Isolation of cobamides from *Methanothrix soehngenii*: 5-methylbenzimidazole as the α -ligand of the predominant cobamide

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Abstract. *Methanothrix soehngenii* was found to contain five different cobamides when grown on vitamin B₁₂ supplemented as well as vitamin B₁₂ free media. In both cases, it was shown by HPLC-chromatography and UV/VIS spectroscopy, that α -5-methylbenzimidazolyl- β -cyanocobamide was the predominant cobamide, accounting for 27% and 23%, respectively, of the total corrinoid content. Vitamin B₁₂ and α -5-hydroxybenzimidazolyl- β -cyanocobamide could also be detected in both cell batches in varying amounts. Cells grown on vitamin B₁₂ free medium contained significantly more baseless cobamides, indicating biosynthesis of cobamides.

Key words: *Methanothrix soehngenii* — Cobamide — 5-Methylbenzimidazole — Methanogen — Corrinoid — Acetotroph — α -5-Methylbenzimidazolyl- β -cyanocobamide — Vitamin B₁₂ — Cobalamin

Cobamide dependent reactions are involved in the anaerobic metabolism of C₁- and C₂-compounds (Ljungdahl and Wood 1982, Wood et al. 1982, Thauer 1985). It was shown that cobamides play an important role in the synthesis of acetate from two CO₂ in acetogenic anaerobes (Hu et al. 1984), in the synthesis of acetyl-CoA from two CO₂ in methanogens (Holder et al. 1985), in the methane formation from methanol (Van der Meijden et al. 1984), and in the methane formation from acetate (Eikmanns and Thauer 1985). Structurally different cobamides in respect to the α -as well as the β -coordinated ligands have been described to be associated with bacteria which carry out the above mentioned reactions (Lezius and Barker 1965; Irion and Ljungdahl 1965; Stadtman and Blaylock 1966; Scherer and Sahm 1981; Shapiro 1982; Pol et al. 1982; Höllriegel et al. 1983; Whitman and Wolfe 1984; Pol et al. 1984; Kräutler 1985; Stupperich et al. 1986). Although exact mechanisms of the action of cobamide enzymes in these reactions are not

fully understood, two cobamides, β -methylcobamide and β -acetylcobamide have been proposed to be active coenzyme forms (Thauer 1985; Kräutler 1985).

In contrast to the specific roles in biochemical reactions assigned to the β -ligands (Golding 1982; Taylor 1982; Wood 1982; Kräutler 1984), the influence of α -ligands on the catalytic properties of the cobalt center in cobamides is poorly understood. Generally β -ligands are known to be specific for different biochemical reactions, in that the biological relevant reactivity of the cobamide coenzymes is reduced to the properties of their cobalt carbon bonds. Important control functions in cobamide coenzyme catalyzed reactions are attributed to α -ligands (Grate and Schrauzer 1979) and the coordination of α -ligands has been reported to stabilize the cobalt carbon bond (Kräutler 1987), but structural differences between them are rather explained in terms of biogenic causes and growth conditions (Bernhauer et al. 1964), than in terms of reaction specificity. Three out of seven naturally occurring cobamides with benzimidazole derived α -ligands (Friedrich 1975) could be assigned to microorganisms (Vitamin B₁₂ can be found in many different microorganisms (Zagalak 1982), (5-HOBza)CNCba occurs in methanogenic bacteria (Lezius and Barker 1965; Kräutler et al. 1987) and (5-MeOBza)CNCba in *Clostridium thermoaceticum* (Irion and Ljungdahl 1965), however it is still obscure, whether distinct bacterial species naturally form cobamides with only one specific α -ligand. *Methanothrix soehngenii* grows on acetate as sole energy and carbon source. It represents a methanogen having to deal with a metabolic problem similar to acetogens, in that it has to split acetate into two C₁-entities — the reverse reaction of acetogenic acetate biosynthesis (Kenealy and Zeikus 1982; Kohler and Zehnder 1984). This study was undertaken to find out, whether the cobamides in *M. soehngenii* contain 5-hydroxybenzimidazole as α -ligand like most other methanogenic bacteria so far examined (Stupperich and Kräutler 1988), or whether similarities to acetogenic bacteria exist.

Materials and methods

Chemicals. All chemicals used were of the highest purity available and were bought from Fluka (Buchs, Switzerland) or Merck (Darmstadt, FRG). Gases were obtained from Carbogas (Zürich, Switzerland) and were purified with Oxisorb cartridges (Messer Griessheim, Dällikon, Switzerland) prior to use.

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Abbreviations: (5-MeBza)CNCba, α -5-methylbenzimidazolyl- β -cyanocobamide, (5-HOBza)CNCba, α -5-hydroxybenzimidazolyl- β -cyanocobamide (factor III), (5-MeOBza)CNCba, α -5-methoxybenzimidazolyl- β -cyanocobamide (factor III_m), (Bza)CNCba, α -benzimidazolyl- β -cyanocobamide

Media and cultivation. Large batch cultures of *Methanothrix soehngenii* were grown in 10 or 20 liter carboys on the mineral medium described by Zehnder et al. 1980 and Huser et al. 1982. Cells were harvested at the end of the logarithmic growth phase (after 2–3 months) as described by Kohler and Zehnder (1984). The cells used for inoculum of the Vitamin B₁₂-free mass cultures were grown on Vitamin B₁₂-free medium for at least one generation.

Synthesis, extraction, and purification of cobamides. (5-MeBza)CNCba and (5-MeOBza)CNCba used as chromatographic references were obtained from *Propionibacterium* fermentations (Friedrich 1975) with 10–20 mg 5-methylbenzimidazole and 5-methoxybenzimidazole, respectively added per liter of fermentation broth. These cobamides were extracted and purified as described below for the cobamides from *M. soehngenii* except that at the end of the purification procedure they could be crystallized from acetone/water. Identification, chemical and physical properties of (5-MeBza)CNCba are described elsewhere (Kräutler et al., unpublished work). (5-HOBza)CNCba (factor III) was a gift from Hoffmann-LaRoche (Basel, Switzerland). To extract and purify the cobamides from *M. soehngenii*, frozen cells were washed with 0.9% NaCl and centrifuged (40,000 × g, 10 min). The pellet was resuspended in methanol/water 80%/20% 0.1% KCN. The cells were broken by passage through a French Press (25 MPa). The extract was then heated to 85°C (15 min) and centrifuged (40,000 × g, 15 min). The cobamide containing supernatant was saved and the pellet was reextracted twice. The pooled supernatants were flash evaporated, taken up in a small amount of water and applied to a XAD-2 (mesh 20–50) column (20 cm × 1.6 cm). The column was washed with 10 l water and then the cobamides were eluted with methanol/water (80%/20%). The eluate was evaporated to a small volume and applied to a CM-cellulose (Whatman CM-52) column (23 cm × 1.6 cm, H⁺-form, eluant: water). The major red fraction passing through the column was collected and concentrated by evaporation. The minor band adsorbing to the column was eluted with 2% acetic acid. Cobamides were further purified by application to a DEAE-cellulose (Whatman DE-52) column (20 cm × 1.6 cm, OH⁻-form, eluant: water). By passing through the column two red bands separated, which were further characterized by HPLC.

HPLC-chromatography. High-performance liquid chromatography was done with two different systems. HPLC-system 1 consisted of two pumps (Pharmacia P-500), a gradient controller (Pharmacia GP-250), a Techsil RP-18 column (2 cm × 15 cm) and a HP-1040A diode array detector connected to a HP-85 computer and a HP-7470A plotter. For best resolution a linear gradient from 40% to 60% B [A = 0.01 M KH₂PO₄/KNaHPO₄, pH 7.0, in water; B = 0.01 M KH₂PO₄/KNaHPO₄, pH 7.0 in methanol/water (80%/20%)] with a flow rate of 0.7 ml min⁻¹ was used. UV-VIS spectra of the eluting compounds could be taken directly and were automatically normalized in respect to the highest absorbance in the individual spectrum. HPLC-System 2 consisted of two pumps (Pharmacia P-500), a gradient controller (Pharmacia LCC-500), a Lichrosorb RP-18 column (4.9 mm × 25 cm), and a Pharmacia UV-monitor (254 nm). A linear gradient from 20% to 45% B (A = 0.05 M NaCH₃COO, pH 4.0, in water; B = 0.05 M NaCH₃COO,

Fig. 1. UV/VIS absorption spectrum of fraction A

pH 4.0, in methanol) with a flow rate of 0.7 ml min⁻¹ was chosen (Frenkel et al. 1979).

Results

Methanothrix soehngenii cells from two different batches — grown on Vitamin B₁₂ supplemented and Vitamin B₁₂ free medium, respectively — were analysed for corrinoids. In both cases a corrinoid content (crude corrinoid extract after XAD-2 chromatography) of 0.011 μmol/g cells (wet weight) was found (Table 1). This corrinoid content is approximately equal to the one found in *Methanosarcina barkeri* by Höllriegel et al. 1983, but is about twenty times less than what has been reported for *M. barkeri* by Pol et al. 1982.

Corrinoids were purified further on CM-cellulose where fraction A could be separated from the main corrinoids. The UV-VIS spectrum (2% acetic acid) of fraction A (Fig. 1) strongly resembles the spectra of cyanoaquocobyrinic acid and partially desaminated forms of it, with maxima at 495, 353–355, and 321 nm.

The remaining corrinoids could be separated on DEAE-cellulose into two fractions; fraction B being the minor, and fraction C being the major fraction (Table 1). Fractions A, B, and C could be found in both cell batches (Vitamin B₁₂ supplemented, Vitamin B₁₂ free). The fractional composition of the two batches varied considerably, although the total corrinoid content stayed constant. In the first case (Vitamin B₁₂ supplemented) fraction C (43.9%) made up the largest part of the total corrinoid content with fraction A (14.8%) and fraction B (10.3%) being considerably smaller (extraction efficiency: 69%). In the second case (Vitamin B₁₂ free) fraction A (31%) was the largest one followed by C (29.1%) and B (15.8%) (extraction efficiency: 75.9%). This shift of composition toward an increase of fraction A,

Table 1. Corrinoid extraction from *Methanothrix soehngenii*. Corrinoids were extracted as described in materials and methods. Crude corrinoid extract was obtained after XAD-2 adsorption. A refers to the fraction eluted from the CM-cellulose column with 2% acetic acid. B and C refer to fractions separated on the DEAE-cellulose column

	Cells (wet weight) g	Crude corrinoid extract μmol		A μmol	B μmol	C μmol
			μmol/g			
Grown on Vitamin B ₁₂ supplemented medium	14.2	0.155 (100%)	0.011	0.023 (14.8%)	0.016 (10.3%)	0.068 (43.9%)
Grown on Vitamin B ₁₂ free medium	15.6	0.165 (100%)	0.011	0.050 (31.0%)	0.026 (15.8%)	0.048 (29.1%)

Table 2. HPLC-Analysis of fraction C (HPLC-system 1)

Cobamide	identified as ^a	t _r ^b (min)	r _i ^c	Vitamin B ₁₂ -free		Vitamin B ₁₂ -supplemented	
				%	% ^d	%	% ^d
X ₄	Vitamin B ₁₂	29.9	1.000	4.2	1.2	19.5	8.6
X ₁	(5-MeBza)CNCba	28.0	0.936	79.4	23.1	61.1	26.8
X ₂	—	26.6	0.890	15.5	4.5	12.4	5.4
X ₃	(5-HOBza)CNCba	25.1	0.839	1.0	0.3	7.0	3.0

^a On grounds of cochromatography with and UV/VIS-spectroscopy of authentic material

^b Absolute retention time

^c Relative retention time with Vitamin B₁₂ as reference

^d Percentage of total corrinoid content

containing mainly cobyrinic acid and derivatives thereof, is in agreement with the need of these cells for complete biosynthesis of cobamides, as cobyrinic acid is known to be an intermediate in the their biosynthesis (Huennekens et al. 1982). The finding that less biosynthetic intermediates could be found in Vitamin B₁₂ supplemented batches, implies that *M. soehngenii* is able to take up Vitamin B₁₂ and convert it to different cobamides.

Fraction C was analyzed using HPLC. This fraction, from both cell batches, was shown to contain the same four different cobamides. Whereas the relative content differed considerably (Table 2) between the two cell batches, cobamide X₁ made up a quite constant proportion of the total corrinoid content in both cases (27%, 23%). Two cobamides (X₄ and X₃) could be readily identified by their UV/VIS absorption properties and their chromatographic behaviour to be Vitamin B₁₂ and (5-HOBza)CNCba, respectively. Also, using the same criteria, it could be excluded, that (5-MeOBza)CNCba was one of them. Figures 2 and 3 show the results of a HPLC-cochromatography experiment with UV/VIS spectra taken of the eluting peaks. The main cobamide of fraction C (X₁) cochromatographed with (5-MeBza)CNCba and also showed an identical UV/VIS spectrum. Cobamide X₂ did not cochromatograph with any of the reference cobamides, but showed a UV/VIS spectrum almost identical to that of Vitamin B₁₂ and (5-MeBza)CNCba. The data presented strongly suggest, that cobamide X₁ is identical to (5-MeBza)CNCba. In order to confirm these findings, fraction C was also analysed on HPLC-system 2. The relative retention times with Vitamin B₁₂ as the internal standard were as follows: (5-HOBza)CNCba, 0.864; (5-MeBza)CNCba, 0.935; X₁, 0.935; Vitamin B₁₂, 1.000. The previous findings could be

repeated, as X₁ cochromatographed with (5-MeBza)CNCba.

Fraction B contained a cobamide (X₅) with an UV/VIS spectrum similar to (5-MeBza)CNCba, Vitamin B₁₂, and X₂. On HPLC-System 1 it eluted between (5-HOBza)CNCba and X₂ (Table 2).

Discussion

The main cobamide in *Methanothrix soehngenii* was found to be (5-MeBza)CNCba. This rather surprising finding leads to a negative answer to the question originally posed. The cobamide pattern of *M. soehngenii* neither showed similarities to the one of *Clostridium thermoautotrophicum* (acetogen) nor to the ones of other methanogens. As small amounts of (5-HOBza)CNCba, Vitamin B₁₂, and two unidentified complete cobamides (X₂ and X₅) also could be found, *M. soehngenii* must have the capability to synthesize different α-ligands and incorporate them into cobamides.

Friedrich and Bernhauer (1958b) isolated different Vitamin B₁₂ analogs [(5-MeBza)CNCba, (6-MeBza)CNCba, and (Bza)CNCba] from sewage sludge. For these compounds a physiological significance has not been proposed. They were thought to arise from unspecific exchange reactions or incorporations of different bases into the α-ligand position, as it could be shown (Dellweg et al. 1956, Friedrich and Bernhauer 1958a, Perlman and Barret 1959), that bacteria, upon supplementation of different bases, tended to incorporate them into their cobamides. An asymmetrical base can give rise to two isomeric cobamides. This has been studied in the case of 5-methyl-benzimidazole (Friedrich and Bernhauer 1958a), where the 5-substituted isomer

Fig. 2A–D. HPLC-experiment (HPLC system 1) for the identification of the predominant cobamide in fraction C. Detection was at 360 nm. A Standards mixed in the following ratios: 2.0/1.0/3.7; (5-HOBza)CNCba (1), (5-MeBza)CNCba (2), Vitamin B₁₂ (3). 100% = 163.7 mAU (milli absorbance units). B Fraction C. 100% = 161.7 mAU. C Fraction C together with (1) and (3). 100% = 178.9 mAU; X₃ cochromatographs with (1), X₄ cochromatographs with (3). D Fraction C together with (2). 100% = 284.1 mAU. X₁ cochromatographs with (2)

predominated over the 6-substituted one (96% to 4%). Also, the raw corrinoid fraction of *Methanobacterium thermoautotrophicum* was found to contain besides (5-OHBza)CNCba about 10% of the isomeric (6-OHBza)CNCba (Kräutler et al. 1987). Upon finding (5-MeBza)CNCba one would also expect to find (6-MeBza)CNCba in smaller quantities. In both cell batches analyzed, X₂ comprised 20% of (5-MeBza)CNCba (X₁) and possessed a UV/VIS spectrum (diode array) undistinguishable from the spectra of (5-MeBza)CNCba Vitamin B₁₂. The possibility of X₂ being (6-MeBza)CNCba could neither be excluded nor confirmed, although the spectral data suggest it to be a benzimidazole derived (un-, mono-, or dimethylated) cobamide. As X₅ also showed an identical spectrum, but a different chromatographic behaviour, assignments to the two cobamides in question [(Bza)CNCba, (6-MeBza)CNCba] could not be made.

Fig. 3A, B. UV/VIS difference-spectra (diode array) of the cobamides from figure 2A and 2C. A Spectra of the eluting peaks of Figure 2A. (1) (5-HOBza)CNCba, (2) (5-MeBza)CNCba, (3) Vitamin B₁₂. Spectra were normalized with respect to the peak at 361 nm which is 82.3 mAU for (1), 27.3 mAU for (2), and 135.6 mAU for (3). B Spectra of the eluting peaks of Fig. 2C. (1) X₃ + (5-HOBza)CNCba, (2) X₁, (3) X₄ + Vitamin B₁₂. The peak at 361 nm is 50.5 mAU for (1), 134.4 mAU for (2) and 115.7 mAU for (3)

The findings, that (5-MeBza)CNCba is the major cobamide present in *M. soehngenii* strongly suggests a physiological role to be associated with it. The question to whether this role is in methyl transfer to coenzyme M, for which a corrinoid enzyme has been proposed in *M. barkeri* (Eikmanns and Thauer 1985), or in other cobamide dependent reactions can not be answered yet. The importance of α -ligands for the catalytic properties of cobamides should be reconsidered, as the known complete cobamides isolated from distinct bacterial strains also seem to be associated with different biochemical reactions. In the case of *M. soehngenii* five cobamides, three of which were identified, could be detected. It would seem reasonable to assume that they were active in different enzymic reactions, as their presence can not be explained by assuming that they are biosynthetic intermediates (Scherer et al. 1984). Finding different complete cobamides in one bacterial strain supports the view, that α -ligands are important in the reactivity patterns of cobamides.

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