Biochimica et Biophysica Acta 905 (1987) 435–446
Elsevier

BBA 73812

Comparison of the membrane-bound hydrogenases from *Alcaligenes eutrophus* H16 and *Alcaligenes eutrophus* type strain

Hans G. Podzuweit [a], Klaus Schneider [b] and Heinz Knüttel [c]

[a] *Abteilung Immunologie der Universität Kiel, Kiel,* [b] *Lehrstuhl für anorganische Chemie I der Universität Bielefeld, Bielefeld, and* [c] *Institut für Mikrobiologie der Universität Göttingen, Göttingen (F.R.G.)*

(Received 16 July 1987)

Key words: Hydrogenase; Methyl viologen; Membrane protein; Electron acceptor; Enzyme localization; (*A. eutrophus*)

Whereas the membrane-bound hydrogenase from *Alcaligenes eutrophus* H16 is an integral membrane protein and can only be solubilized by detergent treatment, the membrane-bound hydrogenase of *Alcaligenes eutrophus* type strain was found to be present in a soluble form after cell disruption. For the enzyme of *A. eutrophus* H16 a new, highly effective purification procedure was developed including phase separation with Triton X-114 and triazine dye chromatography on Procion Blue H-ERD-Sepharose. The purification led to an homogeneous hydrogenase preparation with a specific activity of 269 U/mg protein (methylene blue reduction) and a yield of 45%. During purification and storage the enzyme was optimally stabilized by the presence of 0.2 mM $MnCl_2$. The hydrogenase of *A. eutrophus* type strain was purified from the soluble extract by a similar procedure, however, with less specific activity and activity yield. Comparison of the two purified enzymes revealed no significant differences: They have the same molecular weight, both consist of two different subunits (M_r = 62000, 31000) and both have an isoelectric point near pH 7.0. They have the same electron acceptor specificity reacting with similar high rates and similar K_m values. The acceptors reduced include viologen dyes, flavins, quinones, cytochrome *c*, methylene blue, 2,6-dichlorophenolindophenol, phenazine methosulfate and ferricyanide. Ubiquinones and NAD were not reduced. The two hydrogenases were shown to be immunologically identical and both have identical electrophoretic mobility. For the membrane-bound hydrogenase of *A. eutrophus* H16 it was demonstrated that this type of hydrogenase in its solubilized, purified state is able to catalyze also the reverse reaction, the H_2 evolution from reduced methyl viologen.

Introduction

The ability of aerobic hydrogen oxidizing bacteria to yield energy and reduction equivalents from the utilization of molecular hydrogen is mediated by two kinds of hydrogenases, a soluble NAD-linked enzyme [1] and a membrane-bound one, linked to the respiratory chain [2]. Most hydrogen bacteria contain only the latter enzyme and just a few organisms e.g. *Alcaligenes eutrophus* contain both enzymes. On the other hand, only *Nocardia opaca* 1b was found to contain just the soluble NAD-reducing hydrogenase [3].

Within the recent years, the membrane-bound hydrogenases from several hydrogen bacteria have been isolated and highly purified. These were the enzymes from *Alcaligenes eutrophus* H16 [2],

Abbreviation: DCPIP, 2,6-dichlorophenolindophenol.

Correspondence: K. Schneider, Institut für Mikrobiologie der Universität Göttingen, Grisebachstrasse 8, 3400 Göttingen, F.R.G.

Pseudomonas pseudoflava GA3 [4], *Paracoccus denitrificans* [5] and from the hydrogen oxidizing and nitrogen fixing bacteria *Xanthobacter autotrophicus* GZ29 [6], *Rhizobium japonicum* [7–9] and *Alcaligenes latus* [9,10]. All these hydrogenases are integral membrane proteins and catalyze the reduction of methylene blue with high velocity. The viologen dyes, however, are rather poor substrates in the most cases and pyridine nucleotides cannot be reduced at all.

Some remarkable properties led our attention to the type strain of *A. eutrophus*: (i) its membrane associated hydrogenase activity was described to be very low [11]; (ii) in contrast to other hydrogen oxidizing bacteria *A. eutrophus* type strain was found to require always NADH for the electron transport from H_2 to O_2 [12].

It was therefore one aim of this work to answer the question whether this organism contains only small amounts of the membrane-bound hydrogenase in vivo or a hydrogenase that is only weakly attached to the membrane surface or even not at all. We also compare the properties of this enzyme with those of the membrane-bound hydrogenase isolated from *A. eutrophus* H16.

Materials and Methods

Organisms and cultivation. Alcaligenes eutrophus H16 (ATCC 17699) and *Alcaligenes eutrophus* type strain (ATCC 17697) were cultivated in a mineral salts medium as described [13] in a 10-l fermenter (Braun, Melsungen, F.R.G). The gas atmosphere contained 10% O_2, 10% CO_2 and 80% H_2. Cells were harvested at the beginning stationary growth phase at an A_{436nm} of 40, washed twice with 50 mM potassium phosphate buffer (pH 7.0) and kept frozen at $-25\,^{\circ}C$ in a suspension adjusted to A_{436nm} of approx. 300.

Preparation of membranes and soluble crude extract. The cell suspensions were thawed and disrupted by an ultrasonicator (Schoeller, Frankfurt/Main, F.R.G.). The sonication time was 30 s per ml at 20 kHz and 60% amplitude. The sonication vessel was cooled continuously to $-20\,^{\circ}C$. Membrane particles and whole cells were removed by centrifugation for 1 h at $100\,000 \times g$ (Micron ultracentrifuge, Christ, Osterode, F.R.G). For the purification of the type strain membrane-hydrogenase the cell-free supernatant was used. Membranes from *A. eutrophus* H16 were isolated by removing the upper part of the pellet with a spatula and suspended in 50 mM potassium phosphate buffer (pH 7.0) (30 ml per g wet weight).

Solubilization and purification of enzymes. To the membrane suspensions of *A. eutrophus* H16 1% Triton X-114, 10% sucrose and 0.2 mM $MnCl_2$ were added. The mixture was stirred for 30 min at $0\,^{\circ}C$ before the membranes were removed by centrifugation (1 h at $100\,000 \times g$). The supernatant, which contained the solubilized proteins, was used for the phase separation procedure [14]. The protein solution was incubated for 10 min at $30\,^{\circ}C$ and then centrifuged for 3 min at 4000 rpm (also at $30\,^{\circ}C$). Under these conditions the crude solubilizate separated itself into an oily, reddish detergent phase at the bottom of the tube and an aqueous upper phase. The detergent phase was discarded and the aqueous phase was further purified by ammonium sulfate precipitation (30–60% saturation).

The ammonium sulfate precipitate was dissolved and dialysed against 25 mM potassium phosphate buffer (pH 7.0) + 0.2 mM $MnCl_2$ and then applied to a DEAE-Sephacel column (2.5×40 cm, 200 ml volume), which was equilibrated with the same buffer. Hydrogenase did not bind to DEAE and could be washed off the column with the equilibration buffer. The vast majority of proteins, however, bound tightly and had to be washed off the column by treatment with 1 M KCl. At a flow rate of 48 ml per h, 6-ml fractions were collected. The most active fractions were combined and concentrated to 5 ml.

This partially purified hydrogenase was then applied to a small affinity-chromatography column (1.6×20 cm) with 20 ml Procion Blue H-ERD-Sepharose 4B. The column was equilibrated with 50 mM potassium phosphate buffer (pH 7.0) + 0.2 mM $MnCl_2$. Contaminating protein was removed by washing with the equilibration buffer. Hydrogenase was eluted with a linear 0–2 M KCl gradient prepared in the equilibration buffer. Flow rate and fraction volume were 20 ml per h and 3 ml, respectively. Fractions that contained pure hydrogenase were combined and concentrated by ultrafiltration in an Amicon diaflo cell using a PM 30 membrane.

The purification of the hydrogenase from the *A. eutrophus* type strain started with the cytoplasmic supernatant obtained after cell disruption and ultracentrifugation.

To the DEAE-Sephacel and the Procion Blue-Sepharose column, which were performed as described, an FPLC-step was added. The FPLC-column contained the strong anionic exchanger Mono Q HR 5/5 and was equilibrated with 15 mM potassium phosphate buffer (pH 8.0). The hydrogenase sample (17 mg protein in 0.5 ml) was eluted from the column with a phosphate gradient (15–30 mM, pH 8.0, 30 ml total volume). The pressure was held constantly at 3 MPa. Flow rate and fraction size were 2 ml per min and 1 ml, respectively. The most active fractions were combined and concentrated to 5 mg protein per ml.

Preparation of lysozyme-membranes. Lysozyme protoplasts were prepared and osmotically disrupted as described [15].

Enzyme assays. Hydrogenase activity was measured photometrically in anaerobic cuvettes at 30 °C. Each assay contained 2 ml H_2-saturated buffer, electron acceptor as listed below, 50–100 μM sodium dithionite and hydrogenase. The following buffers and electron acceptors were used: 0.3 mM FMN and 0.3 mM FAD in 50 mM potassium acetate buffer (pH 3.5); 20 mM benzyl viologen in 50 mM potassium phosphate buffer (pH 7.5); 20 mM methyl viologen in 50 mM glycine potassium hydroxide buffer (pH 9.5); 0.2 mM methylene blue, 0.2 mM ferricyanide and 0.5 mM DCPIP in 50 mM potassium phosphate buffer (pH 5.5); 0.2 mM cytochrome *c* in 35 mM potassium citrate phosphate buffer (pH 4.0); 0.70 mM menadione in 50 mM potassium acetate buffer (pH 5.5); 0.2 mM duroquinone in 10 mM potassium acetate buffer (pH 3.5); and 0.26 mM phenazine methosulfate in 50 mM potassium citrate phosphate buffer (pH 5.0). The wavelengths used were: FMN and FAD, 446 nm; DCPIP, methyl viologen und benzyl viologen, 546 nm; methylene blue, 578 nm; ferricyanide, 405 nm; cytochrome *c*, 354 nm; phenazine methosulfate, 388 nm; menadione, 360 nm; duroquinone, 255 nm. H_2 uptake activities with methylene blue and various quinones were also measured manometrically. The Warburg vessels contained: 50 mM buffer, 6 mM acceptor and an appropriate amount of enzyme. Quinones were added from 120 mM stock suspensions in 0.5% Triton X-100. Naphthoquinones were assayed in 50 mM acetate buffer (pH 5.5), while 10 mM potassium acetate buffer (pH 3.5), was used with the benzoquinones. The hydrogenase activity with methylene blue was measured in 50 mM potassium phosphate buffer (pH 5.5). The Warburg vessels were equilibrated with pure H_2 for 30 min and the reaction was started by tipping the enzyme from the side arm into the main compartment. H_2 production was monitored by gas chromatography [16,17]. A Warburg vessel contained: 5 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 3 mM methyl viologen in the main compartment, 200–250 μg *A. eutrophus* H16 membrane-bound hydrogenase in one sidearm and 250 μmol sodium dithionite in the second side arm. The vessel was gassed with pure N_2. After the reduction of the methyl viologen by dithionite, the reaction was started with the enzyme. Samples were withdrawn from the gas atmosphere and injected into a gas chromatograph (Packard-Becker, Delft, The Netherlands).

Protein determination. Protein contents were determined with a modified biuret method [18].

Polyacrylamide electrophoresis and determination of the isoelectric point. Polyacrylamide electrophoresis was carried out as described [3]. The isoelectric point was determined using the chromatofocussing method. The hydrogenase sample (5 mg protein in 1 ml volume was applied to a column (1 × 40 cm) with 30 ml chromatofocussing gel equilibrated with 25 mM Tris acetate buffer (pH 8.3). The column was eluted with a mixture of 15 ml 'polybuffer 96', 35 ml 'polybuffer 74' and 450 ml H_2O. The 'polybuffers' were commercial, preprepared solutions from Pharmacia. The pH of this mixture was adjusted to 5.0. Flow rate and fraction volume were 18 ml/h and 0.3 ml, respectively.

Preparation of ubiquinone Q-8. Ubiquinone Q-8 was extracted from H16 membrane material as described [19].

Preparation of anti-hydrogenase-antiserum. Pure membrane hydrogenase from H16 (3.5 mg protein) was mixed with 0.5 ml Freunds complete adjuvant and injected into the leg of a young rabbit. The booster injection was performed after 8 days with 1.8 mg hydrogenase, injected in-

travenously. The antiserum was obtained after one month and the Ig G fraction was purified on a Protein A Sepharose column as described [20].

Immunodiffusion test. Ouchterlony-immunodiffusion tests were conducted in 50 mM diethylbarbiturate/acetate buffer, (pH 8.2), solidified with 1% agarose, on microscopic slides [21].

Chemicals. Barbital, ubiquinone Q-10, Nitroblue Tetrazolium, Triton X-114, Coomassie brilliant blue R-250 and Hepes were obtained from Serva (Heidelberg, F.R.G.). FMN, phenazine methosulfate, duroquinone and Tris were bought from Sigma (München) and DEAE-Sephacel, chromatofocussing gel, polybuffers and Sepharose 4B were from Pharmacia (Uppsala, Sweden). Methyl-*p*-benzoquinone, obtained from Merck (Darmstadt, F.R.G.), was recrystallized twice from ethanol to remove impurities. The Procion dyes were a gift from ICI Deutschland (Frankfurt/Main). The Procion Blue Sepharose was prepared as described [22]. The sources of the other chemicals are listed elsewhere [1].

Results

Localization of the NAD-independent hydrogenase in Alcaligenes eutrophus type strain

After ultrasonic disruption of the *A. eutrophus* type strain cells and centrifugation at 100000 × *g*, the H_2-dependent NAD-reducing activity was located exclusively in the soluble fraction (Fig. 1). This activity was caused by a soluble NAD-linked hydrogenase, which has been found also in other *A. eutrophus* strains [1]. H_2-dependent methylene blue reducing activities could be detected in the membrane pellet and in the soluble fraction. The relatively low membrane-bound activity was undoubtly due to a NAD independent and membrane-bound hydrogenase because the membranes did not reduce NAD. The high methylene blue-reducing activity in the supernatant, however, could be caused by both hydrogenases because both react with methylene blue. The NAD-independent membrane-bound hydrogenase could have been solubilized by the ultrasonic treatment. To assign the methylene blue-reducing activity of the supernatant to one or the other hydrogenase, it was necessary to separate both hydrogenases. This separation was achieved by chromatography on small

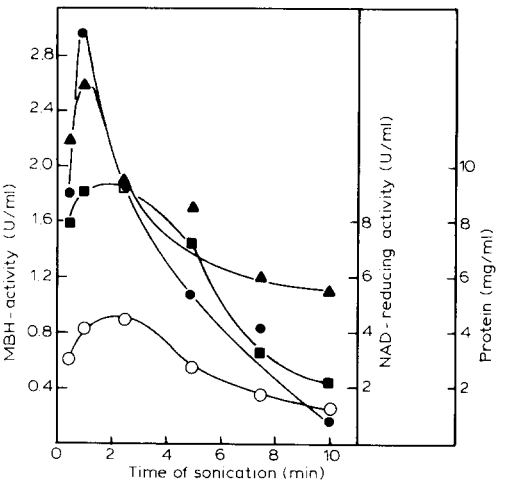

Fig. 1. Sonication kinetics of *Alcaligenes eutrophus* type strain cells. 10-ml portions of a cell suspension from *A. eutrophus* type strain were sonicated at 20 kHz and 60% amplitude. The sonicated extracts were centrifuged for 1 h at 100000 × *g*. The supernatant was tested for NAD-reducing hydrogenase activity (■) and protein content (▲). The membranes were resuspended in 25 mM potassium phosphate buffer (pH 7.0) and tested for membrane-bound, NAD-independent hydrogenase (○) as methylene blue reduction. The two hydrogenases in the supernatant were separated by columns with 10 ml DEAE-Sephacel in 25 mM potassium phosphate buffer (pH 7.0). 5 ml of the supernatant were loaded on each column and the NAD-independent hydrogenase could be eluted quantitatively by 5 ml of the same buffer and was tested for methylene blue reduction (●). No reduction could be found in the eluates. MBH, membrane-bound hydrogenase.

DEAE-Sephacel columns, which bound the NAD-linked hydrogenase but did not bind the NAD-independent enzyme. And indeed, the amount of NAD-independent hydrogenase, eluted from this column was surprisingly large. Of the total MB-reducing activity, caused by the NAD-independent enzyme, 70–90% were localized in the soluble fraction (Fig. 1, Trace 3) and in good accordance, 5–20% of this activity was still bound to the membranes. Prolonged sonication led to inactivation of all activities and a decreasing protein content in the supernatant due to denaturation of proteins.

In some preparations the membranes were even void of methylene blue-reducing activity. Even more gentle methods of cell disruption, like French press or lysozyme treatment, did not alter the distribution of the activities significantly. At least

60% of the activity could always be recovered in the supernatant.

Immunological comparison by the Ouchterlony double-diffusion technique showed, that the NAD-independent hydrogenase from the membranes and from the soluble fractions were identical proteins. Therefore the enzyme present in the soluble fractions does not represent a new type of a soluble NAD-independent hydrogenase. It is only a soluble form of the originally membrane-bound enzyme.

Stabilization and purification of membrane-bound hydrogenase

For the membrane-bound hydrogenase of *A. eutrophus* H16, two purification procedures have been described [2,23] and final specific activities of 170 and 40 units/mg protein have been reported. We tried both procedures several times and obtained specific activities of 30–90 units/mg protein. In our hands the membrane-bound hydrogenase was not (particularly) O_2-sensitive but generally labile. It lost the complete activity in the crude solubilizate within 5 days even at 4°C. Therefore, in order to stabilize the enzyme during the purification, we added different reagents to the crude solubilizate. Protease inhibitors were not effective. Some metal ions (0.5 mM $NiCl_2$, 2 mM $CoCl_2$) and high ionic strength (1 M phosphate buffer or ammonia sulfate) did not stabilize sufficiently (residual activity 50–60%). A perfect and apparently specific stabilizing effect was observed in the presence of 0.2–2 mM $MnCl_2$. The hydrogenase did not loose any activity within the experimental time period under these conditions.

For the purification procedure we added therefore 0.2 mM $MnCl_2$ to all buffers used. We additionally included three further improvements: (i) We replaced the nonionic detergent Triton X-100 by Triton X-114, which has the same solubilizing abilities but separates itself at room temperature into a hydrophilic and a hydrophobic phase [14]. Using this phase separation procedure, 95% of the hydrogenase activity could be recovered in the hydrophilic phase which contained only 25% of the total solubilized protein, resulting in a 4-fold increase of the specific activity. The appearance of membrane-bound hydrogenase in the hydrophilic phase in an unexpected behavior, because this type of hydrogenase is without any doubt an integral membrane protein. Obviously, this enzyme contains hydrophilic regions which are predominant over the hydrophobic ones. (ii) With DEAE-Sephacel a kind of negative chromatography was used. The MBH did not bind to this ion exchanger and came off the column with the equilibration buffer, but, as the majority of the proteins were firmly bound, this method was not only very rapid but also quite effective. (iii) Affinity chromatography on Triazine-dye-agarose has been successfully used with other hydrogenases [23] and also the membrane-bound hydrogenase of H16 could be purified effectively with Procion Red HE-3B-agarose. But to improve the purification procedure, we tested 24 other triazine dyes for affinity to the membrane-bound hydrogenases. Among them, Procion Blue H-ERD had the highest affinity and consequently the best resolving power as affinity-chromatography gel. Applied as the last purification step, several remaining minor contaminants could be removed. The final enzyme preparation was homogeneous and had a

TABLE I

PURIFICATION OF THE MEMBRANE-BOUND HYDROGENASE OF *A. EUTROPHUS* H16

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)	Yield (%)
Membrane fraction	2506	7592	3.0	1.0	100
Solubilization	1571	7269	4.6	1.5	96
Phase separation	359	6505	18.1	6.0	86
$(NH_4)_2SO_4$ (30–60% satd.)	119	5446	45.6	15.2	75
DEAE-Sephacel	28	3738	133	44.3	51
Procion Blue H-ERD-Sepharose	12.6	3392	269	89.7	45

specific activity of 269 units/mg protein and a yield of 45% of the total activity. Both values surpassed those of other procedures, described earlier, significantly. We feel that this was mainly due to the stabilizing effect of $MnCl_2$. The data of the whole purification procedure are summarized in Table I.

Purification of the NAD-independent hydrogenase from the *A. eutrophus* type strain turned out to be more difficult (and time-consuming), mainly, because the purification had to start with the cytoplasmic fraction after the cell disruption. Therefore, the specific activity in the crude extract was low (0.6 units/mg), compared to the membrane-bound hydrogenase activity in the membrane fraction of H16 (3.2 units/mg) and consequently more chromatography steps were required for the purification of the type strain enzyme (Table II). Moreover, the type strain hydrogenase could not be stabilized as successfully with $MnCl_2$ as the H16 membrane-bound hydrogenase. Following the same procedure as described for the H16 membrane-bound hydrogenase, the type strain enzyme was only about 25% pure. Therefore, we introduced as additional purification step an FPLC-column with Mono Q and gained a preparation with a purity grade of about 80%. We disclaimed to purify this hydrogenase further, because it appeared to be sufficiently pure for the electrophoretic and other studies described in this paper.

Molecular weight, subunit structure and isoelectric point

The H16 membrane-bound hydrogenase and the NAD-independent hydrogenase of the type strain had the same molecular weight (95 000 ± 3 000) as determined by electrophoresis. Both showed identical electrophoretic mobility in the presence and absence of SDS and both had the same dimeric structure. The molecular weights were 61 000 ± 3 000 per larger subunit and 30 000 ± 2 000 per small subunit. The variations of these values were dependent on experimental conditions, i.e. in the kind of SDS treatment or in the protein or acrylamide concentration, respectively. Our data on molecular weights agree fairly well with those published earlier for the membrane-bound hydrogenases of H16 [2,27] and of other hydrogen oxidizing bacteria [4,9,10]. Fig. 2 shows the elution profile of the type strain hydrogenase from the chromatofocussing column. The enzyme came off the column in two peaks at 6.9 and 6.5, respectively. Because the fractions around 6.9 were contaminated with cytochrome *c*, the value of 6.5 appears to be more realistic and it is in fact more in agreement with the isoelectric point found for the H16 membrane-bound hydrogenase [2].

pH and temperature optima

The methylene blue-reducing activity of the H16 membrane-bound hydrogenase had a broad optimal pH region (7–8.5), when the enzyme is bound to the membrane and undergoes a shift to a more pronounced optimum at pH 5.5 after solubilization [2]. While we were able to reproduce this phenomenon with H16 membrane-bound hydrogenase, the type strain hydrogenase behaved differently (Fig. 3). The soluble form had a sharp pH optimum of 5.3, while the membrane form had an optimum between pH 5.7 and 6.1. This is significantly more acidic and less broad than the optimal region of H16 membranes.

As reported for other hydrogenases the enzyme

TABLE II

PURIFICATION OF THE MEMBRANE-BOUND HYDROGENASE OF *A. EUTROPHUS* TYPE STRAIN

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)	Yield (%)
Crude extract	7686	4361	0.6	1.0	100
$(NH_4)_2SO_4$(30–60% satd.)	3940	3597	1.1	1.8	82
DEAE-Sephacel	857	2259	2.6	4.4	52
Procion Blue H-ERD-Sepharose	117	554	4.7	7.9	13
Mono Q (FPLC)	17	310	18.2	30.4	7

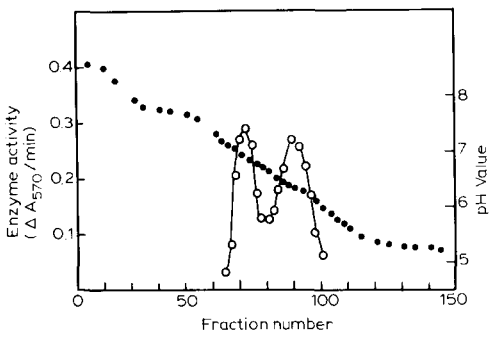

Fig. 2. Determination of the isoelectric point of the solubilized type strain hydrogenase. Type strain hydrogenase was adsorbed to a 30 ml chromatofocussing column and eluted by a buffer mixture, described in Materials and Methods. 0.3 ml fractions were collected and measured for pH value (●) and hydrogenase activity with methylene blue (○).

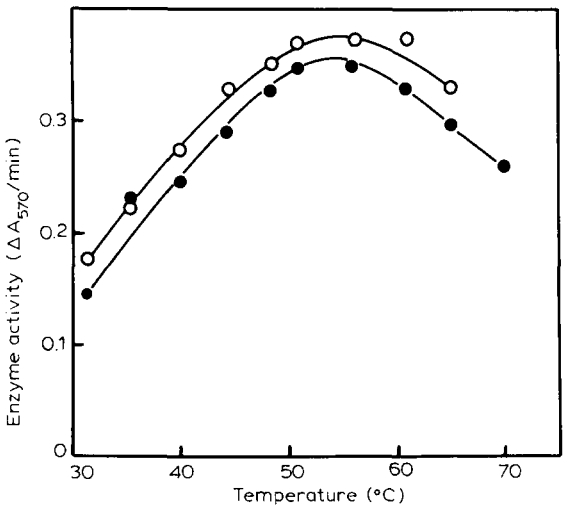

Fig. 4. Temperature dependence of the activity (methylene blue reduction) of the membrane-bound hydrogenases of H16 and type strain. The assays were performed as described in Materials and Methods at a reaction temperature as indicated. ●, type strain hydrogenase (partially purified from the soluble crude extract); ○, H16 hydrogenase (partially purified sample, DEAE-eluate).

reactions of the membrane-bound hydrogenases from H16 and type strain were remarkably stable at high temperatures and their highest activity were at 55 °C (Fig. 4). The enthalpies of activation were calculated to be 10.8 kcal/mol for the type strain hydrogenase and 9 kcal/mol for the H16 membrane-bound hydrogenase.

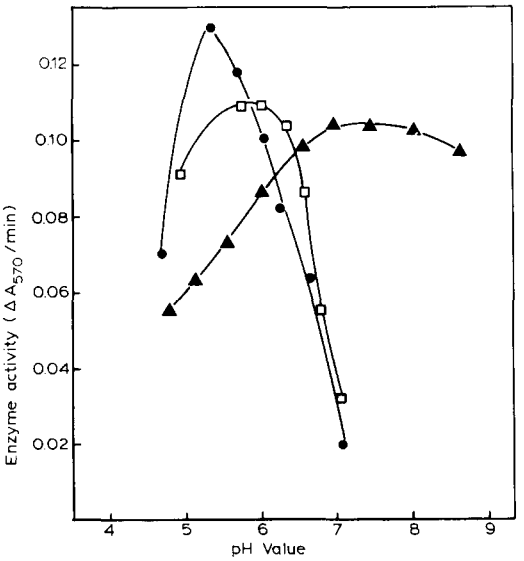

Fig. 3. Dependence of hydrogenase activities from the pH value. Activities were measured in 50 mM potassium phosphate buffer using methylene blue as electron acceptor. Assay conditions were as described in Materials and Methods. The pH values were varied as indicated in the figure. Curve 1 (●), activity of solubilized type strain hydrogenase. Curve 2 (□), activity of membrane-bound type strain hydrogenase. Curve 3 (▲), activity of membrane-bound H16-hydrogenase.

Reduction of electron acceptors with H_2

The purified membrane hydrogenase from *A. eutrophus* H16 has been reported earlier to react only with the artificial electron acceptors methylene blue, phenazine methosulfate, menadione and pyocyanine [2]. With ferricyanide, dependent on the enzyme preparation, no or only traces of activity were found [2]. Later on, conditions were described, which enabled a cytochrome *c* reduction although at a low rate (1.5% of the methylene blue reduction) [24]. In this paper we studied the electron carrier specificity in more detail and by improving the reaction conditions (buffer system, pH, substrate concentration) we enhanced the reaction rates with ferricyanide and cytochrome *c* and we demonstrated that the membrane hydrogenases of H16 and type strain also reacted with 2,6-dichlorophenolindophenol (DCPIP), flavins, methyl viologen and benzyl viologen, menadione and duroquinone (Table III). The pH optima, the reaction rates, and K_m values of the different

TABLE III

REDUCTION OF ELECTRON ACCEPTORS BY PURIFIED HYDROGENASES

The hydrogenase activity with different electron acceptors was measured photometrically. Wavelengths, buffer systems, acceptor concentrations and further details of the enzyme assays are described in Materials and Methods. For these studies the DEAE eluates were used. 100% activity corresponds to a specific activity of 133 U/mg of protein (H16) and 8 U/mg of protein (type strain).

Electron acceptor	pH optimum	Activity (%)		K_m (mM)	
		H16	type strain	H16	type strain
Methylene blue	5.5	100	100	1.3	1.3
Ferricyanide	5.5	1.55	n.d.	n.d.	n.d.
2,6-Dichlorophenolindophenol	5.5	28	n.d.	n.d.	n.d.
Phenazine methosulfate	5.0	87	75	0.004	0.004
Cytochrome *c*	4.0	8	7	0.1	0.1
FAD	3.5	13	10	0.3	0.3
FMN	3.5	11	10	0.2	0.2
Benzyl viologen	8.0	9	12	4.4	9.5
Methyl viologen	9.5	0.9	0.5	2.8	6.3
Menadione	5.5	40	31	2.0	1.0
Duroquinone	3.5	0.96	0.8	0.08	0.08

n.d., not determined.

electron acceptors were remarkably similar for both enzymes. It is interesting that the pH optima of the different substrates can be subdivided into three groups: cytochrome *c*, flavins and duroquinone had pH optima at a distinctly acidic pH (3.5–4), while methylene blue, DCPIP, menadione and ferricyanide reacted best at pH values around 5.5. The pH optima for benzyl viologen and methyl viologen were, however, found to be alkaline (pH 7.5–9.5). We did not observe an inactivation of the hydrogenase at high pH values as observed with the hydrogenase from *Azotobacter vinelandii* [25]. In contrast, the affinity of the H16 membrane-bound hydrogenase to methyl viologen increased with increasing pH. For example, the K_m for methyl viologen was 26 mM at pH 7.0 but only 2.8 mM at pH 9.5. The lowest K_m value was determined for phenazine methosulfate (4 μM) but despite its high affinity its reaction rate just reached 70–80% of the reduction rate of methylene blue which had a rather high K_m value (1.3 mM). Among the substrates, DCPIP and ferricyanide were the most difficult to handle, because: (i) the reactions with these acceptors were strongly inhibited by high substrate concentrations. The acceptor concentrations had to be chosen as low as possible. (ii) These reactions were markedly dependent on the grade of reductive activity of the enzyme. The hydrogenase had to be preincubated for at least one hour to achieve maximal reaction rates. The immediate reduction of the enzyme with H_2 and 50 μM dithionite directly in the assay mixture was not sufficient. If measured at a concentration between 0.1–1.2 mM ferricyanide, the initial rate of reduction increased with decreasing acceptor concentration. However, because of the low absorption coefficient of ferricyanide (0.9 at 405 nm), we could not perform sufficient reliable measurements below 0.2 mM. But even at this concentration we reached with ferricyanide the highest activity of all acceptors, tested. This was 155% of the methylene blue reduction rate (see Table III). With DCPIP similar results were obtained, but the reduction rates were lower. The most convenient DCPIP concentration was 0.13 mM.

The reactivity of the H16 membrane-bound hydrogenase towards quinones was examined by

TABLE IV

H_2-UPTAKE ACTIVITY OF THE MEMBRANE-BOUND HYDROGENASE OF *A. EUTROPHUS* H16 WITH QUINONES

Hydrogenase activity with different quinones was measured manometrically as described in Materials and Methods. As enzyme preparation partially purified hydrogenase (DEAE eluate) was used and methylene blue served as reference.

Electron acceptor	H_2 uptake (μl/min)	Relative activity (%)
Methylene blue	800	100
1,4-Naphthoquinone	520	65
2-Hydroxy-1,4-naphthoquinone	160	20
5-Hydroxy-1,4-naphthoquinone	160	20
Menadione	320	40
p-Benzoquinone	70	9
Methyl-*p*-benzoquinone	180	23
Ubiquinone Q-10 (bovine heart)	0	0
Ubiquinone Q-8 (*A. eutrophus* H16)	0	0

measuring the H_2-uptake rates manometrically (Table IV) and for two representatives, duroquinone and menadione also photometric activity assays have been established (Table III). It was found, that several quinone derivatives reacted with hydrogenase. The naphthoquinone derivatives were in general more reactive than the benzoquinones and the highest activities were observed with 1,4-naphthoquinone and menadione. Remarkably, the only quinone which did not react was ubiquinone, which has been proposed to be the electron acceptor of the MBH in vivo [26]. The hydrogenase was neigher active with the commercially available ubiquinone Q-10 nor with Q-8, isolated from H16 membranes. Attempts to demonstrate hydrogenase activity with Q-8 photometrically at 275 nm varying the pH values between 3.5 and 7.0 were also unsuccessful.

H_2 evolution from reduced methyl viologen

Although the membrane-bound hydrogenase of H16 has been assumed to be an 'unidirectional' enzyme, we could demonstrate, that the purified enzyme is also capable of evolving molecular H_2 from reduced methyl viologen. The rate of H_2 production was linearly dependent on the enzyme concentration. The pH optimum of this reaction was at 7.0 and differed distinctly from the optimal pH of the H_2-uptake reaction with methyl viologen as electron acceptor, which was at pH 9.5. The rates of the H_2 evolution were low; specific activities of about 1 U/mg protein were determined, which was only 1% of the H_2-uptake reaction,

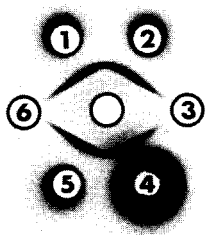

Fig. 5. Immunological comparison of the membrane-bound hydrogenase (MBH) enzymes of H16 and type strain by double diffusion. The central well contained 0.48 mg anti-H16-MBH antibodies; wells 1, 2 and 5 each contained 23 μg H16-MBH; well 4 contained 92 μg protein of a partially purified sample of the type strain-MBH; wells 3 and 6 remained empty.

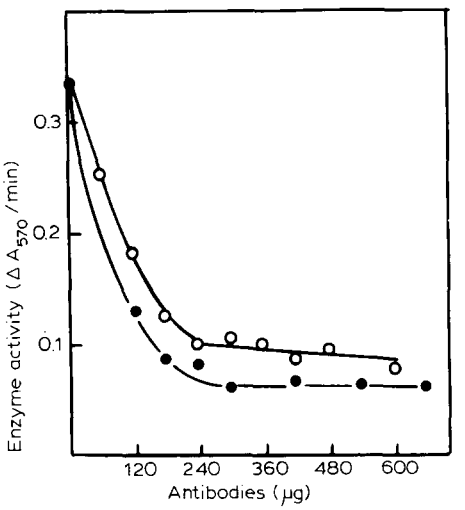

Fig. 6. Inhibition of hydrogenase activity by anti-H16-MBH antibodies. To 100 μl purified H16 (46 μg) or type strain-MBH (80 μg) anti-H16-MBH-antiserum and 0.15 M NaCl were added to give a total volume of 125 μl. The hydrogenase-antibody mixtures were preincubated for 50 min at 30 °C, shaken and measured for methylene blue reduction. ○, membrane-bound hydrogenase of type strain; ●, membrane-bound hydrogenase of H16.

obtained with methylene blue as electron acceptor. It has to be emphasized, that only the solubilized enzyme, independent of the purity grade, was able to catalyze the H_2 evolution from methyl viologen. No H_2 evolution could be catalyzed by the membrane material.

Immunological comparison

Rabbit antibodies were raised against the pure H16 membrane-bound hydrogenase to compare the relationship between the H16 membrane-bound hydrogenase and the type strain hydrogenase. In the Ouchterlony double-diffusion test [21], the precipitation lines were completely fused and no spurs were observed (Fig. 5). Moreover, both enzymes were inhibited by the antibodies and the degree of inhibition as well as the K_i values of the antiserum were almost identical (Fig. 6). These results indicate that the two hydrogenases are serologically identical proteins.

Discussion

Among the H_2-oxidizing bacteria the *A. eutrophus* strains belong to a small group that

contains two catalytically, structurally and immunologically different hydrogenases. One is located in the cytoplasmic fraction, contains FMN and reacts specifically with NAD, while the other hydrogenase is coupled to the respiratory chain, of less complex structure and unable to reduce NAD. The existence of both enzymes has been demonstrated in several *A. eutrophus* strains [27]. In this stains, so far investigated, the cytoplasmic fraction catalyzes the H_2-dependent reduction of methylene blue and NAD due to the presence of the soluble hydrogenase, and the membrane particles just catalyze the reduction of methylene blue caused by the membrane-bound hydrogenase. In the most cases, the methylene blue-reducing activities of the membranes and the cytoplasmic fractions were about the same and in H16 the membrane-bound methylene blue-reducing activity was even predominant (70% of the total activity [2]). A completely different result, however, was obtained with the *Alcaligenes eutrophus* type strain in an early investigation [11]. 90% of the total methylene blue-reducing activity could be found in the supernatant. Our observations are in agreement with this result; we also found most of the methylene blue-reducing activity in the supernatant. In addition, by chromatography with DEAE-Sephacel we could separate two different hydrogenase activities from the cytoplasmic fraction and show that most of the methylene blue-reducing activity was caused by a NAD-independent hydrogenase.

We compared this soluble NAD-independent hydrogenase with the H16 membrane-bound hydrogenase and we could not detect any significant difference between them; they had the same molecular weights, subunit structure and immunological properties and the isoelectric points and kinetic properties were comparable within experimental limits. In an earlier publication, the EPR spectra of the two hydrogenases were already shown to be very similar indicating that they have the same types and arrangements of redox centres [28]. From this accordance of properties, we conclude that the soluble NAD-independent hydrogenase of the type strain and the membrane-bound hydrogenase of H16 represent the same type of enzyme and that in the *A. eutrophus* type strain the originally membrane-bound hydrogenase is not integrated into the membrane. This finding would explain the results of an investigation by Ishaque and Aleem [12], who found, that the presence of NAD was necessary to maintain an electron flow in *A. eutrophus* type strain membranes under autotrophic conditions. The lacking or incomplete integration of the type strain enzyme into the membrane could have four reasons: (i) the enzyme has a modified conformation and/or an altered primary structure in the lipophilic region. Such modifications of an enzyme, however, should influence the immunological, biochemical and catalytical properties, which was not the case. (ii) A second possible difference in the protein structure could be the absence of a subunit which normally functions as a membrane intrinsic anchor protein. The membrane-bound hydrogenases consists of a large 60-kDa subunit and a small 30-kDa subunit, the latter of which could be this anchor. However, the type strain enzyme does not lack any of these subunits as could be demonstrated electrophoretically and immunologically (Lorenz and Schneider, unpublished results). It cannot, however, be completely excluded, that both subunits just represent the catalytic part of the enzyme whereas the anchor protein got lost during solubilization in H16 or did not work at all in the type strain cells. The existence of those hypothetical anchor polypeptides has never been proved, but it is possible that they are bound to tightly to the membranes to become solubilized together with the 60-kDa and the 30-kDa subunit upon detergent treatment. Such an anchor protein was found in the fumarate reductase and succinate dehydrogenase of *Escherichia coli* [29]. The fumarate reductase, for example, consists of a membrane extrinsic domain composed of a catalytic dimer ('catalytic head') with 69-kDa and 27-kDa subunits and a membrane intrinsic or anchor portion composed of 15-kDa and 13-kDa subunits [30]. Provided, the membrane-bound hydrogenases are of an analogous structure, the absence of such a low-molecular-weight anchor protein or a defect at the binding site of this protein or of the catalytic dimer might prevent the binding of the active enzyme to the membrane. (iii) Alternatively, the non-integration or incomplete integration into the membrane might be due to a deficient membrane structure with the enzyme staying intact. (iv) A further possible explanation is that the insertion into the

cytoplasmic membrane is not spontaneous but actively regulated by a leader polypeptide sequence, which binds to a specific receptor in the membrane and becomes cleaved off the enzyme after the integration into the membrane [31]. If such a regulator system exists for the membrane-bound hydrogenase, it might be defective in *A. eutrophus* type strain. In *Azotobacter vinelandii* a short DNA-sequence has been found that is assumed to encode for the hydrogenase leader sequence [32]. At present it cannot be answered, which of these four possibilities is true.

The isolated and purified membrane-bound hydrogenase of *A. eutrophus* H16 reduced many electron acceptors but it did not react with the ubiquinones Q-8 and Q-10. These components are part of the respiratory chains in hydrogen oxidizing bacteria. In the case of *Paracoccus denitrificans* ubiquinone has been proposed to accept the electrons directly from the membrane-bound hydrogenase [26]. Obviously this direct reduction of ubiquinone does not occur in vitro. It would be premature, however, to conclude just from this result that the reduction could not take place in vivo, because the hydrogenase might undergo a conformational shift upon solubilization which could prevent the reaction with ubiquinone.

It has also to be considered that respiratory chains are of very complex structure. For example, the mitochrondrial succinate dehydrogenase has a function comparable to that of membrane-bound hydrogenase, it is, however, a much more complex enzyme system containing iron-sulfur centres, FAD and an ubiquinone binding subunit [33]. It is possible therefore that the membrane-bound hydrogenase requires a similar binding protein to react with ubiquinone. Such a protein, which of course is lacking in the isolated hydrogenase, might contain (an) additional electron transferring component(s). Recent studies with respiratory membranes of *A. eutrophus* H16 [34] and *A. latus* [35] that included the use of inhibitors and redox-spectroscopy of the ubiquinone did not give any evidence for the direct reaction of the membrane-bound hydrogenase with ubiquinone. It has been postulated that at least one electron carrier 'X' is located between hydrogenase and ubiquinone [34].

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft and by the Max Buchner-Forschungsstiftung. The cooperation of Dr. R. Brinkmann is providing cells from *A. eutrophus* type strain and H16 and the technical assistance of Mrs. B. Vogt are gratefully acknowledged.

References

1 Schneider K. and Schlegel, H.G. (1976) Biochim. Biophys. Acta 452, 66–80
2 Schink, B. and Schlegel, H.G. (1979) Biochim. Biophys. Acta 567, 315–324
3 Schneider, K., Schlegel, H.G. and Jochim, K. (1984) Eur. J. Biochem. 138, 533–541
4 Weiss, A.R., Schneider, K. and Schlegel, H.G. (1980) Current Microbiol. 3, 317–320
5 Sim, E. and Vignais, P.M. (1979) Biochim. Biophys. Acta 570, 43–55
6 Schink, B. (1982) FEMS Microbiol. Lett. 13, 289–293
7 Arp, D.J. and Burris, R.H. (1979) Biochim. Biophys. Acta 570, 221–230
8 Harker, A.R., Lambert, G.R., Hanus, F.J. and Evans, H.J. (1985) J. Bacteriol. 164, 187–191
9 Arp, D.J., McCollum, L.C. and Seefeldt, L.C. (1985) J. Bacteriol. 163, 15–20
10 Pinkwart, M., Schneider, K. and Schlegel, H.G. (1983) Biochim. Biophys. Acta 745, 267–278
11 Wittenberger, H. and Repaske, R. (1961) Biochim. Biophys. Acta 47, 542–552
12 Ishaque, M. and Aleem, M.I.H. (1970) Biochim. Biophys. Acta 223, 388–397
13 Schlegel, H.G., Kaltwasser, H. and Gottschalk, G. (1961) Arch. Mikrobiol. 39, 209–222
14 Bordier, C. (1981) J. Biol. Chem. 256, 1604–1607
15 Probst, I. and Schlegel, H.G. (1979) Biochim. Biophys. Acta 440, 412–428
16 Van Dijk, C., Mayhew, S.G. and Veeger, C. (1981) Eur. J. Biochem 114, 201–207
17 Kuhn, M., Steinbüchel, A. and Schlegel, H.G. (1984) J. Bacteriol. 159, 633–639
18 Beisenherz, G., Bolze, H.J., Bücher, T., Czok, R., Garbade, K.H., Mayer-Ahrend, E. and Pfleiderer, G. (1953) Z. Naturf. 8B, 555–577
19 Pfitzner, J. (1972) Zbl. Bact. Hyg. I Abt. Orig. A 220, 396–401
20 Miller, T.J. and Stone, H.O. (1978) J. Immunol. Methods 24, 111–125
21 Oakley, C.L. (1971) in Methods in Microbiology (Norris, J.R. and Ribbons, D.W., eds.), pp. 173–218, Academic Press, New York
22 Atkinson, T., Hammond, P.M., Hartwell, R.D., Hughes, P., Scawen, M.D., Sherwood, R.F., Small, D.A.P., Bruton, C.J.,

Harvey, M.J. and Lowe, C.R. (1981) Biochem. Soc. Trans. 9, 290–93
23 Schneider, K., Pinkwart, M. and Jochim, K. (1983) Biochem. J. 213, 391–398
24 Weiss, A.R. and Schlegel, H.G. (1980) FEMS Lett. 8, 173–176
25 Seefeldt, L.C., Fox, C.A. and Arp, D.J. (1986) J. Biol. Chem. 261, 1688–1694
26 Henry, M.F. and Vignais, P.M. (1983) Arch. Microbiol. 136, 64–68
27 Schneider, K. and Schlegel, H.G. (1977) Arch. Microbiol. 112, 229–238
28 Schneider, K., Patil, D.S. and Cammack, R. (1983) Biochim. Biophys. Acta 748, 353–361
29 Ingledew, W.J. and Poole, R.K. (1984) Microbiol. Rev. 48 222–271
30 Lemire, B.D., Robinson, J.J., Bradley, R.D., Scraba, G.G and Weiner, J.H. (1983) J. Bacteriol. 155, 391–397
31 Inouye, M. and Halegoua, S. (1980) Crit. Rev. Biochem. 7 339–371
32 Prickvil, B.C., Czechowski, M.H., Przybyla, A.E., Peck H.D. and Le Gall, J. (1986) J. Bacteriol. 167, 722–725
33 Mitchell, P. (1985) J. Biochem. 97, 1–18
34 Podzuweit, H.G., Arp, D.J., Schlegel, H.G. and Schneider K. (1986) Biochimie 68, 103–111
35 Podzuweit, H.G. and Arp, D.J. (1987) Arch. Microbiol 146, 332–337