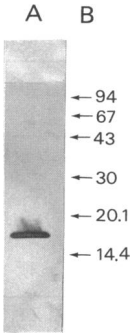

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cob(II)yrinic acid *a,c*-diamide reductase. (A) A sample of 100 ng of purified cob(II)yrinic acid *a,c*-diamide reductase was applied. (B) Arrows show the migration distance of protein standards (molecular weights shown on right, 10^3). The gel was stained with silver.

shown to ensure completely satisfactory reproducibility without interference. All buffers and reagents were extensively deoxygenated by bubbling argon in the solution for 8 h just before use.

Assay 1. Cob(II)yrinic acid *a,c*-diamide reductase was assayed as follows. A sample of enzyme (about 10 U) was mixed at 20°C under an argon atmosphere with 300 nmol of NADH, 24 nmol of flavin mononucleotide (FMN), 80 nmol of [2-^{14}C]iodoacetic acid (8.4 μCi μmol^{-1}; Amersham), 10 U of glucose oxidase (grade II; Boehringer Mannheim), 200 U of catalase (crystalline suspension; Boehringer Mannheim), and 12 μmol of D-glucose in a total volume of 570 μl of 0.2 M Tris hydrochloride buffer (pH 8.0) containing 15% (vol/vol) glycerol. Cob(II)yrinic acid *a,c*-diamide reduction was started by adding 30 μl of 400 μM cob(II)yrinic acid *a,c*-diamide [or another Co(II)-corrinoid], and the incubation was carried out under argon for 15 min at 30°C in the dark. The reaction was stopped by heating to 80°C for 10 min. After centrifugation at 15,000 × *g* for 10 min, the radioactive carboxymethyl-corrinoid formed during incubation was assayed by high-performance liquid chromatography (HPLC) (14). A Gilson HPLC system coupled to a Berthold LB 506 HPLC radioactivity detector (Berthold, Wildbad, Germany) equipped with a GT 200 glass scintillator cell was used. Standards of carboxymethyl-corrinoids were prepared by alkylation of their Co(I) counterparts with iodoacetic acid (17, 18).

Assay 2. Incubations were performed as in assay 1, except that iodoacetic acid was replaced by cob(I)alamin adenosyltransferase (2 U; purified from *P. denitrificans*) (11)–400 μM [8-^{14}C]ATP (2.5 μCi μmol^{-1})–0.8 mM manganese chloride. The Ado-corrinoid formed during incubation was identified and quantitated by HPLC as previously described (11).

One unit of cob(II)yrinic acid *a,c*-diamide reductase activity was defined as the amount of enzyme necessary to generate 1 nmol of Co(I)-corrinoid per h under the conditions described above. Both assays gave comparable values (Table 1).

TABLE 1. Purification of cob(II)yrinic acid *a,c*-diamide reductase from *P. denitrificans*

Purification step	Vol (ml)	Amt of protein (mg)	Sp act[a] (kU mg of protein^{-1})		Recovery[b] (%)	Purification[b] (fold)
			Assay 1	Assay 2		
Crude extract	150	1,050	0.072	0.051	100	1.0
MonoQ 10/10 eluate	145	6.60	5.6	6.7	82	130
FMN-agarose eluate	16.5	0.100	150	ND[c]	ND	ND
MonoQ 5/5 eluate	0.80	0.030	300	320	18	6,300

[a] Assayed with cob(II)yric acid.
[b] Determined with assay 2.
[c] ND, not determined.

Cell extracts from *P. denitrificans* SC510 Rif^r (3) were prepared by ultrasonication. Bacteria (10 g [wet weight] of cells) were resuspended in 50 ml of 0.1 mM Tris hydrochloride (pH 8.0) and sonicated at 4°C with 30 30-s bursts from a Branson B 30 disintegrator. Cell debris was separated by centrifugation (50,000 × *g* for 1 h), and the supernatant was desalted at a flow rate of 2 ml min^{-1} on a Sephadex G-25 column (4.7 by 18 cm; Pharmacia) equilibrated with 50 mM Tris hydrochloride (pH 8.0)–15% (vol/vol) glycerol–250 μM dithiothreitol (buffer A). The protein extract (210 mg of protein per run) was loaded at a flow rate of 3 ml min^{-1} onto a Mono Q HR 10/10 column (Pharmacia) equilibrated with buffer A. Cob(II)yrinic acid *a,c*-diamide reductase activity was eluted with buffer A as a broad fraction after the peak of unbound material. Active fractions were pooled, concentrated to 16 ml in Centriprep 10 concentrators (Amicon), and applied at a flow rate of 1 ml min^{-1} to a column (1.0 by 10 cm) of FMN-agarose (Sigma) equilibrated with buffer A. The column was washed with 10 ml of buffer A, and cob(II)yrinic acid *a,c*-diamide reductase activity was eluted with 16.5 ml of buffer A containing 50 μM FMN. The protein fraction was concentrated to 1.9 ml in Centricon 10 microconcentrators and injected on a MonoQ HR 5/5 column equilibrated with 50 mM Tris hydrochloride (pH 8.6)–15% glycerol–250 μM dithiothreitol (buffer B). Cob(II)yrinic acid *a,c*-diamide reductase activity was eluted at a flow rate of 1 ml min^{-1} with a 15-ml linear 0 to 0.2 M gradient of potassium chloride in buffer B. The enzyme was purified about 6,300-fold with an overall yield of 18% (Table 1).

With previously described procedures (3, 13), the purified protein was shown to be homogeneous and to exhibit the following structural characteristics: a molecular weight of 16,000 ± 1,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3), an apparent molecular weight of 30,000 by gel permeation HPLC on a Bio-Sil SEC-125 column (indicating a homodimeric structure), and the N-terminal amino acid sequence H_2N-MEKTRL. This sequence was not found on any of the four DNA fragments previously reported to encode cobalamin biosynthetic enzymes in *P. denitrificans* (5–10). This observation was confirmed by the finding that unamplified levels of cob(II)yrinic acid *a,c*-diamide reductase activity were present in genetically engineered *P. denitrificans* strains that carry cloned genes involved in cobalamin synthesis (5–10).

TABLE 2. Substrate specificity of cob(II)yrinic acid *a,c*-diamide reductase from *P. denitrificans*

Substrate[a]	Activity (% of cobyric acid)	
	Assay 1	Assay 2
Cob(II)yrinic acid *a,c*-diamide	52	75
Cob(II)yric acid	100	100
Cob(II)inamide	67	48
Cob(II)inamide phosphate	66	ND[b]
GDP-cob(II)inamide	71	ND
Cob(II)alamin	41	ND

[a] Generated by anaerobic photolysis of the coenzyme form (1, 2, 4) immediately before use and assayed at a concentration of 20 μM as described in the text.
[b] ND, not determined.

Cob(II)yrinic acid *a,c*-diamide reductase catalyzed the reduction of all of the Co(II)-corrinoids studied (Table 2). Steady-state kinetic analysis gave K_m values of 5.9 ± 1.2 μM for FMN and 105 ± 18 μM for NADH and a V_{max} of about 300 kU mg^{-1} at a concentration of 20 μM cob(II)yric acid. The enzyme displayed negligible activity with NADPH. It was not specific for FMN and also functioned with flavin adenine dinucleotide, although at half the velocity. Partially or extensively purified preparations were completely inactive without an added flavin cofactor. This indicated that this reductase is an easily dissociable flavoprotein and suggested that its natural cofactor is FMN.

Our previous studies with *P. denitrificans* showed that the adenosyl group is inserted just after cobyrinic acid *a,c*-diamide formation (1, 11). We have since demonstrated that cobalt insertion occurs with hydrogenobyrinic acid *a,c*-diamide as the substrate to give cob(II)yrinic acid *a,c*-diamide (12). Therefore we suggest that the physiological substrate of the presently reported reductase is cob(II)yrinic acid *a,c*-diamide.

An enzyme showing cob(II)alamin reductase activity (Fig. 2, reaction 2) has been partially purified (100-fold) from *Clostridium tetanomorphum* (16, 19). Like cob(II)yrinic acid *a,c*-diamide reductase, this enzyme was shown to be an easily dissociable flavoprotein (flavin adenine dinucleotide or FMN) with NADH as the source of reducing equivalents (19). Moreover, since no assay of cob(II)alamin reductase with a corrinoid substrate other than cob(II)alamin has been reported and since *C. tetanomorphum* is a cobalamin producer, one cannot exclude the possibility that cob(II)alamin reductase is identical with the Co(II)-reductase of the cobalamin pathway considered here, i.e., cob(II)yrinic acid *a,c*-diamide reductase.

We are grateful to D. Faucher and F. Cuine for sequencing of cob(II)yrinic acid *a,c*-diamide reductase.

REFERENCES

1. **Blanche, F., M. Couder, L. Debussche, D. Thibaut, B. Cameron, and J. Crouzet.** 1991. Biosynthesis of vitamin B_{12}: stepwise amidation of carboxyl groups *b*, *d*, *e*, and *g* of cobyrinic acid *a,c*-diamide is catalyzed by one enzyme in *Pseudomonas denitrificans*. J. Bacteriol. **173**:6046–6051.
2. **Blanche, F., L. Debussche, A. Famechon, D. Thibaut, B. Cameron, and J. Crouzet.** 1991. A bifunctional protein from *Pseudomonas denitrificans* carries cobinamide kinase and cobinamide phosphate guanylyltransferase activities. J. Bacteriol. **173**: 6052–6057.
3. **Blanche, F., L. Debussche, D. Thibaut, J. Crouzet, and B. Cameron.** 1989. Purification and characterization of *S*-adenosyl-L-methionine:uroporphyrinogen III methyltransferase from *Pseudomonas denitrificans*. J. Bacteriol. **171**:4222–4231.
4. **Brady, R. O., and H. A. Barker.** 1961. Influence of oxygen on the photolysis of coenzyme B_{12}. Biochem. Biophys. Res. Commun. **4**:373–378.
5. **Cameron, B., F. Blanche, M.-C. Rouyez, D. Bisch, A. Famechon, M. Couder, L. Cauchois, D. Thibaut, L. Debussche, and J. Crouzet.** 1991. Genetic analysis, nucleotide sequence, and products of two *Pseudomonas denitrificans cob* genes encoding nicotinate-nucleotide:dimethylbenzimidazole phosphoribosyltransferase and cobalamin (5′-phosphate) synthase. J. Bacteriol. **173**:6066–6073.
6. **Cameron, B., K. Briggs, S. Pridmore, G. Brefort, and J. Crouzet.** 1989. Cloning and analysis of genes involved in coenzyme B_{12} biosynthesis in *Pseudomonas denitrificans*. J. Bacteriol. **171**:547–557.
7. **Cameron, B., C. Guilhot, F. Blanche, L. Cauchois, M.-C. Rouyez, S. Rigault, S. Levy-Schil, and J. Crouzet.** 1991. Genetic and sequence analysis of a *Pseudomonas denitrificans* DNA fragment containing two *cob* genes. J. Bacteriol. **173**:6058–6065.
8. **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.
9. **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.
10. **Crouzet, J., S. Lévy-Schil, B. Cameron, L. Cauchois, S. Rigault, M.-C. Rouyez, F. Blanche, L. Debussche, and D. Thibaut.** 1991. Nucleotide sequence and genetic analysis of a 13.1-kilobase-pair *Pseudomonas denitrificans* DNA fragment containing five *cob* genes and identification of structural genes encoding cob(I)alamin adenosyltransferase, cobyric acid synthase, and bifunctional cobinamide kinase-cobinamide phosphate guanylyltransferase. J. Bacteriol. **173**:6074–6087.
11. **Debussche, L., M. Couder, D. Thibaut, B. Cameron, J. Crouzet, and F. Blanche.** 1991. Purification and partial characterization of cob(I)alamin adenosyltransferase from *Pseudomonas denitrificans*. J. Bacteriol. **173**:6300–6302.
12. **Debussche, L., M. Couder, D. Thibaut, B. Cameron, J. Crouzet, and F. Blanche.** 1992. Assay, purification, and characterization of cobaltochelatase, a unique complex enzyme catalyzing cobalt insertion in hydrogenobyrinic acid *a,c*-diamide during coenzyme B_{12} biosynthesis in *Pseudomonas denitrificans*. J. Bacteriol. **174**:7445–7451.
13. **Debussche, L., D. Thibaut, B. Cameron, J. Crouzet, and F. Blanche.** 1990. Purification and characterization of cobyrinic acid *a,c*-diamide synthase from *Pseudomonas denitricians*. J. Bacteriol. **172**:6239–6244.
14. **Djalali, M., J.-L. Gueant, D. Lambert, S. El Kholty, M. Saunier, and J.-P. Nicolas.** 1990. High-performance liquid chromatographic separation and dual competitive binding assay of corrinoids in biological material. J. Chromatogr. **529**:81–91.
15. **Fenton, W. A., and L. E. Rosenberg.** 1981. The defect in the *cbl B* class of human methylmalonic acidemia: deficiency of cob(I)alamin adenosyltransferase activity in extracts of cultured fibroblasts. Biochem. Biophys. Res. Commun. **98**:283–289.
16. **Huennekens, F. M., K. S. Vitols, K. Fujii, and D. W. Jacobsen.** 1982. Biosynthesis of cobalamin coenzymes, p. 145–167. *In* D. Dolphin (ed.), B_{12}, vol. 1. John Wiley & Sons, Inc., New York.
17. **Schrauzer, G. N., E. Deutsch, and R. J. Windgassen.** 1968. The nucleophilicity of vitamin B_{12s}. J. Am. Chem. Soc. **90**:2441–2442.
18. **Vitols, E., G. A. Walker, and F. M. Huennekens.** 1966. Enzymatic conversion of vitamin B_{12s} to a cobamide coenzyme, α-(5,6-dimethylbenzimidazolyl)deoxyadenosylcobamide (adenosyl-B_{12}). J. Biol. Chem. **241**:1455–1461.
19. **Walker, G. A., S. Murphy, and F. M. Huennekens.** 1969. Enzymatic conversion of vitamin B_{12a} to adenosyl-B_{12}: evidence for the existence of two separate reducing systems. Arch. Biochem. Biophys. **134**:95–102.