Cobamide-containing membrane protein complex in *Methanobacterium*

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In *Methanobacterium thermoautotrophicum*, most of the sole cellular cobamide, 5-hydroxybenzimidazolyl-cobamide (factor III [(1986) Anal. Biochem., submitted]) is bound to a membrane protein. This corrinoid protein is solubilized as a protein complex of M_r ~500 000 by 5% nonpolar detergents. The complex is stable in 7 M urea. In SDS, two smaller fractions of M_r ~30 000 and ~60 000 carrying the cobamide are found. The unusual properties of the corrinoid enzyme complex suggest a novel function in the metabolism of CO_2 and H_2 in methanogens.

(Methanobacterium) *Methanogenesis* *Cobamide* *Corrinoid enzyme* *Vitamin B_{12}* *Membrane protein*

1. INTRODUCTION

Methanogenic bacteria gain energy by synthesizing CH_4 from CO_2 and H_2, C_1-compounds and acetate, respectively [1]. They contain appreciable amounts of cobamides, but their role in metabolism is little understood [2–6]. In *Methanosarcina* sp. two soluble corrinoid-containing proteins act as methyltransferases in $*CH_4$ formation from $*CH_3OH$ [7–9] and probably from $*CH_3COOH$ [10]. There is indirect evidence that a corrinoid is involved in methyl ($*CH_3$-) transfer during synthesis of acetyl-CoA ($*CH_3$-COSCoA) from two CO_2 [11–14]; this process serves for autotrophic cell carbon assimilation in methanogens as well as in many other strict anaerobes [15].

Before the discovery of coenzyme M ($HSCH_2CH_2SO_3H$) [16], corrinoids were thought to act as CH_3-carriers in methanogenesis from CO_2 and H_2 [17]. However, methyl coenzyme M reductase was shown to catalyze the exergonic final step of $*CH_4$-formation from $*CH_3SCoM$ and reducing compounds [18]. This enzyme contains a nickel tetrapyrrole rather than a cobamide [19,20]; thus the role of corrinoids in methanogenesis was questionable.

This is the first report of a corrinoid-containing membrane protein which in *Methanobacterium thermoautotrophicum* accounts for most of the cellular cobamide. This bacterium will only grow on CO_2 and H_2. Therefore, the unusual corrinoid protein almost certainly plays an important and novel role in growth of methanogenic bacteria on H_2 and CO_2.

2. EXPERIMENTAL

M. thermoautotrophicum (Marburg strain) was routinely grown as in [21]; ^{57}Co-labelled cells were grown in presence of 0.3 μM $^{57}CoCl_2$ (1.1×10^4 Bq/nmol), ^{32}P-labelled cells were grown in 50 mM Pipes-Na rather than in phosphate-buffered mineral medium (pH 7.0) in the presence of 1 mM $KH_2{}^{32}PO_4$ (8.5×10^6 Bq/mmol). Cells were harvested at $\Delta A_{578} = 3$ ($d = 1$ cm; ≙1.2 g dry wt/l) and stored in liquid nitrogen. The cell extract was made by passage of cell suspensions (1 g fresh cells + 1 g water) through a French pressure cell at 137.6 MPa. Membranes were prepared by centrifugation of a 5000 × g supernatant for 60 min at 100000 × g. The ^{57}Co-membrane protein was solubilized by stirring the suspended 100000 × g pellet for 2 h in buffered detergent solutions of dif-

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ferent concentrations at 20°C, followed by centrifugation. 5-Hydroxybenzimidazolylcobamide (factor III) was isolated, identified and quantitated as in [6]. Gel-filtration experiments were performed on a 2.1 × 75 cm Sepharose CL-6B column (Pharmacia) using as elution buffers (4 ml·cm^{-2} h^{-1} flow rate): (I) 50 mM Tris-HCl, pH 7.5, containing 0.1% deoxycholate, 5 mM dithioerythritol, 0.1 mM phenylmethylsulfonyl fluoride, 0.02% NaN_3; (II) as I, but additional 7 M urea. Calibration was done with a high molecular mass protein calibration kit (Pharmacia) with both elution buffers I and II. CsCl density-gradient centrifugation (2.5 M CsCl in 10 mM Tris-HCl, pH 7.5; T = 20°C) was performed with a Beckman VTi 50 rotor at 50000 rpm (206000 × g) for 16 h. The gradients were fractionated in ~20 fractions. The density was determined by measuring the refraction index. Protein was determined by the Bradford method. ^{57}Co was quantitated in a γ-counter, ^{32}P in an LSC counter or, in double-labelling experiments, by measuring the continuous radiation in a γ-counter. Polyacrylamide-SDS gel electrophoresis in gels containing 12.5% acrylamide was calibrated using a low molecular mass standard protein kit (Pharmacia). Gels were stained with Coomassie brilliant blue G 250 and sliced in 0.5 cm slices.

3. RESULTS

M. thermoautotrophicum contains 0.1 μmol of the Co-corrinoid factor III per g dry wt as the only Co-corrinoid detectable by refined analytical methods [6] (E. Stupperich, personal communication). When cells were grown with $H_2:CO_2$ (80:20) gas as the sole energy and carbon source in the presence of the γ-radioisotope $^{57}Co^{2+}$, three quarters of the tracer incorporated could be centrifuged down at 100000 × g. The remaining one quarter of ^{57}Co in the supernatant was free $^{57}Co^{2+}$ and ^{57}Co in Co-corrinoids (table 1). A minimum of 66% of the bound ^{57}Co was isolated as ^{57}Co-cyano factor III (table 2), which was identified by UV/VIS and FAB spectroscopy after HPLC purification [6]. Factor III and ^{57}Co comigrated exactly in one single peak on HPLC. When the ^{57}Co-labelled, membrane containing 100000 × g pellet was resuspended and centrifuged in a CsCl density gradient, ^{57}Co appeared as a symmetrical peak at a density of 1.23 g·cm^{-3} (fig.1A). Since phospholipids (determined as ^{32}P) banded with ^{57}Co (not shown), the Co-protein(s) is most likely a membrane protein. In support of this conclusion the ^{57}Co-corrinoid protein complex could only be solubilized by relatively high (5–10%) concentrations of nonpolar detergents (fig.2). The solubilized ^{57}Co-protein gave a single peak on gel filtration with Sepharose CL-6B in the presence of 0.1% deoxycholate, corresponding to an apparent M_r of 600000–800000. This large complex was rather stable, since a single peak corresponding to an apparent M_r of 500000 was obtained on gel filtration in the presence of 7 M urea (fig.1B). When either

Table 1

Distribution of ^{57}Co in cell fractions of *Methanobacterium thermoautotrophicum*, which was grown for 3–4 generations in the presence of $^{57}Co^{2+}$

Cell fractionation step	^{57}Co (cpm)	Percentage of ^{57}Co
Cell extract	254500	100
5000 × g supernatant	242000	95
100000 × g pellet	183400	72
100000 × g supernatant	69600	27

Table 2

^{57}Co-corrinoid isolation [6] from 100000 × g pellet (membrane fraction) of *Methanobacterium thermoautotrophicum*

Purification step	^{57}Co (cpm)	Percentage of ^{57}Co in pellet
^{57}Co in 100000 × g pellet (≙72%)	183400	100
Extraction by boiling	142300	78
XAD-4 column pass through (non-corrinoid)	15900	9
Al_2O_3 column pass through (corrinoid)	120800	66
HPLC corrinoid fraction (cyano-factor III)	95000	52

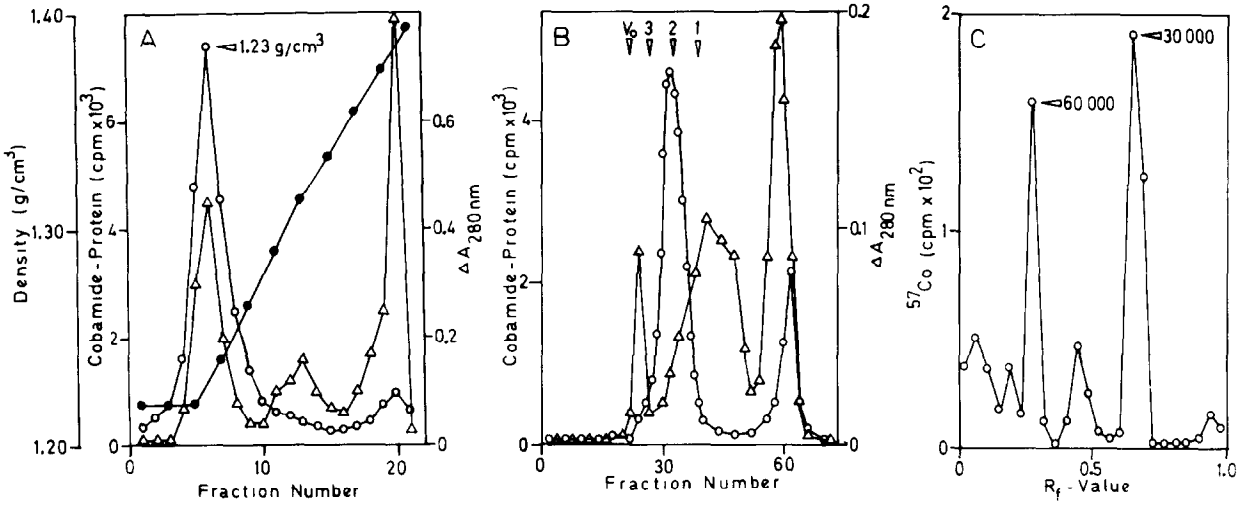

Fig.1. Determination of physical properties of the cobamide protein complex from *Methanobacterium thermoautotrophicum:* (A) CsCl density-gradient centrifugation of resuspended 100000 × *g* pellet. (○) ^{57}Co in the fractions, (△) ΔA_{280nm}, (●) density gradient. (B) Sepharose CL6B gel filtration of the solubilized cobamide protein complex in the presence of 7 M urea. (○) ^{57}Co in the fractions, (△) ΔA_{280nm}. Arrowheads (calibration proteins): 1, aldolase (158 kDa); 2, ferritin (440 kDa); 3, thyroglobulin (669000 g). V_0, void volume. (C) SDS-polyacrylamide gel electrophoresis of the 500 kDa fraction of Sepharose CL6B gel filtration shown in B. (○) ^{57}Co per slice.

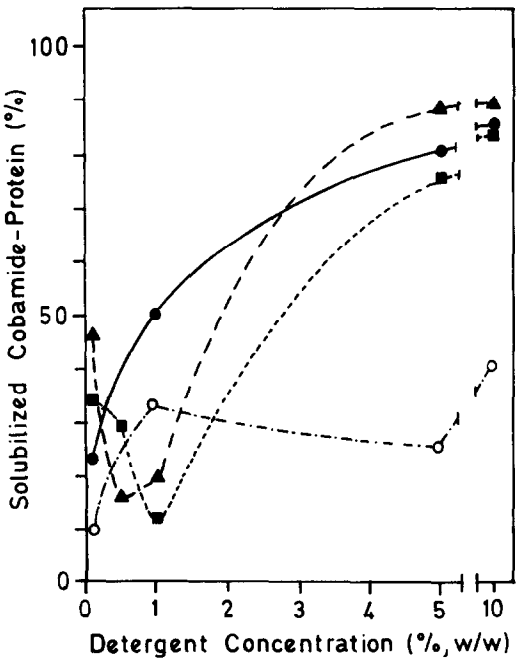

Fig.2. Solubilization of the cobamide protein complex from the 100000 × *g* pellet of cell extract from ^{57}Co-labelled *Methanobacterium thermoautotrophicum*: dependence on detergent concentration. (●) Deoxycholate, (▲) Triton X-100, (■) Lubrol, (○) cholate (less polar).

crude extract or the 500 kDa Sepharose fraction were analyzed on SDS-polyacrylamide gel electrophoresis, two clearly separated ^{57}Co-carrying bands corresponding to ~30 kDa and ~60 kDa were observed (fig.1C). They had a similar Co content and accounted for more than 70% of the ^{57}Co applied to the gels. Excessive heating in the presence of SDS resulted in the disappearance of ^{57}Co from the two protein bands. Since most ^{57}Co was recovered in the cyano factor III, these findings indicate that the proteins contain factor III firmly, but not covalently bound.

4. DISCUSSION

It was shown that most of the corrinoid factor III, which is the sole cobamide detected in *M. thermoautotrophicum* [6] is bound here to a large stable protein complex. By different preliminary criteria this was characterized best as an integral membrane protein complex, with the smallest corrinoid-containing subunit of molecular mass ~30 kDa. Until now no enzymatic function can be ascribed to this corrinoid protein. However, in vitro studies of autotrophic acetyl-CoA synthesis

(unpublished) seem to indicate that it is not involved in CO_2 fixation into cell carbon. On the other hand, reduced free vitamin B_{12} (B_{12s}) was the most effective electron donor for methyl coenzyme M reduction to CH_4 catalyzed by the purified methyl coenzyme M reductase [22]. This enzyme is membrane associated (G. Gottschalk, Göttingen, personal communication) and its physiological electron donor unknown. Furthermore, the stimulatory effects of corrinoids on the methyl-CoM reductase system using H_2 as the electron donor were described [23]. When actively growing cultures were pulse labelled with $^{14}CO_2$, only very little (~4%) of cellular factor III was methylated (the light-sensitive CH_3-B_{12} was added as internal standard for control; Stupperich, E. and Rühlemann, M., unpublished). This indicated that indeed a corrinoid enzyme is involved in methyl transfer, e.g. in acetyl-CoA and, perhaps, methionine synthesis; but most of the cobamide may fulfill a different function in the membrane. In view of our and others' findings we suggest a role in electron transport and, hence, in the generation of an electrochemical gradient [24] (cf. B_{12a} (Co^{III})/B_{12r} (Co^{II}), $E_0' = 242$ mV; B_{12r} (Co^{II})/B_{12s} (Co^{I}), $E_0' = -556$ mV; cf. CH_3OH/CH_4, $E_0' = 169$ mV) [25]. Future studies of methanogenesis from CO_2 and H_2 may have to account for the role of the corrinoid membrane protein.

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