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THE MEMBRANE-BOUND HYDROGENASE OF *ALCALIGENES EUTROPHUS*

I. SOLUBILIZATION, PURIFICATION, AND BIOCHEMICAL PROPERTIES

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Summary

The membrane-bound hydrogenase of *Alcaligenes eutrophus* was solubilized from washed membranes of autotrophically grown cells. The enzyme consists of two types of subunits and is an iron-sulfur protein. A flavin compound was not detected. The enzyme reacts only with few artificial electron acceptors.

Introduction

In aerobic hydrogen-oxidizing bacteria, two types of hydrogenases have been found: a soluble hydrogenase, which reduces NAD^+, mainly for autotrophic CO_2 fixation, (hydrogen:NAD^+ oxidoreductase, EC 1.12.1.2), and a tightly membrane-bound hydrogenase which is unable to reduce pyridine nucleotides and provides electrons for energy generation in a modified electron transport chain.

The majority of the hydrogen-oxidizing bacteria contain a single membrane-bound hydrogenase [1]. These strains have to generate the NADH necessary for autotrophic growth by reversed electron transport. *Nocardia opaca* has a single NAD^+-reducing hydrogenase, which is soluble or loosely associated with the membrane [2,3]. Only few strains of hydrogen bacteria contain both types of hydrogenase [4—7].

The soluble hydrogenase of *Alcaligenes eutrophus* has been purified to electrophoretic homogeneity [8]. It differs from the soluble hydrogenases of the anaerobic bacteria, e.g. *Clostridium pasteurianum* [9] primarily by its

tolerance to oxygen, its high molecular weight, its complex structure and content of FMN [10].

For characterization of the membrane-bound hydrogenase, so far only washed membranes have been used [11]. It was not clear whether the soluble and the membrane-bound enzyme are different entities or only one enzyme with two functions and different localization. In this paper, the biochemical properties of the purified membrane-bound hydrogenase of *A. eutrophus* H 16 are described and compared to those of the soluble hydrogenase. A short report on the purification procedure has been given before [12].

Materials and Methods

Growth of the organism and membrane preparation. A. eutrophus H 16 (DSM 428, ATCC 17699) was grown autotrophically in the medium of Schlegel et al. [13] under an atmosphere of 80% H_2, 10% CO_2 in a Braun Biostat Fermenter (Braun, Melsungen, F.R.G.). Cells were harvested at $E_{436} = 10—14$, washed in 50 mM potassium phosphate buffer (pH 7.0) and resuspended in the same buffer at 1 g wet weight/4 ml buffer. The cells were disintegrated by sonication for 1 min (Schoeller TG 250, Schoeller, Frankfurt/M, F.R.G.), by a French pressure cell at 1875 bar, or by osmotic shock after lysozyme treatment [11]. Cell debris and membranes were sedimented at 100 000 × *g* for 50 min (Christ Omikron centrifuge, Heraeus-Christ, Osterode, F.R.G.), and the upper ruby layer of membranes was collected. Membranes were washed once with 50 mM potassium phosphate buffer (pH 7.0) and for a second time with the same buffer containing 0.15 M NaCl and 0.25 M sucrose. Finally, the membranes were resuspended in 50 mM potassium phosphate buffer (pH 7.0) at 4—5 mg protein/ml.

Solubilization. The membrane suspension was mixed with additives to final concentrations of 10% sucrose, 10 mM EDTA, 0.1% sodium deoxycholate, and 0.5% Triton X-100. The suspension was stirred at room temperature for 30 min and centrifuged at 100 000 × *g*. The clear yellow supernatant could be stored at 4° C overnight or, for longer periods, at −18° C under air, without the addition of any reducing agents.

$(NH_4)_2SO_4$ fractionation. $(NH_4)_2SO_4$ was added to the membrane extract at 0°C to give a 25% saturated solution. Before centrifugation, a thin layer of light petroleum or hexane was added to retain the apolar membrane constitutents and detergent at the surface. After centrifugation at 20 000 × *g* for 10 min, the aqueous solution containing the hydrogenase was sucked off by a pipette. After adding $(NH_4)_2SO_4$ to 30% saturation, centrifugation in the presence of hexane was repeated until the aqueous solution was clear and almost free from lipids and detergents. Thereafter hydrogenase was precipitated between 40 and 55% $(NH_4)_2SO_4$ saturation. The precipitate was dissolved in 50 mM potassium phosphate buffer (pH 7.0) and dialysed overnight at 4° C against the same buffer. A second $(NH_4)_2SO_4$ fractionation step resulted in precipitation of hydrogenase between 45 and 60% saturation. The precipitate was dissolved in potassium phosphate buffer and dialysed for at least 6 h against 25 mM potassium acetate buffer (pH 5.5).

Carboxymethyl-cellulose chromatography. The dialysed protein solution was

applied to a 1.6 × 30 cm CM-cellulose (Whatman, CM-52) column pre-equilibrated with 25 mM potassium acetate buffer (pH 5.5). The protein was eluted by a linear gradient of 0—0.5 M KCl (flow rate, 12 ml/h). The most active fractions of both hydrogenase peaks were pooled separately and concentrated by ultrafiltration in an Amicon Diaflo cell to 1—2 mg protein/ml.

Protein determination. Protein was determined by the method of Hartree [14]. Calibration curves with bovine serum albumin as standard in the presence and absence of 0.5% Triton X-100 differed by only about 10%.

Enzyme assays. Activity of soluble hydrogenase was assayed by NAD^+ reduction [8]. Membrane-bound hydrogenase was assayed with methylene blue as hydrogen acceptor either manometrically [5] or, for rapid measurement, by an optical test system. 2.9 ml hydrogen-saturated 50 mM potassium phosphate buffer (pH 7.0) containing 200 μM methylene blue were pipetted into 3-ml cuvettes continuously flushed with hydrogen. Glucose (0.2 μmol/assay), glucose oxidase and catalase (each 1 I.U./assay) were added as oxygen trap. The reaction was started by adding hydrogenase. The decrease in absorption was followed at 570 nm by a Zeiss PM 4 photometer. Calculations were based on a molar extinction coefficient of 13.1 $cm^2/\mu mol$. The results were in accordance with those derived from manometric measurements.

Electrophoresis. Electrophoretic analysis of enzyme purity was performed in 0.5 × 10 cm glass tubes with gels containing 5, 7.5, or 10% acrylamide at pH 8.9 at 4 mA/tube [15]. Protein bands were stained with Coomassie brilliant blue G 250 [16]. The activity bands of hydrogenase on the gel slabs were located [8] without adding NAD. Determination of subunit structure was performed by the method of Weber et al. [17].

The molecular weight was determined by gel filtration on a Sephadex G-200 column [18] and also by sucrose density gradient centrifugation [19].

Isoelectric focussing of hydrogenase was carried out as recommended in the LKB manual, using a 110 ml column (LKB), with ampholytes of pH 3.5—10.0. After 1 ml purified enzyme preparation was focussed for 64 h at 310 V and at 6° C, the column was eluted (120 × 1-ml fractions).

Quinones were extracted from membranes of autotrophically grown cells by the method of Kröger et al. [20] as modified [11].

Extraction of flavins was performed with trichloroacetic acid [8].

Determination of iron [21] was done after dialysis of the protein sample overnight against 50 mM potassium phosphate buffer (pH 7.0).

Determination of acid-labile sulfur was performed according to Brumby et al. [22].

Chemicals

The sources of biochemicals are those mentioned in [8]; Triton X-100 and lysozyme were from Serva (Heidelberg, F.R.G.).

Results

Solubilization and purification

The membrane-bound hydrogenase of *A. eutrophus* proved to be tightly bound to the membrane. Significant amounts of hydrogenase were solubilized

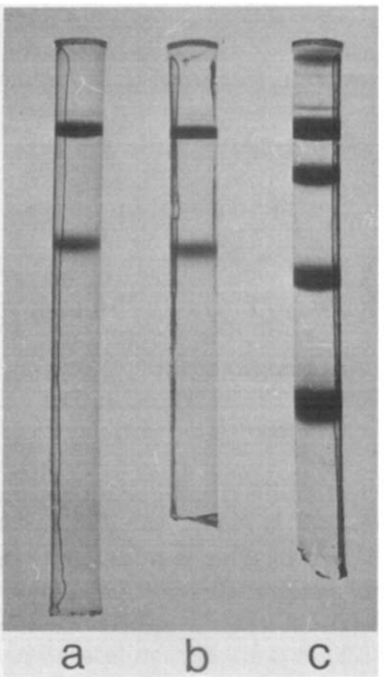

Fig. 1. Sodium dodecyl sulfate gel electrophoresis of purified hydrogenase. (a) The 'greater' enzyme preparation; (b) the 'smaller' enzyme preparation; (c) calibration gel with bovine serum albumin, ovalbumin, chymotrypsinogen A, and cytochrome *c*.

by phospholipase D and by the detergents, Triton X-100 and deoxycholate. Optimal solubilization was finally achieved with 0.5% Triton, 0.1% deoxycholate, 10% sucrose, and 10 mM EDTA [12,23]. The supernatant was stable under air at 4°C, or, better, at 18°C. In the presence of reducing agents (e.g. mercaptoethanol), the enzyme was rapidly inactivated.

Purifying the hydrogenase was easy due to two properties of the enzyme: (i) the enzyme did not stick to those lipophilic membrane constituents, which were extruded by raising the ionic strength; (ii) the enzyme had a relatively high isoelectric point resulting in optimal conditions for separation on a cation exchanger. Starting from washed membranes with a specific activity of 0.99 μmol H_2 oxidized/min per mg protein, the specific activity was increased 172-fold to 170 μmol H_2 oxidized/min per mg protein with a yield of 35.8%.

Chromatography on CM-cellulose resulted in resolution of two adjacent peaks. The protein of each peak was homogeneous in polyacrylamide gel electrophoresis run at different polyacrylamide concentrations, but the R_F values differed slightly. These were the only differences between the two fractions that could be detected [12].

Subunit structure, molecular weight and isoelectric point

Sodium dodecyl sulfate gel electrophoresis resulted in two types of subunits, one of M_r = 67 000, and one of M_r = 31 000 in equal molar concentration (Fig. 1). The weights of the two subunits add up to a total molecular weight of

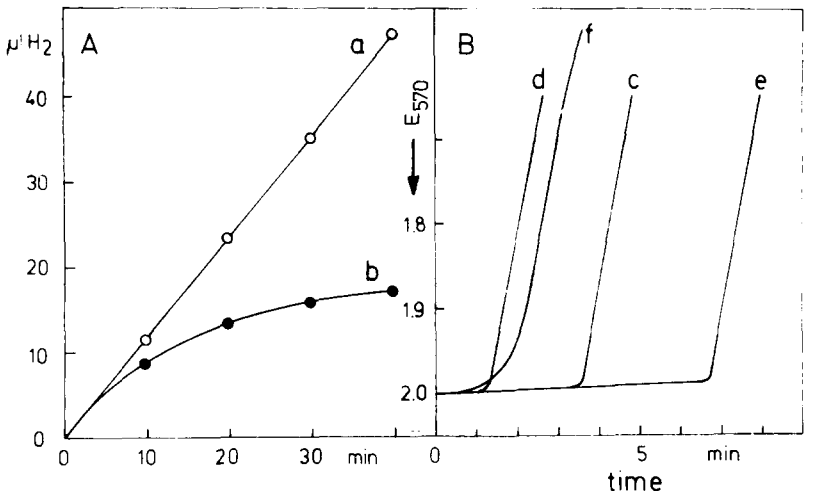

Fig. 2. Comparison of the reaction kinetics of membrane-bound hydrogenase under different conditions with methylene blue as electron acceptor. (A) Manometric measurement of H_2 uptake in the presence of methylene blue; (B) spectrophotometric measurement of the decrease of absorption at 570 nm. (a) Suspension of intact hydrogenase-containing membranes; (b) hydrogenase-containing supernatant after treatment of membranes by sodium deoxycholate; (c) suspension of intact hydrogenase-containing membranes without addition of any oxygen trap; (d) same as (c) with the addition of glucose oxidase and glucose; (e) same as (c) with the addition of 0.2 ml potassium phosphate buffer saturated with air; (f) membrane suspension treated with Triton and deoxycholate.

98 000 daltons, which is in accordance with the value obtained by sucrose gradient sedimentation (97 000—102 000 daltons). The low values found with gel filtration on Sephadex G-200 and G-150 (75 000—77 500 daltons) can be explained by a probable low degree of hydration, which is compensated in sedimentation experiments by a higher buoyant density of the resulting particle [24]. The isoelectric point of hydrogenase was found to be 6.5 when examined by electrofocussing in an LKB column containing ampholytes (pH 3.5—10.0).

Kinetics of enzyme reaction

Intact membrane particles (Fig. 2A) and membrane extracts prepared by Triton X-100 reduced methylene blue at a constant reaction rate for more than 1 h. If the enzyme had been solubilized by sodium deoxycholate or after Triton had been removed during the purification procedure, the reaction rate decreased after a few minutes. By adding Triton or phosphatidylcholine the decrease of the reaction rate could be avoided. Presumably the enzyme protein is impaired by reduced methylene blue at its apolar domain.

The spectrophotometric assay system showed always reaction rates retarded by a lag phase (Fig. 2B). This lag phase was short when glucose and glucose oxidase had been added to the assay mixture and was long when buffer saturated with air had been added. With intact membranes, the reaction rate was initially zero and then suddenly reached the maximum rate. With the solubilized hydrogenase preparation, the reaction rate increased continuously from the start to the maximum rate. The hydrogenase was apparently partially

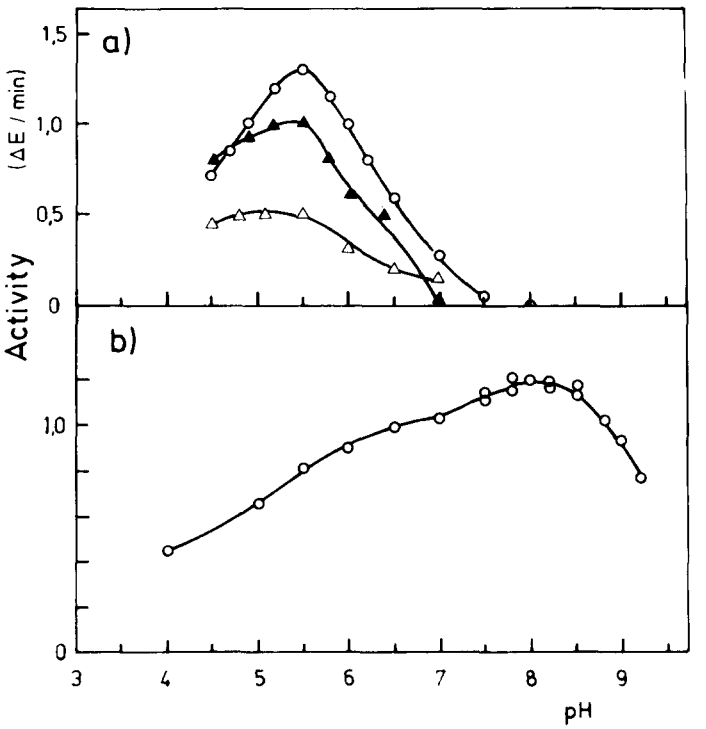

Fig. 3. Dependence of hydrogenase activity on the pH value. (a) Purified enzyme preparation; (b) intact membrane/suspension. Measurement took place in optical test system at 30°C with different buffers ○- - - - - -○, 50 mM potassium phosphate buffer; ▲- - - - - -▲, 50 mM Tris-HCl buffer; △- - - - - -△, 50 mM triethanolamine-HCl buffer. 10—100 μg protein were applied to each cuvette.

inhibited by oxygen and recuperates its activity gradually when oxygen is being removed.

The saturation curve of hydrogenase with respect to hydrogen as substrate was hyperbolic and followed Michaelis-Menten kinetics. The K_m value for hydrogen was 32 μM at a methylene blue concentration of 0.2 mM.

Optimum conditions for enzyme activity

The optimum pH for the reduction of methylene blue and menadione by the purified hydrogenase was pH 5.5. It was identical in different buffers; however, in Tris-HCl 75% and in triethanolamine only 35% of the enzyme activity compared to that in potassium phosphate buffer were measured (Fig. 3a). In intact membrane particles, high dehydrogenase activities were measured at pH 7.0—8.5 (Fig. 3b).

Maximal rates of methylene blue reduction were measured at 52°C in potassium phosphate buffer (pH 5.5). From the slope of the Arrhenius plot, which showed no bend, an activation energy of 26.2 kJ/mol (6.25 kcal/mol) was calculated. Maximal rates of the hydrogen-oxygen reaction in intact membranes were reached at 45°C [25]. The Arrhenius plot of this reaction showed a bend at 30°C apparently due to phase changes in the surrounding lipids [26]. An essential requirement for dissociable cofactors could not be detected. The reac-

TABLE I

REDUCTION OF ELECTRON ACCEPTORS BY THE PURIFIED HYDROGENASE OF *A. EUTROPHUS* H 16

Values are given in percent reduction rate found with methylene blue as acceptor.

Acceptor	% activity
Menadione	62
Methylene blue	100
Phenazine methosulfate	40—60
Pyocyanine	9
$K_3Fe(CN)_6$	4.5

tion was slightly inhibited by Mg^{2+}, sodium citrate, and $FeCl_3$, and totally inhibited by sulfhydryl reagents, such as 0.1 μM $CuCl_2$, 1.2 μM $NiCl_2$, 5 μM $HgCl_2$, and 150 μM *p*-chloromercuribenzoate. No inhibition was observed with NaN_3 or CN^-. Inhibition by CO was slight: 50% inhibition occurred at 0.8 bar CO.

Reactivity with electron acceptors

Only a few artificial electron acceptors were found to react with the purified hydrogenase, both in the presence or absence of phosphatidylcholine: methylene blue, phenazine methosulfate, menadione, pyocyanine, and potassium ferricyanide (Table I). No reaction occurred with methyl or benzyl

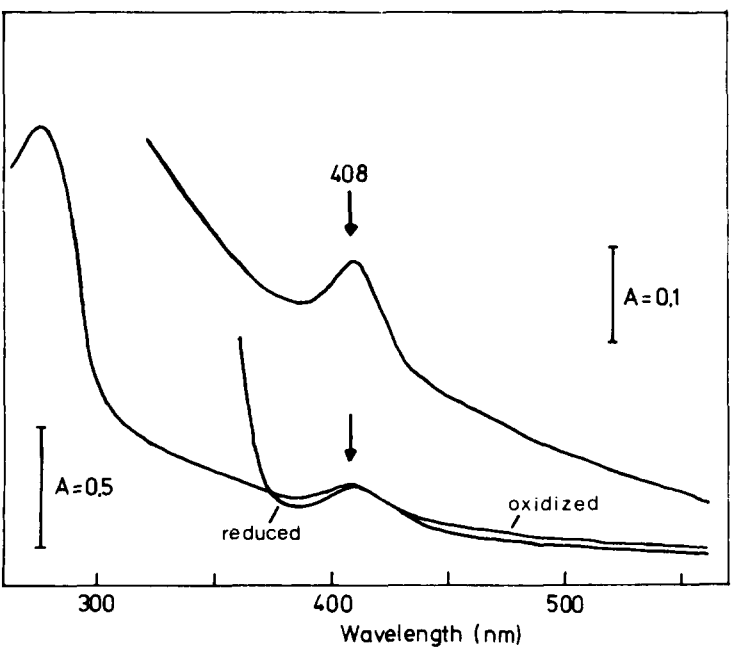

Fig. 4. Absorption spectrum of purifed hydrogenase in 1 cm cuvettes. Protein content, 1.56 mg/ml.

viologen, NAD(P), FAD, FMN, dichlorophenol indophenol, cytochrome *c* (horse heart), or with ubiquinone and menaquinone extracted from *A. eutrophus* membranes. The physiological acceptor of the enzyme is unknown.

Content of functional groups

The absorption spectrum of the purified enzyme showed an absorbance peak at 408 nm (Fig. 4). The ratio of absorbance at 280 nm: 408 nm was 4.76.

Neither the absorption spectrum of the native enzyme nor the trichloroacetic acid extract suggested that flavin was a constituent of the enzyme; FMN had previously been found in the soluble hydrogenase [8,10]. The absence of flavin from the membrane-bound hydrogenase is in agreement with its narrow spectrum of electron acceptors [27].

Determination of iron and acid-labile sulfur resulted in 6.2 ± 0.5 mol sulfur and 6.1 ± 1.2 mol iron/mol enzyme, characterizing the enzyme as an iron-sulfur protein.

Discussion

Comparison of the two purified hydrogenases of *A. eutrophus* on the basis of the results of the present work now demonstrates that these two enzymes are in fact completely different enzyme proteins (Table II). Remarkable differences concern molecular weight, subunit composition, isoelectric point, oxygen tolerance under reaction conditions, pH and temperature optima, acceptor specificity, presence of flavin, and quantity of labile iron and sulfur. With respect to some properties, both hydrogenases are similar: they are both stable during storage under air and unstable under reducing conditions; both are

TABLE II

COMPARISON OF BIOCHEMICAL DATA OF SOLUBLE AND MEMBRANE-BOUND HYDROGENASE OF *A. EUTROPHUS* H 16

Values for the soluble hydrogenase from Schneider and coworkers [8,10,36].

Property	Soluble hydrogenase	Membrane-bound hydrogenase
Localization	Cytoplasm	Membranes, tightly bound
Physiological acceptor	NAD	Quinones (?)
Molecular weight	205 000	98 000
Subunits	68 000	67 000
	60 000	31 000
	29 000	
Isoelectric point	4.85	6.5
Iron content	12.00	6.0
Acid-labile sulfur content	12.00	6.0
Flavin content	FMN	—
pH optimum	8.0	5.5
Temperature optimum	33°C	52°C
Activation energy	14.3 kcal/mol	6.25 kcal/mol
	(59.9 kJ/mol)	(26.2 kJ/mol)
K_m for H_2	37 μM	32 μM
Stability, oxidized	+	+
Inhibition by oxygen	—	+

activated by the removal of oxygen, and the K_m values of both enzymes towards H_2 as substrate are almost identical. Both enzymes contain one subunit of about 67 000 daltons and one of about 30 000 daltons. Previous experiments revealed tight relatedness of both hydrogenases with respect to mutability and regulation [28]. Immunological comparison revealed no cross-reaction between the two hydrogenase enzymes (unpublished data), confirming assumptions made on the basis of mutant studies [28,29].

A. eutrophus is the first organism for which the existence of two different hydrogenase systems could be proven. Recently, in *Clostridium pasteurianum*, a second hydrogenase has been detected which has an uptake function only [30].

The membrane-bound hydrogenase of *A. eutrophus* is obviously involved in energy generation during autotrophic growth [5,29]. A scheme for the electron transport has been proposed including an unknown acceptor X, quinones, and cytochromes of the *c*, *b*, *a*, and *o* type, as electron carriers [31]. The primary acceptor has not yet been identified. Inhibitor studies on *Paracoccus denitrificans* are suggestive that hydrogenase interacts with the electron transport chain between the rotenon- and the antimycin A-sensitive site [32]. Cytochromes have been identified as electron acceptors of other membrane-bound hydrogenases [33—35].

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