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CORRINOIDS FROM *METHANOSARCINA BARKERI*: STRUCTURE OF THE α-LIGAND

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5-Hydroxybenzimidazole is the only base detected in cobamide compounds from methanol-grown *Methanosarcina barkeri*. 5-Hydroxybenzimidazolylcobamide accounted for about 83 and 90% of the total corrinoids of whole cells and cell-free extracts, respectively. Probably, the rest of the corrinoids are base-less.

The possible involvement of corrinoids in the metabolism of methanogenic bacteria was studied by Blaylock and Stadtman (1,2) who observed that methylcobalamin could be used as a substrate for CH_4 formation in cell-free extracts of these bacteria. The identification of methylcoenzyme M (3-7) as the substrate of the final step of methanogenesis made the role of methylcobalamin as methyl donor in methanogenesis very questionable and Gunsalus (8) and Wolfe (9) pointed out that also no evidence exists for a physiological role of corrinoids in methyl transfer leading to the formation of methane in hydrogen-grown cells. Recently, Shapiro (10) reported indications that such a transfer is not involved in the biosynthesis of methylcoenzyme M or CH_4 from methanol by *Methanosarcina barkeri*. However, many authors (2,10,11) have pointed to the high amounts of corrinoids present in *M. barkeri*, and recent studies may implicate a possible involvement in the final steps of methanogenesis (12-14) or in acetate synthesis or catabolism (15).

ABBREVIATIONS: HBI, 5-hydroxybenzimidazole; B_{12}-HBI, 5-hydroxybenzimidazolylcobamide; B_{12}-mHBI, 5-methoxybenzimidazolylcobamide; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; UV, ultraviolet; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; CCE, crude corrinoid extract.

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Friedrich and Bernhauer (16-19) reported that the α-ligand of a predominant corrinoid present in sewage sludge differed from the common corrinoids: 5-hydroxybenzimidazole (HBI) was present instead of 5,6-dimethylbenzimidazole. This corrinoid, called Factor III, was identified by Lezius and Barker to be present in *Methanobacillus omelianskii* (20), a syntrophic association of *Methanobacterium bryantii* and the S organism (21).

This study is the first to describe the isolation and structural analysis of the α-ligand of the corrinoids from a pure culture of methanogenic bacteria. 5-Hydroxybenzimidazole is the only ligand found in *M. barkeri*.

The term Factor III is also used to denominate 20-methylsirohydrochlorin (22), an intermediate in cobyrinic acid biosynthesis. Because of this ambiguity we will use the term B_{12}-HBI instead of Factor III in this report.

MATERIALS AND METHODS. *M. barkeri* strain MS (DSM 800) was cultured on methanol as substrate and cell-free extracts were prepared as described by Hutten *et al.* (23); CN-B_{12} was omitted from the growth medium.

Isolation of the corrinoids and conversion to their cyano-form. 36 g cells (wet weight) of *M. barkeri* were mixed with 11 ml of water. To the suspension 0.01% KCN in methanol was added up to a final methanol concentration of 80%. The mixture was heated for 20 min at 80°C and then centrifuged at 10,000 x *g* for 20 min at 4°C. The pellet was suspended in 175 ml of 80% methanol containing 0.01% KCN, heated and centrifuged twice more as described above, after which the third pellet and supernatant were colorless. The combined supernatants were concentrated to dryness by flash evaporation. The residue was dissolved in water and loaded on an Amberlite XAD-2 column (15 x 1.5 cm). The column was washed with water and the adsorbed corrinoids were eluted with 50% aqueous methanol. The corrinoid fraction was flash evaporated to dryness and dissolved in a few ml of water. The resulting corrinoid solution, which will be referred to as the crude corrinoid extract (CCE), was applied to a QAE-Sephadex A-25 (Cl^- form) column (50 x 2.5 cm), equilibrated with water. The predominant compound present in CCE appeared to be CN-B_{12}-HBI, which was eluted with water. Elution was continued with a linear gradient of NaCl (0-0.5 M). Besides some faint red bands only two purple bands were observed, which slowly turned red and became very diffuse while moving down the column. These compounds, which presumably are corrinoids, were not further investigated. CN-B_{12}-HBI was further purified by chromatography on a SP-Sephadex C-25 column (30 x 2.6 cm) equilibrated with 10 mM sodium citrate, pH 4.0, and was then desalted on Amberlite XAD-2 as described above.

For HPLC analyses, corrinoid preparations of cell-free extracts were obtained essentially as described above for whole cells; a Sep-pak C_{18} cartridge (Waters Associates, Inc.) was used instead of the Amberlite XAD-2 column.

Acid hydrolysis of CN-B_{12}-HBI. 4.6 μmol of purified CN-B_{12}-HBI were hydrolyzed in 4 ml 6 N HCl for 4 h at 150°C in an evacuated fused glass tube (10 x 1 cm). The hydrolysate was flash evaporated to dryness; to remove HCl 5 ml of water was added and evaporated three times. The residue, containing HBI, was dissolved in water and applied to a Dowex 50 W (H^+) column (12 x 0.7 cm). The base was eluted by an increasing concentration of HCl, as described for the nucleotide obtained from B_{12}-mHBI, which contains 5-methoxybenzimidazole as the α-ligand (18). Corrinoids were eluted just after the base and a brown band

stuck to the column. The concentrated solution of the base was pink, which indicates that a small amount of contaminating corrinoids was still present. This solution was used for the spectroscopic studies.

Analytical procedures. Ultraviolet-visible light spectra were obtained with a Cary model 118 spectrophotometer. For quantitative determinations the following extinction coefficients at the indicated wavelengths were used: $\epsilon_{580} = 10.2 \times 10^3$ $M^{-1}cm^{-1}$ for $(CN)_2$-B_{12}-HBI and total corrinoid in 0.01% KCN; $\epsilon_{550} = 8.65 \times 10^3$ $M^{-1}cm^{-1}$ for CN-B_{12}-HBI; $\epsilon_{286} = 7.1 \times 10^3$ $M^{-1}cm^{-1}$ for HBI (pH $\leq$ 2). ^{1}H-NMR spectra were obtained with a Bruker model WH 90 spectrometer. HPLC was performed on a reverse-phase C_{18} column eluted with a gradient of 0-50% methanol in 10 mM potassium acetate, pH 6.0, as described previously (24) Phosphate was determined by the method of Fiske and SubbaRow as described by Bartlett (25). Protein was determined by the method of Lowry (26).

Synthesis of 5-hydroxybenzimidazole. 1.5 g of 4-methoxy-O-phenylenediamine hydrochloride (Aldrich Chemical Co.) was refluxed with 10 ml formic acid (98%) for 2 h at 105°C. The resulting reaction mixture was concentrated by flash evaporation to a dark brown oil. To remove residual formic acid 5 ml of water was added and evaporated three times. This crude preparation of 5-methoxy-benzimidazole was hydrolyzed in 5 ml 6 N HCl for 2 h at 150°C in an evacuated fused glass tube (10 x 1 cm). HBI formed was purified on a Dowex 50 W (H^+) column (15 x 1.5 cm) as described above. The free base of HBI was prepared by passing the HCl form through a Amberlite IR-4B (OH^- form) column in water and was purified further by three-fold crystallization from hot water. Elemental analysis gave C, 62.6%; H, 4.5%; N, 20.6%. Calculated values for $C_7H_6N_2O$ were C, 62.7%; H, 4.5%; N, 20.9%. Ultraviolet-visible light spectra, isosbestic points and extinction coefficients in 0.1 N HCl and 0.1 N NaOH were the same as those reported for HBI by Friedrich and Bernhauer (17).

RESULTS AND DISCUSSION. Extraction of corrinoids. Extraction of 36 g wet cells of *M. barkeri* with methanol and subsequent isolation of the corrinoids by Amberlite XAD-2 yielded 7.2 µmol of total corrinoid in the crude corrinoid extract (CCE); the presence of contaminating compounds absorbing at 580 nm may result in an overestimation. This amount is equivalent to about 1.3 nmol corrinoid per mg cell (dry weight) and was one third of that found by Krzycki and Zeikus (11) for methanol-grown *M. barkeri*. Further purification of CCE on QAE- and SP-Sephadex yielded 6 µmol of CN-B_{12}-HBI; this represents a 83% molar recovery as a compound with HBI as the α-ligand. The purity of CN-B_{12}-HBI was checked by TLC and HPLC (detection at 220 nm) and was estimated to be over 98%. Probably the rest of the corrinoids are base-less. In agreement with this view purple compounds were observed in CCE. These compounds are most probably dicyanocorrinoids and turned red on chromatographic treatment by the loss of one cyano group; in contrast to base-containing dicyanocorrinoids, base-less dicyanocorrinoids turned out to be rather stable under the conditions applied.

Properties of CN-B_{12}-HBI. CN-B_{12}-HBI and $(CN)_2$-B_{12}-HBI, which was obtained by conversion of the former in 0.01% KCN, exposed spectra (Fig. 1) which were

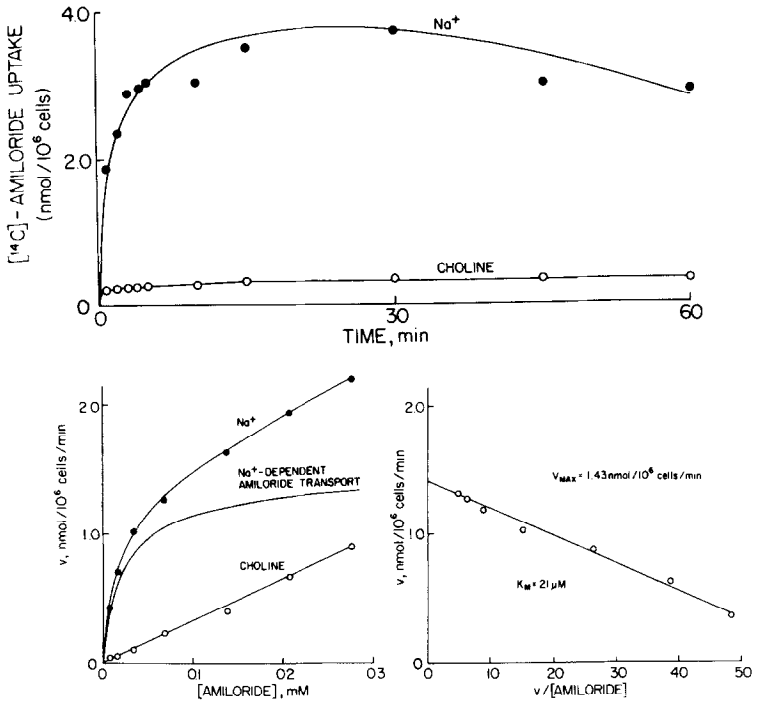

Fig. 2. Isolated adult rat hepatocytes: Kinetics of [^{14}C]amiloride uptake (top). 1.5 x 10^6 cells/ml were incubated at 37°C with 0.04 mM [^{14}C]amiloride (0.05 μCi/tube) in Krebs-Ringer bicarbonate buffers (10,11) ±Na^+. At varying times, cells were centrifuged, resuspended in 4°C NaCl, recentrifuged and counted. Concentration dependence of amiloride uptake (lower left). 1.5x10^6 cells/ml were incubated at 37°C for 2 min in buffer ±Na^+. The Na^+-dependent component of amiloride transport was obtained by subtracting, at each substrate concentration, the Na^+-insensitive part from the total. (lower right) Woolf-Augustinsson plot of the Na^+-dependent part of amiloride transport.

present during incubations, the measurements reflected relative rates of de novo protein synthesis (11,16). Extracellular amiloride concentrations of 0.004 mM amiloride did not affect overall rates, in contrast to drug levels of 0.04 mM and 0.4 mM which did ($p<0.02$ and $p<0.0001$, respectively). The latter dose reduced overall rates ca. 50% (Fig. 4, top left).

Amiloride did not block cellular protein synthesis immediately. More than 1 hr elapsed before overall rates changed (Fig. 4, right). Low concentrations of cycloheximide (0.0002-0.002 mM), by contrast, abolished synthesis within 30 min.

Amiloride reduced relative rates of cellular albumin synthesis; the ID_{50} was ~0.028 mM (Fig. 4, bottom left). Higher doses (0.04 and 0.4 mM) lowered synthesis rates between 1-2 hr ($p<0.003$ and $p<0.0001$, respectively), whereas

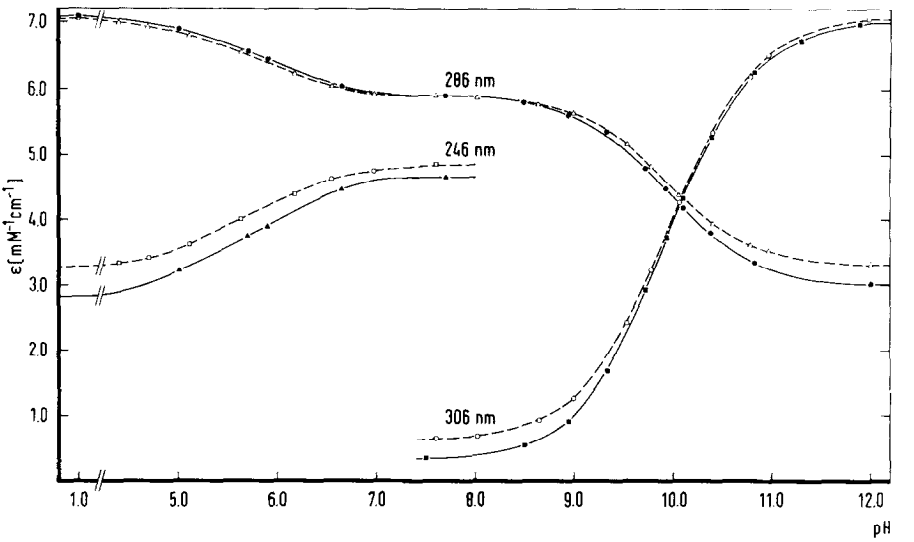

Fig. 2 Extinction coefficients of the absorption maxima as a function of pH for authentic (solid line) and isolated (dashed line) HBI.

was prepared from cell-free extracts and HPLC analysis was performed. 3 nmol total corrinoid per mg protein was obtained, a value also found by Shapiro (10). By the use of CN-B_{12}-HBI as a standard 90% of the corrinoids could be identified as CN-B_{12}-HBI, but CN-B_{12}, which separates well from CN-B_{12}-HBI in the HPLC-system, could not be detected at all.

Identification of 5-hydroxybenzimidazole. By the method outlined in Materials and Methods 4.6 µmol HBI was obtained from 4.6 µmol of purified CN-B_{12}-HBI. At all pH values between 1 and 12, the spectra of the isolated HBI were nearly identical to those of HBI synthesized as described above. The spectrophotometrically estimated pK_a values were the same for both materials (pK_1 = 5.7 ± 0.1, pK_2 = 9.9 ± 0.1) (Fig. 2) and are in agreement with the values determined electrometrically by Friedrich and Bernhauer (17). Fig. 3 shows the ^{1}H-NMR spectrum, signal assignment and coupling constants of authentic HBI, which appeared to be identical to those of isolated HBI, apart from minor resonances below 5 ppm (data not shown). The coupling constants $J_{6,7}$ and $J_{4,6}$ agree well with those found for the corresponding protons in 8-hydroxy-5-deazaflavins (28-30). The coupling corresponding to $J_{4,7}$ was not reported for the latter group.

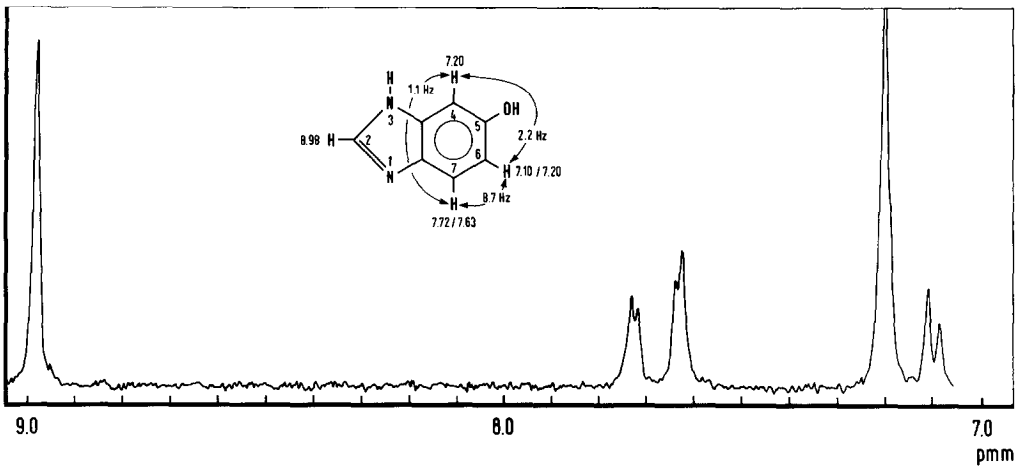

Fig. 3 ^{1}H-NMR spectrum of 4 µmol authentic HBI in 0.6 ml D_2O. Values adjacent to the protons indicate the chemical shift from TMS in ppm. The arrows give the coupling constants (Hz) between the indicated protons.

The C'-5 instead of a C'-6 position of the hydroxy group in B_{12}-HBI may be derived from a comparison with the characteristic spectral data (18,31) of 5- and 6-hydroxy (or methoxy) substituted 1-methylbenzimidazoles.

REFERENCES

1. Blaylock, B.A., and Stadtman, T.C. (1963) Biochem.Biophys.Res.Commun. 11, 34
2. Blaylock, B.A., and Stadtman, T.C. (1966) Arch.Biochem.Biophys. 116, 138.
3. McBride, B.C., and Wolfe, R.S. (1971) Biochemistry 10, 2317.
4. Taylor, C.D., and Wolfe, R.S. (1974) J.Biol.Chem. 249, 4879.
5. Gunsalus, R.P., and Wolfe, R.S. (1977) Biochem.Biophys.Res.Commun. 76, 790.
6. Gunsalus, R.P., and Wolfe, R.S. (1978) J.Bacteriol. 135, 851.
7. Shapiro, S., and Wolfe, R.S. (1980) J.Bacteriol. 141, 728.
8. Gunsalus, R.P., Eirich, D., Romesser, J., Balch, W., Shapiro, S., and Wolfe, R.S. (1976) in Proceedings of the Symposium on Microbial Production and Utilization of Gases, (Schlegel, H.G., Gottschalk, G., and Pfennig, N., eds.) Akademie der Wissenschaften zu Göttingen, Goltze K.G., Göttingen.
9. Wolfe, R.S., and Higgins, I.J. (1979) in Microbial Biochemistry, 21 (Quayle, J.R., ed.) University Park Press, Baltimore.
10. Shapiro, S. (1982) Can.J.Microbiol. 28, 629.
11. Krzycki, J., and Zeikus, J.G. (1980) Curr.Microbiol. 3, 243.
12. Hutten, T. (1982) Methanogenesis. Studies of the process and micro-organisms involved in methane formation. Ph.-D. thesis. University of Nijmegen.
13. Wood, J.M., Moura, I., Moura, J.J.G., Santos, M.H., Xavier, A.V., LeGall, J., and Scandellari, M. (1982) Science 216, 303.
14. Whitman, W.B., and Wolfe, R.S. (1982) Fed.Proc. 41, 1152.
15. Kenealy, W., and Zeikus, J.G. (1981) J.Bacteriol. 146, 133.
16. Friedrich, W., and Bernhauer, K. (1953) Angew.Chem. 65, 627.
17. Friedrich, W., and Bernhauer, K. (1956) Z.Naturforsch. 11B, 68.
18. Friedrich, W., and Bernhauer, K. (1956) Chem.Ber. 89, 2030.
19. Friedrich, W., and Bernhauer, K. (1956) Angew.Chem. 68, 439.
20. Lezius, A.G., and Barker, H.A. (1965) Biochemistry 4, 510.
21. Bryant, M.P., Wolin, E.A., Wolin, M.J., and Wolfe, R.S. (1967) Arch.Microbiol. 59, 20.

22. Scott, A.I. (1979) in Vitamin B_{12}, Proceedings of the Third European Symposium on Vitamin B_{12} and Intrinsic Factor (Zagalak, B., and Friedrich, W., eds.) p. 247, De Gruyter, Berlin.
23. Hutten, T.J., de Jong, M.H., Peeters, B.P.H., van der Drift, C., and Vogels, G.D. (1981) J.Bacteriol. 145, 27.
24. Van Beelen, P., Geerts, W.J., Pol, A., and Vogels, G.D. (1982) Anal. Biochem., submitted.
25. Bartlett, G.R. (1959) J.Biol.Chem. 234, 466.
26. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, A.J. (1951) J.Biol.Chem. 193, 265.
27. Friedrich, W., and Bernhauer, K. (1959) in Medizinische Grundlagenforschung, (Bauer, K.F., ed) Band II, p. 661, G. Thieme Verlag, Stuttgart.
28. Eirich, L.D., Vogels, G.D., and Wolfe, R.S. (1978) Biochemistry 17, 4583.
29. Pol, A., van der Drift, C., Vogels, G.D., Cuppen, T.J.H.M., and Laarhoven, W.H. (1980) Biochem.Biophys.Res.Commun. 92, 255.
30. Yamazaki, S., Tsai, L., and Stadtman, T.C. (1982) Biochemistry 21, 934.
31. Shunk, C.D., Robinson, F.M., McPherson, J.F., Gasser, M.M., and Folkers, K. (1956) J.Am.Chem.Soc. 78, 3228.