ENZYMIC SYNTHESIS OF COBINAMIDE PHOSPHATE FROM COBINAMIDE BY EXTRACTS OF PROPIONIBACTERIUM SHERMANII

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From studies with whole cells of Nocardia rugosa Barchielli et al (1960) and Boretti et al (1960) proposed the following pathway for the synthesis of cobalamin from cobinamide:

1. cobinamide + ATP ⟶ cobinamide phosphate + ADP
2. cobinamide phosphate + GTP ⟶ GDP-cobinamide + PP_i
3. GDP-cobinamide + α-ribazole ⟶ cobalamin + GMP

An indication that this pathway may also be operative in Propionibacterium shermanii is the fact that under suitable conditions this organism produces large amounts of cobinamide phosphate and GDP-cobinamide (compounds y1 and y2, Pawelkiewicz, 1956).
The enzymic synthesis of GDP-cobinamide (step 2) has been shown by labelling experiments with extracts of P.shermanii by Ronzio et al (1967), and independently with unlabelled material on a more preparative scale in this laboratory (Renz, unpublished).
For the present system step 3 of the Barchielli scheme has to be replaced by the following steps (Renz, 1967, and unpublished data):

3a. GDP-cobinamide + α-ribazole 5'-phosphate ⟶ cobalamin 5'-phosphate + GMP
3b. cobalamin 5'-phosphate ⟶ cobalamin + P_i

Abbreviations: GDP-cobinamide = P(1)-cobinamide-P(2)-guanosine 5'-pyrophosphate; 5,6-DMBIA = 5,6-Dimethylbenzimidazole; α-ribazole = 1-α-D-ribofuranosido-5,6-dimethylbenzimidazole; AS = ammonium sulfate; GSH = reduced glutathion.

In this publication results are given illustrating the enzymic synthesis of cobinamide phosphate from cobinamide.

Materials and Methods:

ATP-Na_4 and reduced glutathion were purchased from Zellstoff-Fabrik Waldhof, Mannheim, other nucleotides from Boehringer, Mannheim. Terminally labelled ATP-^{32}P was purchased from the Radiochemical Center, Amersham, England. The radioactivity measurements were done on paper in the Packard Tri Carb Liquid scintillation spectrophotometer. Values are corrected for background.

P. shermanii was grown according to Bernhauer et al(1959). Highest enzymic activities were usually obtained using bacteria from 2 day-cultures. Cells were broken at -30°C in the X-press (AB Biox, Nacka, Sweden). Pressed bacteria were suspended in 20 mM Tris/HCl buffer, pH 7.5 containing 1 mM EDTA, and treated with a small amount of DNAase. This suspension was either used as "cell homogenate" or it was centrifuged at +2°C for 20 minutes at 20 000 rpm. The supernatant was decanted and the precipitate eluted again with Tris/EDTA-buffer. The combined supernatants usually contained 25-40 mg of protein/ml (biuret).

For the preparation of AS-fractions this crude extract was diluted to 10 mg protein/ml and supplied with 1 mM GSH to preserve the cobinamide phosphate activity.

In order to show a GSH-effect, the precipitated protein was dissolved in 20 mM Tris/HCl, pH 7.5 containing no GSH.

Enzyme assays: experimental conditions are given with the tables. Cobinamide was added in the dicyanoform to crude extracts, since freshly prepared extracts convert dicyanocobinamide into the Co-5'-deoxyadenosyl-derivative within 5 minutes or less.

After incubation 5 mg KCN were added to each assay, the pH brought to 6-7 with 0.2 ml N acetic acid and the mixture heated in a boiling water bath for 8 minutes. After centrifugation (5 min., 12 000 rpm) the supernatant was decanted, and the precipitate was resuspended in 2 ml water and centrifuged again. The corrinoids were isolated from the supernatants by phenol extraction and separated by descending paper chromatography on Schleicher u. Schüll

paper 2043a with sec.butanol/water/acetic acid/KCN = 70/30/1/0.01. Average $R_{cobinamide}$-values in this solvent system are: GDP-cobinamide 0.05, cobalamin 5'-phosphate 0.14, cobinamide phosphate 0.23, cobalamin 0.75, ATP 0.

Results:

Table 1 shows the dependence of the cobinamide phosphate-production on the cobinamide/ATP-ratio.

Table 1 Synthesis of cobinamide phosphate as a function of the amount of ATP added to extracts of P. shermanii

cobinamide:ATP	1:0	1:10	1:50	1:100	1:500
cobinamide phosphate formed (% of total corrinoids)	3.9	4.5	12.5	15.8	2.2

Reaction mixtures (4.3 ml) contained: cobinamide 0.25 µmoles, ATP 0, 2.5, 12.5, 25, and 125µmoles respectively, Magnesium chloride 2 µmoles, Tris/HCl-buffer, pH 7.5 300 µmoles, and 57 mg protein of crude extract of P. shermanii. Incubation 10 hrs., 37°C.

The extinction of the corrinoid bands on the paper chromatograms was recorded with the "Extinktionsschreiber Fraktograph", Fa. Bergmann, Berlin (precision ±3 %).

The cobinamide phosphate synthesis was further demonstrated in experiments with terminally labelled ATP-^{32}P (Table 2). The radioactivity from ATP-γ-^{32}P was found in cobinamide phosphate, GDP-cobinamide and cobalamin 5'-phosphate. In many crude extracts, which were supplied with 5,6-DMBIA (expt.3, Table 2), the synthesis stopped at cobalamin 5'-phosphate. But if the reaction mixture was also supplied with 2.5 µmoles of GTP, some extracts produced cobalamin, and only very little cobalamin 5'-phosphate.
The cobalamin 5'-phosphate of expt.3, Table 2 was converted into cobalamin by a "cell homogenate" of P.shermanii, in which the enzymic activity for step 3b is preserved to a great extent. The resulting cobalamin contained the ^{32}P-activity.

Table 2 Incorporation of radioactivity (counts/20 min.) from ATP-γ-32P into various corrinoids catalysed by P. shermanii extracts

	Experiment Number				
	1	2	3	4	5
GDP-cobinamide	9,040	6,770	790	0	0
Cobalamin-5'-phosphate	0	0	36,750	0	0
Cobinamide phosphate	7,140	4,590	4,120	500	0

Complete reaction mixtures (2.7 ml) contained: cobinamide 0.25 μmoles, ATP-γ-32P 2.5 μC, ATP 12.5 μmoles, magnesium chloride 5 μmoles, Tris-HCl-buffer, pH 7.5 500 μmoles, 60 mg protein of crude P. shermanii-extract. Incubation 10 hrs., 37°C.
Expt.1: unlabelled ATP omitted,
Expt.3: unlabelled ATP omitted, 2.5 μmoles 5,6-DMBIA added,
Expt.4: extract boiled, Expt.5: extract omitted.
The chromatograms, on which the total amount of corrinoids of each experiment were applied as a 4.5 cm broad band, were run for 60 hrs. Sections of the chromatogram (1.5×1 cm) were cut and activity measured by scintillation counting for 20 min. Thus the numbers represent about a third of the total counts in each corrinoid band. In expt.1 and 3 10,000 counts/20 min. are equivalent to about 0.01 μmoles.

Table 3 Radioactivity of 20 μg cobinamide phosphate after several purification steps

	counts/100 min.
1. descending paper chromatography	1534
2. carboxymethylcellulose chromatography	926
3. electrophoresis at pH 2.5	518
4. electrophoresis at pH 7.5	721

250 μg of labelled cobinamide phosphate from experiments described in Table 2 were added to 500 μg of unlabelled cobinamide phosphate. After each purification procedure 20 μg corrinoid were applied to a filter paper as a spot of about 6-8 mm diameter and counted.

Table 3 shows that the radioactive cobinamide phosphate eluted from descending chromatograms retained most of its radioactivity through several purification steps.
The pH-optimum of the cobinamide phosphate formation with crude extracts lies between 7 and 8. Under the conditions of expt.2, Table 2 the time course of cobinamide phosphate formation shows linearity within the first thirty minutes

of incubation. Maximal cobinamide phosphate formation is reached after 4-6 hrs.

In experiments with AS-fractions it can be demonstrated that an SH-compound is essential for the cobinamide phosphate formation (Table 4), as is the case for step 2 (Ronzio et al, 1967).

Table 4 Cobinamide phosphate synthesis with AS-fractions of P. shermanii extracts (counts/10 min. found in the cobinamide phosphate band after descending paper chromatography)

Expt.	AS-fraction (% saturation)	GSH	counts/10 min. in the cobinamide phosphate region
1	40-60 %	-	380
2	40-60 %	+	2,130
3	60-100 %	-	580
4	60-100 %	+	255
5	40-100 %	-	540
6	40-100 %	+	550
7	40-100 %	+ and GTP	1,310

Complete reaction mixtures (2.45 ml) contained: Co-5'-deoxyadenosyl-cobinamide 0.25 μmoles, ATP 2 μmoles, ATP-γ-32P 1.25 μC, GSH 20 μmoles, GTP 2.5 μmoles(Expt.7), Magnesium chloride 3 μmoles, Tris-buffer,pH 7.5 400μmoles, 24.6 mg protein of the 40-60 % AS-fraction and/or 39.4 mg protein of the 60-100 % AS-fraction.
Incubation 4 hrs., 37°C.

Discussion: The data given here show that contrary to results of Ronzio et al (1967) crude extracts of P.shermanii or the 40-60 % AS-fraction thereof catalyze the formation of cobinamide phosphate from cobinamide and ATP. For this reaction a SH-compound e.g. glutathion, is necessary.
The cobinamide phosphate synthesis is inhibited in reaction mixtures containing the 40-100 % AS-fraction. This inhibition can be overcome partly by addition of GTP (Table 4). GTP also activates the cleavage of cobalamin 5'-phosphate to cobalamin. These effects could be due to a regulatory function of GTP in the cobalamin biosynthesis.

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