Hydrogenase from *Vibrio succinogenes*, a nickel protein

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1. INTRODUCTION

The anaerobic rumen bacterium *Vibrio succinogenes* [1] gains the ATP required for growth according to:

$$H_2 + \text{fumarate} \rightarrow \text{succinate} \qquad (a)$$

when grown with H_2 and fumarate as sole energy sources. Reaction (a) is catalyzed by a membrane-bound electron transport chain, the initial part of which is a hydrogenase. This enzyme has been purified to homogeneity and found to contain approximately 1 Ni atom per enzyme molecule.

2. MATERIALS AND METHODS

2.1. *Growth of* V. succinogenes

V. succinogenes was grown at 37°C in an anaerobic medium containing 80 mM formate, 100 mM fumarate, 0.57 mM cysteine, 0.68 mM glutamate, 50 mM Tris, 20 mM K_2HPO_4, 5 mM ammonium chloride, 5 mM ammonium sulfate, 1 mM $MgCl_2$, 0.05 mM $CaCl_2$, 0.01% thioglycolate and the trace elements used earlier [2]. The pH was adjusted to 7.5 with KOH. The cells were harvested in the stationary phase of growth and stored in liquid N_2.

2.2. *Enzymic activity*

Hydrogenase activity was measured photometrically at 546 nm as the reduction of benzyl viologen by H_2, at 37°C (ε_{546} = 9.75 $mM^{-1} \cdot cm^{-1}$). The enzyme, in a solution of 150 mM glycine and 1 mM dithiothreitol at pH 9.5, was made anaerobic by evacuation and flushing with H_2 in a stoppered cuvette. The reaction was started by adding 1.5 mM benzyl viologen. The unit of activity (U) corresponds to 1 μmol H_2 oxidized per min.

2.3. *Purification of hydrogenase*

The purification was performed under anaerobic conditions at 0°C essentially as described earlier for formate dehydrogenase [3].

2.3.1. Triton extract

The cells (2.5 g protein) were lysed and extracted with Tween 80 and Triton X-100 as described [3], except that the buffers contained 2 mM dithiothreitol, and formate, $Na_2S_2O_4$ and NaN_3 were omitted.

2.3.2. Chromatography on hydroxyapatite

The Triton extract was passed through a hydroxyapatite column, and the hydrogenase eluted with a linear K-phosphate gradient (30–350 mM). The buffers used were those described earlier [3], except that $Na_2S_2O_4$ and NaN_3 were replaced by 2 mM dithiothreitol. The fractions containing hydrogenase were pooled and concentrated by pressure dialysis [3].

2.3.3. Sucrose gradient centrifugation

The solution obtained from the preceding step was layered onto a buffer containing 0.05% Triton X-100, 20 mM Tris and 2 mM dithiothreitol pH 7.6 (buffer I) and a linear sucrose gradient (7.5–25%, w/v). Centrifugation for 3 h was done in a VTi 50 rotor (Beckman Instr.) at 206 000 × *g*. The frac-

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tions containing hydrogenase were concentrated by pressure dialysis.

2.3.4. Chromatography on DEAE–Sephadex

The enzyme (2 mg protein/ml column volume) was passed through a DEAE–Sephadex A-25 column which was equilibrated with buffer I. The pass through contained the hydrogenase and was concentrated by pressure dialysis.

2.3.5. Chromatography on Sephacryl S-300

The concentrated enzyme (2 ml) was subjected to gel filtration. The column (1 m long, inner diameter 1 cm) was equilibrated with buffer I. The flow velocity was 1.5 ml/h.

2.4. *Ni determination*

Ni was determined by counting the radioactivity of ^{63}Ni. The specific radioactivity of Ni (86 500 dpm/nmol) was calculated by measuring the Ni content and the radioactivity of a culture supernatant on which the bacteria had been grown. Ni was extracted from 200 ml medium with 5 ml of 70 mM 8-hydroxyquinoline in $CHCl_3$ and determined by atomic absorption [4].

2.5. *Protein*

Protein was measured by the biuret method with KCN [5].

3. RESULTS

3.1. *Purification of hydrogenase*

The specific activity of the hydrogenase from *V. succinogenes*, as measured with benzyl viologen, was the same in bacteria grown either with H_2 or with formate as electron donor. This indicates that hydrogenase is a constitutive enzyme in *V. succinogenes*. After fractionation of the cells, following treatment with EDTA plus lysozyme and osmotic shock [3], the activity was found only in the membrane fraction of the bacterium.

The enzyme was solubilized by treatment of the membrane fraction with Triton X-100 (table 1). The Triton extract was passed through a hydroxyapatite column which absorbed the enzyme. On application of a linear phosphate gradient the enzyme eluted as a single band with a peak at about 0.2 M phosphate. In density gradient centrifugation hydrogenase activity was sometimes found in

Table 1

Purification of hydrogenase

Purification step	Activity (H_2 → benzyl viologen)	
	U	U/mg
Cell homogenate	1650	0.7
Triton extract	1180	1.2
Chromatography on hydroxyapatite	770	6.1
Density gradient centrifugation	470	53
Chromatography on DEAE–Sephadex A-25	190	194
Chromatography on Sephacryl S-300	170	660

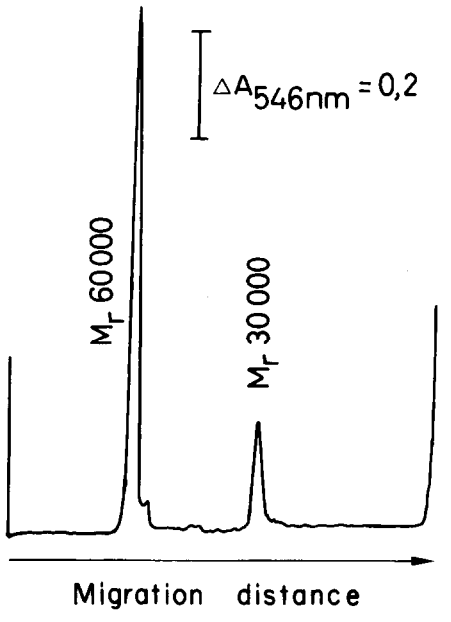

Fig.1. SDS-gel electrophoresis of the hydrogenase of *V. succinogenes*. Electrophoresis [3] was carried out with 12 μg of the enzyme preparation obtained from the experiment of table 1 according to Neville [6]. The gel was stained with Coomassie Blue G-250 and scanned photometrically at 546 nm.

2 bands. Only the major band was used for further purification using DEAE–Sephadex and Sephacryl S-300. The final specific activity of the preparation was 660 U/mg in the experiment of table 1, indicating nearly a 1000-fold purification. The overall yield was 10%. Chromatography of this preparation on phenyl–Sepharose CL-4B, hydroxyapatite or DEAE–Sephadex did not give an increase of the specific activity of the enzyme.

As shown by SDS-gel electrophoresis (fig.1), the hydrogenase preparation obtained from the purification procedure (table 1) consisted of 2 different peptides (M_r 60 000 and M_r 30 000). The M_r-values were determined from the electrophoretic mobilities in comparison with those of peptides of known molecular weights (not shown). The relative molar content of M_r 60 000 to M_r 30 000 peptide was estimated as 1:0.6 from the protein stain with Coomassie Blue G-250 (fig.1) and Amido Black 10B (not shown). The ratio remained the same during the last 3 steps of the purification procedure. With other preparations ratios up to 1:0.8 were found.

The hydrogenase of the minor peak obtained by density gradient centrifugation was also purified further. The enzyme contained at least 2 more peptides in addition to the M_r 60 000 and M_r 30 000 subunits, and catalyzed the reduction of 2,3-dimethyl-1,4-naphthoquinone by H_2. This reaction was not catalysed by the hydrogenase consisting only of the 2 peptides. It is, therefore, believed that the latter enzyme is derived from a hydrogenase complex which is characterized by its reaction with quinones.

3.2. *Ni content*

When grown in the presence of 0.6 μM Ni, *V. succinogenes* (0.25 g protein/l) contained 24 nmol Ni/g protein after washing of the cells. More than 90% of this Ni was separated from the hydrogenase activity during fractionation of the cell in the first step of the purification procedure (table 1). In each of the following chromatographic steps at least 70% of the Ni eluted coincidently with hydrogenase activity (not shown). The Ni contents of various hydrogenase preparations which differed by the degrees of purity were plotted against the specific activity of the enzyme (fig.2). The Ni content was a linear function of the specific activity of the preparations. From the slope of the line the turnover number based on the Ni content was calculated as 1.2×10^5 min^{-1}. The purest preparation contained 7.9 μmol Ni/g protein. About 80% of this preparation consisted of the M_r 60 000 and M_r 30 000 peptides, as judged from the protein stain of the corresponding SDS-gel (not shown). Inspite of the greater specific activity (970 U/mg protein) this preparation appeared to be less pure than that obtained in the experiment of table 1. This discrepancy may be explained by partial denaturation of the latter enzyme.

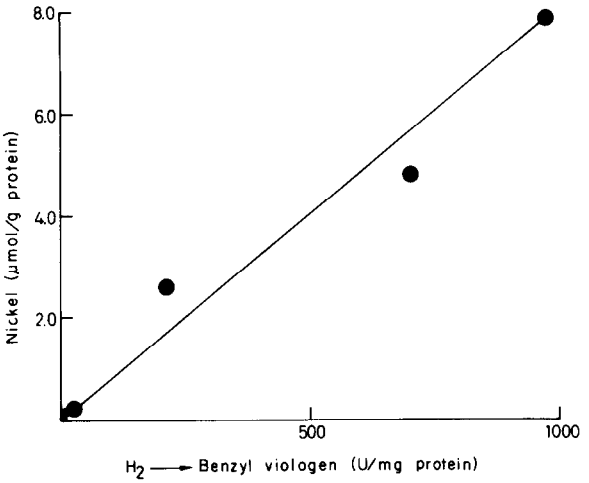

Fig.2. Correlation of the Ni contents and the specific enzymic activities of various preparations of the hydrogenase of *V. succinogenes*. The preparations analyzed were obtained at different steps of the purification procedures.

The hydrogenase preparation contained non-heme iron and acid-labile sulfur (table 2). The

Table 2

Composition of hydrogenase

Component	Content (μmol/g protein)
Nickel	7.9
Non-heme iron	90–160
Sulfide	62
Cytochrome *c*	< 0.8

Cytochrome *c* [7], non-heme iron and acid labile sulfur [8] were determined as described previously

content of sulfide was approximately 8 mol/mol Ni, while that of the iron varied from 11 to 20 mol/mol Ni. It is possible that the sulfide content is equivalent to the amount of iron–sulfur clusters in the enzyme and that the excess of iron is an impurity of the preparation. Cytochromes were not present in the preparation.

4. DISCUSSION

From the data presented it is concluded that *V. succinogenes* contains only 1 species of hydrogenase. The enzyme is involved in electron transport from H_2 to fumarate and is probably derived from a hydrogenase complex which catalyzes the reduction by H_2 of the menaquinone present in the membrane of the bacterium. Reoxidation of the reduced menaquinone by fumarate is known to be catalyzed by the fumarate reductase complex [9,10].

Ni appears to be an essential constituent of the hydrogenase. This is concluded from the finding that Ni elutes coincidently with the hydrogenase activity in the various chromatographic procedures used for purification. Furthermore, the specific activity is linearly related to the Ni content of the enzyme preparations obtained at the various purification steps. From the measured Ni content (7.9 μmol/g protein) and the purity of the preparation (80%) the minimum molecular weight of the enzyme can be calculated as 100 000. This value is close to the molecular weight obtained on the basis of the assumption that the enzyme consists of equimolar amounts of the M_r 60 000 and the M_r 30 000 peptide. The sedimentation coefficient (8 S) determined by density gradient centrifugation [11] is consistent with a molecular weight of the functional enzyme of 100 000. Therefore, the enzyme is probably made up of one of each peptide, one of which contains 1 mol Ni/mol.

The presence of non-heme iron and acid-labile sulfur in hydrogenase suggests that the enzyme contains iron–sulfur clusters. This is a common property of all the hydrogenases investigated so far [12]. The sulfide content (8 mol/mol Ni) is sufficient to account for 2 iron–sulfur centres in the enzyme.

The hydrogenase of *Methanobacterium thermoautotrophicum* (Marburg) was first demonstrated to be a Ni enzyme [13]. The Ni was reduced by hydrogen and reoxidized by oxygen [14]. Later this was also shown to be valid for the hydrogenases of *Desulfovibrio gigas* [15,16] and *Chromatium vinosum* [17]. The enzyme from *D. gigas* consists of 2 different peptides (M_r 62 000 and M_r 26 000) and is thus very similar to the enzyme of *V. succinogenes*.

The activity of uptake hydrogenases was shown to be a function of the Ni content of the medium with *M. thermoautotrophicum* [13], *Alcaligenes eutrophus* [18], *Azotobacter chroococcum* [19], *Rhodopseudomonas capsulata* [20] and *C. vinosum* [17]. This it may be concluded from present knowledge that uptake hydrogenases in general are Ni enzymes.

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