HYDROGENASE FROM *METHANOBACTERIUM THERMOAUTOTROPHICUM*, A NICKEL-CONTAINING ENZYME

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Received 23 October 1981

1. Introduction

Growth of methanogenic bacteria has been shown to be dependent on nickel [1,2]. Of the nickel taken up by the cells, 50–70% is used to synthesize factor F_{430}, which has a nickel tetrapyrrole structure [3], and which is the prosthetic group of methyl-CoM reductase [4]. The rest is incorporated into the protein fraction, where most of it can be released as free nickel ions at pH 3 [2]. Factor F_{430} does not release nickel under these conditions. The presence of a second nickel-containing cell component is also indicated by the finding that washed membranes contain an EPR center that can be assigned to a Ni(III) complex [5]. Unlike F_{430}, the EPR center is reduced by dithionite.

Methanogenic bacteria are generally grown on H_2 and CO_2 as sole energy source. Hydrogenase is therefore an essential enzyme for these bacteria. Here, it is shown that the synthesis of active hydrogenase in *Methanobacterium thermoautotrophicum* is dependent on nickel. The enzyme was purified to near homogeneity and found to contain ~1 nickel/molecule. From the results, it is concluded that hydrogenase from methanogenic bacteria is a nickel protein.

2. Materials and methods

2.1. *Growth experiments*

Methanobacterium thermoautotrophicum (strain Marburg; DSM 2133 [6]) was used. The cells were grown on H_2 and CO_2 as sole carbon and energy sources [7]. Growth and uptake of ^{63}Ni were determined as in [8]. For the determination of hydrogenase activity 5 ml samples were anaerobically withdrawn from the culture, mixed with 0.2 ml solution containing 10% sodium desoxycholate and 1 mM benzyl viologen and incubated with H_2 as gas phase for 12 h at room temperature. Aliquots of 10 μl were assayed for hydrogenase activity (see below).

2.2. *Purification of hydrogenase*

All purification steps were performed aerobically at 4°C. The cells were harvested by centrifugation (15 min, 16 000 × *g*) at the time indicated in table 1 and fig.3. One gram (wet wt) of these cells (~100 mg protein) was washed twice with 10 mM imidazole/HCl (pH 6.5) then suspended in 5 ml H_2O containing 3 mg deoxyribonuclease I (Boehringer, Mannheim). Cell extracts were prepared by passing the suspension twice through a french pressure cell at 137 000 kPa (20 000 lb . in^{-2}). Cell debris was removed by centrifugation for 15 min at 27 000 × *g*. The supernatant was dialyzed twice against 500 ml 10 mM imidazole/HCl (pH 6.5) for 8 h (dialysis tubing with an exclusion limit of M_r 20 000–30 000; Roth, Karlsruhe). The dialyzed cell extract was applied to a DEAE cellulose column (1.6 diam; 32 ml DE-52 from Whatman, Maidstone), which was equilibrated with 10 mM imidazole/HCl (pH 6.5). The column was washed with 70 ml of the same buffer containing 0.2 M NaCl before hydrogenase was eluted by increasing to 0.6 M NaCl (linear gradient, 410 ml, 30 ml/h). Fractions of 6 ml were collected. Fraction 32–37, which contained most of the hydrogenase activity, were pooled and applied to a hydroxyapatite column (1 cm diam; 7.9 ml hydroxyapatite prepared as in [9]), which was equilibrated with 100 mM potassium phosphate (pH 7.1) containing 0.2 M NaCl. After washing with 10 ml of this buffer, hydrogenase was eluted by increasing to 0.3 M potassium phosphate (linear gradi-

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

ent, 54 ml, 8 ml/h). Fractions of 2.5 ml were collected. Fractions 5–8 were pooled and applied to a QAE-Sephadex A-25 column (1 cm diam; 8 ml QAE-Sephadex from Pharmacia, Uppsala), which was equilibrated with 10 mM imidazole/HCl (pH 6.5). The column was washed with 10 ml of the same buffer supplemented with 0.3 mol NaCl/l. Hydrogenase was eluted by increasing to 0.5 M NaCl (linear gradient, 54 ml, 8 ml/h). Fractions of 2.5 ml were collected. Fraction 10 contained most of the hydrogenase activity.

For the determination of hydrogenase activity 0.5 ml eluate was mixed with 0.5 ml 10 mM potassium phosphate (pH 7.1) containing 0.5 mM benzyl viologen and incubated for 5 h at 40°C with H_2 as gas phase. Aliquots of 10 μl were then assayed photometrically at 578 nm for benzyl viologen reduction with H_2 ($\epsilon = 8.65\ mM^{-1} \cdot cm^{-1}$). Assays were performed at 40°C under anaerobic conditions in 1.5 ml cuvettes filled with 1 ml 50 mM potassium phosphate (pH 7.1) containing 2 mM benzyl viologen [10]. The gas phase was H_2. One unit corresponds to 2 μmol benzyl viologen reduced by H_2/min. ^{63}Ni was counted in Aqualuma® (Baker Chemicals, Deventer). Protein was determined by the Lowry method [11].

2.3. *Polyacrylamide gel electrophoresis*

Hydrogenase containing fractions (200 μl) were mixed with 200 μl 87% glycerol and 100 μl 0.1% bromophenol blue. The mixture (200 μl) was subject to electrophoresis at 2 mA/gel and 4°C in 7.5% polyacrylamide gels. The pH of the lower and upper buffer (50 mM Tris/glycine) was 8.3, while the gel buffers were at pH 6.9 (spacer gel) and at pH 8.9 (separation gel), respectively [12]. The gels were stained for protein with Coomassie brilliant blue G-250 (Sigma, München), and for hydrogenase activity with benzyl viologen plus H_2. Gels were sliced to locate ^{63}Ni. The slices were dissolved in 30% H_2O_2 for 5 h at 60°C and then analyzed for radioactivity in Aqualuma®.

3. Results

3.1. *Nickel requirement for active hydrogenase formation*

Methanobacterium thermoautotrophicum was grown in a mineral salt medium containing either 2 μM $^{63}NiCl_2$ (fig.1A) or 0.25 μM $^{63}NiCl_2$ (fig.1B). Growth, uptake of nickel, and formation of active hydrogenase were followed. In the cultures with 2 μM Ni growth was not limited by nickel. Growth was exponential (t_d = 1.7 h) and hydrogenase activity increased proportionately with the cell density. In the culture with 0.25 μM Ni growth became limited by

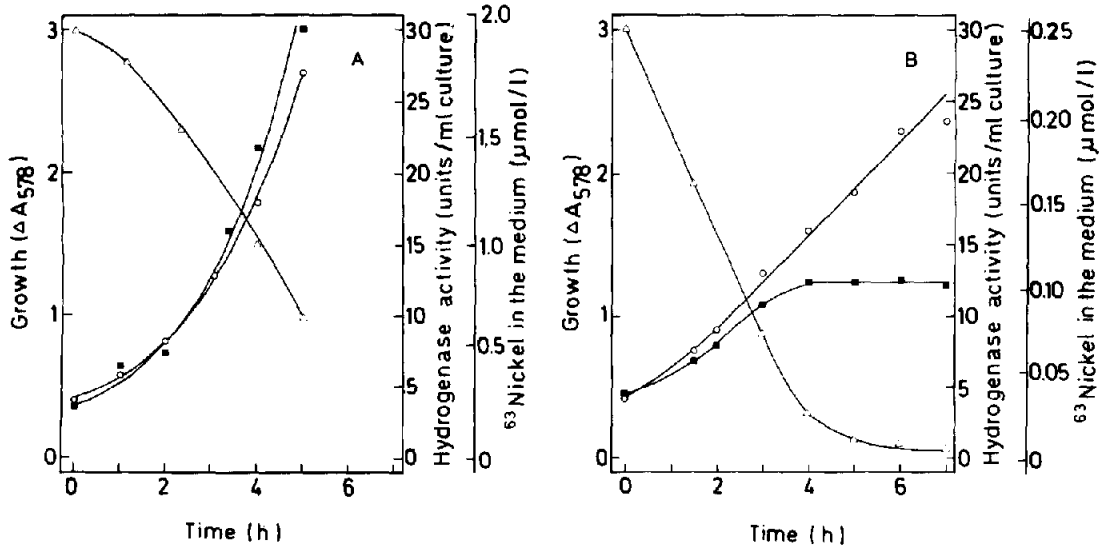

Fig.1. Nickel uptake and hydrogenase formation in growing cells of *M. thermoautotrophicum* (mean values from 3 expt): (A) the medium contained 2 μM $^{63}NiCl_2$ initially; (B) the medium contained 0.25 μM $^{63}NiCl_2$ initially; (○) growth; (■) hydrogenase activity; (△) ^{63}Ni.

nickel after 4 h, when all the nickel was used by the cells. As long as nickel was available, hydrogenase was synthesized and the cells proliferated exponentially. When the medium was depleted of nickel the synthesis of active hydrogenase stopped, although the bacteria continued to grow linearly and thus synthesized protein. When $NiCl_2$ (1 μmol/l) was added to the growing culture after 8 h, active hydrogenase was synthesized again (not shown). Nickel (II) added to non-growing cells or to cell extracts did not affect hydrogenase activity. These findings indicate that nickel is required for active hydrogenase formation in *M. thermoautotrophicum*.

3.2. *Co-purification of hydrogenase activity and nickel*

Extracts prepared aerobically from cells grown with 2 μM Ni contained 18 units hydrogenase activity and 2.3 nmol Ni/mg protein. After dialysis, the specific activity was 15 units/mg and the nickel content was 0.96 nmol/mg (table 1). From the dialyzed extract hydrogenase was purified 12-fold by chromatography on DEAE-cellulose, hydroxyapatite, and QAE-Sephadex A-25. Of the nickel present in the dialyzed extract 25% was found to co-purify with hydrogenase, the rest was associated with proteins, which were separated from the hydrogenase. Therefore the nickel content increased only 3.3-fold rather than 12-fold. In polyacrylamide disc gel electrophoresis almost all of the nickel present in the 12-fold purified hydrogenase migrated together with the hydrogenase activity (fig.2). A quantitative evaluation of the protein profile indicated that the 12-fold purified hydrogenase with a nickel content of 3.2 nmol/mg (table 1) was only 25% pure. When corrected for the impurity,

Table 1
Purification of hydrogenase from *Methanobacterium thermoautotrophicum*

Purification state	Specific activity (U/mg)	Activity yield (%)	Purification (-fold)	Nickel content (nmol/mg)
Dialyzed cell extract	15	100	1	0.96
DEAE-cellulose	54	30	3.6	1.57
Hydroxyapatite	120	18	8	3.0
QAE-Sephadex	180	10	12	3.2

The cells were grown in a medium containing 2 μmol $^{63}NiCl_2$/l (fig.1A) and harvested at $\Delta A_{578} = 3$. The purification procedure is described in section 2

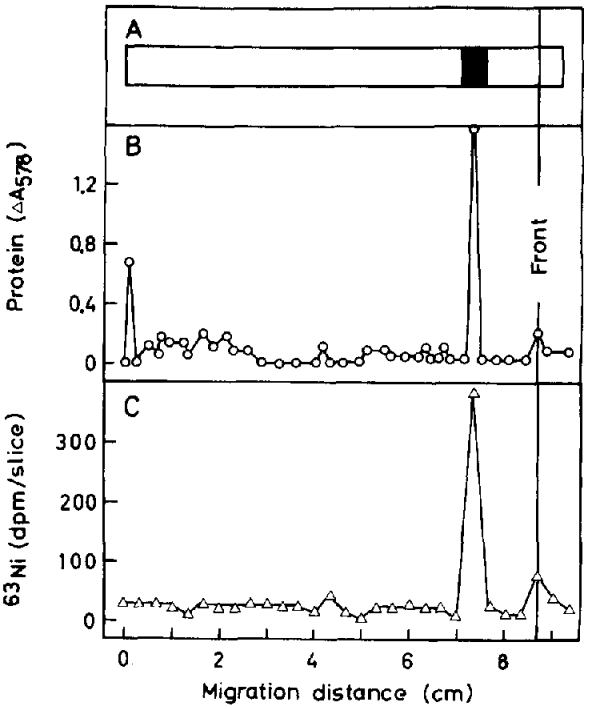

Fig.2. Coincidence of hydrogenase and ^{63}Ni in 7.5% polyacrylamide gels: (A) incubated in benzyl viologen plus H_2 to determine the location of hydrogenase; (B) stained for protein; (C) sliced and analyzed to locate ^{63}Ni. All gels were loaded with 20 μg protein from QAE-Sephadex A-25 (table 1) containing 1500 dpm. The specific radioactivity of ^{63}Ni was 21 500 dpm/nmol. The recovery of ^{63}Ni was 73%.

the nickel content of pure hydrogenase was calculated to be 12.8 nmol/mg or 0.8 mol Ni/mol hydrogenase with M_r 60 000 determined in [13].

Nickel is known to bind non-specifically to proteins. Hydrogenase was therefore purified from cells grown under conditions with limited nickel (fig.1B). The enzyme from these cells also co-purified with nickel (fig.3); the 12-fold purified protein contained the same amount of nickel as the enzyme from bacteria grown in the presence of excess nickel. These findings indicate that the transition metal is specifically associated with the hydrogenase.

From fig.3 it can be seen that hydrogenase activity eluted from the hydroxyapatite column in 2 peaks indicating the presence of 2 hydrogenases or of 2 forms of hydrogenase in *M. thermoautotrophicum*. For both peaks a coincidence of hydrogenase activity and of ^{63}Ni was observed. This indicates that both 'hydrogenases' contained nickel. Only hydrogenase of peak I (fraction 5–8) was further purified.

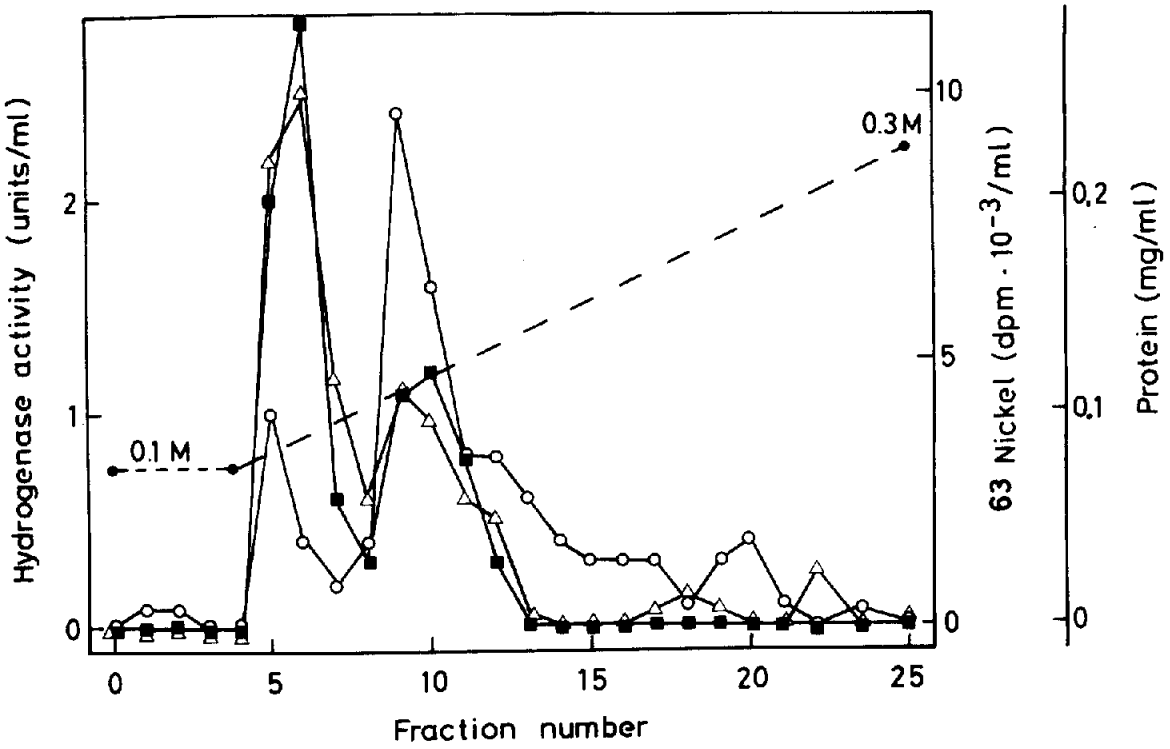

Fig.3. ^{63}Ni (△), protein (○) and hydrogenase (■) elution profiles from hydroxyapatite column (1 × 10 cm). Hydrogenase was purified from 1 g cells (wet wt) grown in a medium containing 0.25 μM ^{63}Ni (106 000 dpm/nmol) (fig.1B), and harvested at ΔA_{578} = 4. Hydrogenase from DEAE-cellulose was applied to the column. A linear potassium phosphate (pH 7.1) gradient from 0.1–0.3 M in 0.2 M NaCl was used to elute the hydrogenase. Recovery of ^{63}Ni was 86%. Fractions of 2.5 ml were collected. When hydrogenase was purified from cells grown in a medium with 2 μM Ni; ~50% of the nickel applied to the column eluted with proteins other than hydrogenase.

4. Discussion

Hydrogenase from *M. thermoautotrophicum* appears to be a nickel protein. This is suggested by the findings that:

(i) The formation of active hydrogenase in the growing bacteria was dependent on the presence of nickel;

(ii) The enzyme contained near to 1 Ni/molecule even when purified from cells grown on media with limited nickel.

In [14] hydrogenase activity of *M. thermoautotrophicum* was stimulated 2-fold by the addition of Ni(II) (0.5 mM) to the assay. The purified enzyme was found to contain Ni, but considered to be an artefact [14].

Methanogenic bacteria are probably not the only organisms that contain a nickel hydrogenase. Formation of active hydrogenase in Knallgas bacteria has been reported to require nickel [15]. For reviews on hydrogenase and on the biological role of nickel see [16] and [17], respectively.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Bonn-Bad Godesberg) and by the Fonds der Chemischen Industrie (Frankfurt/Main).

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