BIOSYNTHESIS OF VITAMIN B_{12}

Formation of free 5,6-dimethylbenzimidazole and α-ribazole from riboflavin by *Propionibacterium freudenreichii*

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1. Introduction

In experiments with radioactive tracers it was shown that the DBI*-moiety of vitamin B_{12} is formed from riboflavin [1]. Thereby the C-1′ of riboflavin is transformed into C-2 of DBI [2]. These studies were carried out with *Propionibacterium shermanii* which produces vitamin B_{12}. In order to isolate the DBI the vitamin B_{12} was degraded by acid hydrolysis. Until now DBI was never found in free form in vitamin B_{12}-synthesizing bacteria but only as a component of vitamin B_{12}.

In this publication experiments are described demonstrating that cells of *P. freudenreichii* but not of *P. shermanii*, grown in the absence of a cobalt salt, transform riboflavin into free DBI, which can be isolated from the incubation medium.

2. Materials and methods

Solvents for chromatography: (1) chloroform/ethanol/acetic acid (85:15:1); (2) Chloroform/ethanol/acetic acid (70:30:2); (3) butanol-2/water/acetic acid (70:30:1); (4) ethanol/water/ammonia (50:50:2); (5) ethanol/conc. ammonia (10:0.5). Descending paper chromatography was performed on Whatman 3 MM paper (19 × 46 cm), paper electrophoresis on Schleicher a. Schuell paper 2043a gew. (18 × 45 cm) in 0.5 M acetic acid at 10 V/cm.

* *Abbreviations:* DBI = 5,6-dimethylbenzimidazole, α-ribazole = 5,6-dimethylbenzimidazole-α-D-ribofuranoside.

For thin-layer chromatography pre-coated silica gel 60 F_{254}-plates (Merck) were used. The radioactivity of aqueous samples was measured in Aquasol (NEN-Chemicals), of ethanolic samples in PPO/toluene (6 g/l) in a Beckman LS 230 liquid scintillation counter. The counting efficiency was determined with an internal standard ([^{14}C]toluene, NEN-Chemicals). Radioactivity on paper and on thin-layer plates was detected with the Dünnschichtscanner II (Fa. Berthold, Wildbad, Germany). The DBI and α-ribazole was quantitatively determined spectrophotometrically [3]. UV-spectra were recorded in an Acta V-spectrophotometer (Beckman).

[1′-^{14}C]Riboflavin (314 000 dpm/μmol) was synthesized in 23% yield according to Kuhn et al. [4] starting from 1 mmol of [1-^{14}C]ribose. A nonradioactive impurity showing a light blue fluorescence under UV-light of 254 nm was removed by boiling three times with a small amount of absolute ethanol.

P. freudenreichii (ATCC 6207) and *P. shermanii* St 33 were grown as described previously [5], but cobalt sulfate was omitted. Thus the bacteria contained less than 2 nmol of corrinoids/g wet weight. The cells were harvested by centrifugation at 7000 × *g* for 1 h and the supernatant decanted. The slimy sediment was recentrifuged at 70 000 × *g* for 1 h. The cells were stored at −20°C.

Incubation of the *P. freudenreichii* cells and isolation of DBI and α-ribazole: 40 g of wet cells were suspended in 400 ml of 0.07 M phosphate buffer (pH 7.0). 4.75 mg (12.6 μmol) of [1′-^{14}C]riboflavin, dissolved in 12.5 ml of water, was added, and incubated aerobically with continuous shaking at 30°C for

48 h. After centrifugation at 70 000 × *g* for 1 h the supernatant was extracted with isobutanol according to Friedmann et al. [6]. The isobutanol was evaporated to a small volume and subjected to descending paper chromatography in solvent III. Radioactive bands of DBI (R_F = 0.73) and α-ribazole (R_F = 0.63) were detected (see also fig.1). These bands were eluted with solvent IV.

The DBI was further purified by thin-layer chromatography in solvent I (R_F = 0.2), eluted from the silica gel with solvent V, subjected to paper electrophoresis, eluted with solvent IV, and rechromatographed on silica gel in solvent I. The UV-spectrum of the DBI thus obtained (68 nmol, 212 000 dpm/μmol) was identical with that of an authentic reference

The α-ribazole was further purified by thin-layer chromatography in solvent II (R_F = 0.12), by paper electrophoresis, and finally by thin-layer chromatography in solvent I, with which the plate was developed three times. The α-ribazole (80 nmol, 170 000 dpm/μmol) then had the same UV-spectrum as authentic α-ribazole.

3. Results and discussion

As shown in fig.1, resting cells of *P. freudenreichii* produce radioactive DBI and α-ribazole on incubation with [1′-^{14}C]riboflavin. The DBI and α-ribazole were identified by their chromatographic and electrophoretic behaviour and by their UV-spectrum. For these experiments bacteria were used which had been grown in the absence of a cobalt salt to prevent the synthesis of corrinoids. Thus the DBI formed from riboflavin could not be used for the vitamin B_{12}-biosynthesis and was partially directly released into the medium and partially after transformation into α-ribazole (presumably via α-ribazole 5′-phosphate [7]). Very little DBI and α-ribazole could be detected within the cells.

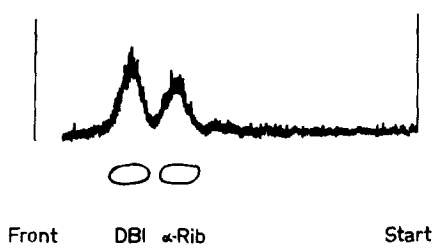

Fig.1. Paper chromatogram with radioactivity scanner trace of the isobutanol extract of the buffer solution, in which *P. freudenreichii* was incubated with [1′-^{14}C]riboflavin.

In some experiments only DBI was found. The addition of ribose or glucose (1% w/v) to the incubation medium had no effect on the production of α-ribazole. When *P. shermanii*, grown in the absence of cobalt sulfate, was used, only minute amounts of DBI could be detected. *P. freudenreichii* and *P. shermanii*, grown in the presence of cobaltous sulfate, did not produce free DBI or α-ribazole, but vitamin B_{12} containing [2-^{14}C]DBI [2].

These experiments suggest that free DBI is an intermediate in the biosynthesis of vitamin B_{12}. But in experiments with *P. freudenreichii* grown with cobaltous sulfate and incubated with 5-^{15}N-riboflavin a vitamin B_{12} is formed, which exhibits only one peak in the ^{15}N-NMR-spectrum [8]. This shows that for the vitamin B_{12}-biosynthesis under normal conditions the DBI remains enzyme bound and that the glycosidic bond is always formed with the same of the two imidazole nitrogens of DBI.

With the experiments described in this publication a system is found which offers the possibility to investigate the transformation of riboflavin into DBI separate from the synthesis of the corrinoid moiety of vitamin B_{12}. Since a considerable rate of synthesis of DBI from riboflavin was also found in broken cells of *P. freudenreichii* [8], the further investigation of this problem seems promising.

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