Biochimica et Biophysica Acta, 452 (1976) 66—80

BBA 67947

PURIFICATION AND PROPERTIES OF SOLUBLE HYDROGENASE FROM *ALCALIGENES EUTROPHUS* H 16

KLAUS SCHNEIDER and HANS G. SCHLEGEL

Institut für Mikrobiologie der Gesellschaft für Strahlen- und Umweltforschung mbH München, in Göttingen, und Institut für Mikrobiologie der Universität Göttingen, Göttingen (G.F.R.)

(Received March 23rd, 1976)

Summary

The soluble hydrogenase (hydrogen: NAD^+ oxidoreductase, EC 1.12.1.2) from *Alcaligenes eutrophus* H 16 was purified 68-fold with a yield of 20% and a final specific activity (NAD reduction) of about 54 μmol H_2 oxidized/min per mg protein. The enzyme was shown to be homogeneous by polyacrylamide gel electrophoresis. Its molecular weight and isoelectric point were determined to be 205 000 and 4.85, respectively. The oxidized hydrogenase, as purified under aerobic conditions, was of high stability but not reactive. Reductive activation of the enzyme by H_2, in the presence of catalytic amounts of NADH, or by reducing agents caused the hydrogenase to become unstable. The purified enzyme, in its active state, was able to reduce NAD, FMN, FAD, menaquinone, ubiquinone, cytochrome *c*, methylene blue, methyl viologen, benzyl viologen, phenazine methosulfate, janus green, 2,6-dichlorophenolindophenol, ferricyanide and even oxygen. In addition to hydrogenase activity, the enzyme exhibited also diaphorase and NAD(P)H oxidase activity. The reversibility of hydrogenase function (i.e. H_2 evolution from NADH, methyl viologen and benzyl viologen) was demonstrated. With respect to H_2 as substrate, hydrogenase showed negative cooperativity; the Hill coefficient was $n = 0.4$. The apparent K_m value for H_2 was found to be 0.037 mM. The absorption spectrum of hydrogenase was typical for non-heme iron proteins, showing maxima (shoulders) at 380 and 420 nm. A flavin component could be extracted from native hydrogenase characterized by its absorption bands at 375 and 447 nm and a strong fluorescence at 526 nm.

Abbreviations: hydrogenase, hydrogen dehydrogenase, EC 1.12.1.2; Cetavlon, cetyltrimethylammoniumbromide; DCPIP, 2,6-dichlorophenolindophenol.

Introduction

The enzyme hydrogenase which catalyzes the reversible activation of molecular hydrogen, was discovered in 1931 by Stephenson and Stickland [1]. It was observed that *Escherichia coli* could use H_2 to reduce a number of different substrates. The enzyme was later found to be widespread in bacteria of different taxonomic and physiological types [2,3]. Hydrogenase is involved in all biological reactions in which hydrogen is consumed and evolved. In the aerobic hydrogen bacteria, the activation of H_2 followed by the transfer to certain acceptors is of physiological significance as hydrogen is used as the sole hydrogen donor and energy source under autotrophic conditions.

There are apparently two types of hydrogenase in hydrogen bacteria: a soluble enzyme able to reduce NAD (hydrogen : NAD^+ oxidoreductase, EC 1.12.12) and a membrane-bound enzyme which does not reduce NAD. In *Nocardia opaca* 1b only the first one is present (Aggag, M., unpublished); in other hydrogen bacteria (e.g. *Pseudomonas facilis*, *Corynebacterium autotrophicum*) only the second enzyme was found [4,5]. In *Alcaligenes eutrophus* [6,7] *Pseudomonas ruhlandii* [8] and *Pseudomonas saccharophila* [9] both enzyme activities have been encountered.

In the past, efforts to purify hydrogenase have not been very successful. The only hydrogenase preparations reported to be at least almost homogeneous were obtained from strictly anaerobic bacteria: *Desulfovibrio vulgaris* [10,11], *Clostridium pasteurianum* [12] and *Chromatium* [13]. Studies to describe hydrogenase systems of hydrogen bacteria were carried out with crude extracts or partial purified enzyme preparations [14—16]. Bone et al. [14] reported on an extensive purification (300-fold) of the soluble hydrogenase from *P. ruhlandii*, however, without indicating the degree of enzyme purity.

Although much information exists concerning the kinetic and catalytic characteristics of hydrogenase from hydrogen bacteria, data dealing with molecular, structural and spectral properties are not available. As this is due to the lack of pure enzyme preparations, the present studies aimed at elaborating a reliable purification procedure to allow a more careful characterization of the soluble hydrogenase from *A. eutrophus* H 16.

Materials and Methods

Chemicals. The chemicals used were obtained from: Boehringer, ATP, NAD, NADH, NADP, NADPH, FMN, cytochrome *c*, aldolase, alcohol dehydrogenase, chymotrypsinogen A and ferritin; from Serva, acrylamide, *N'N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, benzyl viologen, methyl viologen, ubiquinone Q_6; from Sigma Chemical Co., FAD, phenazine methosulfate and Nitro Blue Tetrazolium; from Whatman, DEAE-cellulose (DE 52); from Pharmacia, Sephadex G-200; from Bio-Rad, hydroxyapatite (Bio-Gel HTP); from Merck, all other chemicals.

Organism and cultivation. A. eutrophus (*Hydrogenomonas eutropha*) H 16 (ATCC 17699, DSM 428) was grown autotrophically in mineral medium according to Schlegel et al. [17]. Cells were cultivated in a 10 l fermenter (Bio-

stat: Braun, Melsungen) at 30°C and 600 rev./min. The gas mixture contained 80% H_2, 10% O_2 and 10% CO_2 and the gas flux was 500 ml/min. At the end of the logarithmic growth phase cells were harvested, washed twice with 50 mM potassium phosphate buffer, pH 7.0, resuspended in the same buffer at an absorbance of 200—250 (measured in 1 cm cuvettes at 436 nm) and stored at —20°C.

Enzyme assays. Hydrogenase activity was routinely assayed by measuring the reduction of NAD spectrophotometrically in 1 cm cuvettes at 365 nm and at 30°C. The reaction mixture (3 ml) contained 50 mM H_2-saturated Tris · HCl buffer (pH 8.0), 0.8 mM NAD and an appropriate amount of enzyme. The unit of enzyme activity was defined as the reduction of 1 μmol NAD per min.

To investigate acceptor specificity, hydrogenase activity was also measured manometrically by following the uptake of H_2 at 30°C. The reaction mixture in the Warburg flasks contained 50 mM Tris · HCl buffer (pH 8.0), 2.4 mM hydrogen acceptor and the enzyme sample. The total volume of the reaction mixture was 2.2 ml. The shaking flasks were flushed for 15 min with O_2-free hydrogen, and the reaction was started by tipping the solution of hydrogen acceptor from the sidearm into the main compartment. Hydrogenase was reductively activated either by the presence of 5—25 μM NAD(P)H in the reaction mixture, by preliminary addition of dithionite to the enzyme solution (final concentration: 1 mM) or by ultraviolet irradiation of the enzyme with a germicidal lamp (Schütt, Göttingen) at a distance of 20 cm for 2 h at 4°C under H_2 or N_2. Enzyme activation was also carried out by the addition of glucose (10 mM) and glucose oxidase (18 units) as an O_2-scavenging system to the reaction mixture 5 min before the reaction was started.

H_2 evolution from NADH, dithionite-reduced methyl viologen and benzyl viologen was measured manometrically too. The reaction mixture contained 50 mM Tris · HCl buffer (pH 8.0), 5.5 mM dithionite, 2.4 mM substrate and the enzyme sample. The reaction was started by tipping the substrate from the sidearm into the main compartment. The atmosphere was N_2.

Diaphorase activity was usually measured spectrophotometrically at 30°C under anaerobic conditions by following the reduction of ferricyanide at 405 nm in 1 cm cuvettes. The reaction mixture contained 50 mM Tris · HCl (pH 8.0), 0.6 mM NADH, 0.6 mM ferricyanide and the enzyme sample in a final volume of 3 ml. The reduction of cytochrome *c* was followed at 550 nm, the reduction of methylene blue and DCPIP at 660 nm. For comparison, reduction of hydrogen acceptors was also performed in the absence of NADH, but with H_2 (0.55 mM) as hydrogen donor under corresponding conditions. Reactivation of the enzyme was carried out as described above. In order to pay attention to non-enzymatic substrate reduction, controls without enzyme were always run. NAD(P)H oxidase activity was measured at 365 nm under air.

Enzyme purification. All purification steps were carried out at 0—4°C under aerobic conditions.

Preparation of crude extract: The thawed cell suspension was disrupted by sonication (10 s/ml suspension) in a continuous-flow vessel using a 600 W ultrasonic disintegrator (Schoeller and Co., Frankfurt). The temperature was kept below 5°C by cooling the vessel with ethanol at —10°C (Ultrakryomat TK 30 D 1, Messgerätewerk Lauda). Cell debris and larger particles were removed by cen-

trifugation at 100 000 × *g* for 60 min. The supernatant fraction was called the crude extract.

Cetavlon treatment: 0.15-ml volumes of a 2.5% (w/v) solution of Cetavlon were added dropwise with stirring to the crude extract containing 20–25 mg protein/ml. The precipitate was removed by centrifugation at 20 000 × *g* for 20 min.

Ammonium sulfate fractionation: The supernatant solution from the Cetavlon treatment was fractionated by addition of pulverized $(NH_4)_2SO_4$ to give a 40% saturated solution. After centrifugation at 10 000 × *g* for 10 min the precipitate was discarded. The supernatant solution was then brought to 60% saturation with $(NH_4)_2SO_4$ and centrifugated again at 10 000 × *g* for 10 min. The supernatant fraction was discarded, the pellet dissolved in 16 ml of 20 mM potassium phosphate buffer (pH 7.0) and dialyzed against the same buffer.

DEAE-cellulose chromatography: The dialyzed protein solution was applied to the DEAE-cellulose (DE 52) column (2.5 × 40 cm), pre-equilibrated with 20 mM potassium phosphate buffer, pH 7.0. The protein was eluted with a linear KCl gradient (0—0.5 M) at a flow rate of 20 ml/h. Fractions with a volume of 3 ml were collected. The most active fractions were combined, dialyzed against 50 mM Tris · HCl buffer (pH 8.2) and loaded on a second DEAE-cellulose column (2.5 × 35 cm), pre-equilibrated with the dialysis buffer, and eluted as was the first column.

Sephadex G-200 chromatography: The combined active fractions were dialyzed against 50 mM potassium phosphate buffer (pH 7.0) and concentrated to 1.5 ml by ultrafiltration in an Amicon diaflo cell. The protein solution was then layered on top of a Sephadex G-200 column (2.5 × 45 cm); 50 mM potassium phosphate buffer (pH 7.0) was used for pre-equilibration and for elution. The eluate was directly passed into a second column of the same dimensions and further fractionated by ascending chromatography. Fractions of 2 ml were collected at a flow rate of 12 ml/h.

Hydroxyapatite chromatography: The active fractions from the Sephadex G-200 step were combined, dialyzed against 10 mM potassium phosphate buffer (pH 7.0) and applied to a hydroxyapatite column (1.6 × 15 cm), pre-equilibrated with the dialysis buffer. The protein was eluted with a linear gradient of 10—300 mM potassium phosphate, pH 7.0. Fractions of 2 ml were collected at a flow rate of 12 ml/h. Those fractions, that contained pure hydrogenase, were combined, concentrated by ultrafiltration to 1—1.5 mg protein/ml, dialyzed against 50 mM potassium phosphate buffer (pH 7.0) and stored at —20°C under air.

Polyacrylamide gel electrophoresis. Analytical disc electrophoresis [18] was performed in a WTW (München) apparatus using a current of 2.5 mA per gel. Protein was stained with Coomassie Brilliant Blue [19]. The activity bands of hydrogenase on the gel were located by placing the gel into a test tube filled with a H_2-saturated solution (15 ml) of 50 mM Tris · HCl (pH 8.0), 0.8 mM NAD, 0.06 mM Nitro Blue Tetrazolium and 0.1 mM phenazine methosulfate. During incubation of the test tube at 30°C in the dark a blue-violet band at the site of hydrogenase appeared; a control was run in a H_2-free system.

Protein determination. Protein was determined by the method of Lowry et al. [20].

Determination of molecular weight. The molecular weight of hydrogenase was determined by gel filtration on a Sephadex G-200 column [21] and also by sucrose gradient centrifugation [22].

Isoelectric focusing. The isoelectric focusing of hydrogenase was carried out as recommended in the LKB manual using a column of 110 ml (LKB) with ampholytes pH 3.5–10. After 1 ml of the purified enzyme was focused for 48 h at 310 V and at 6°C, the column was eluted and fractions of 1.8 ml volume were collected.

Extraction of flavin. The enzyme solution was mixed at 0°C with an equal volume of ice-cold 20% trichloroacetic acid. After 15 min the sample was centrifugated at 5000 rev./min for 5 min. The supernatant was neutralized immediately with one-fourth its volume of 4 M K_2HPO_4.

Results

Purification of hydrogenase

The soluble hydrogenase from autotrophically grown *A. eutrophus* H 16 was purified 68-fold with a yield of 20% and a specific activity (NAD reduction) of about 54 μmol H_2 oxidized per min per mg protein. The purification procedure, described in detail in Materials and Methods and summarized in Table I, included: Cetavlon treatment; ammonium fractionation; DEAE-cellulose, Sephadex G-200 and hydroxyapatite chromatography. The elution profile from the hydroxyapatite column, as shown in Fig. 1, revealed exact coincidence of the protein and activity peaks.

Polyacrylamide gel electrophoresis of the hydroxyapatite eluate (fractions 29–32) showed only a single protein band (Fig. 2A, gel 2) having hydrogenase activity as demonstrated by a coupled reduction of nitro blue tetrazolium in the presence of H_2, NAD and phenazine methosulfate. There was no evidence for the existence of isoenzymes. However, if the crude extract was subjected to electrophoresis, three different activity bands (Fig. 2B, gel 1) could be demonstrated, but electrophoresis of the protein solution obtained from ammonium sulfate fractionation resulted in only a single active band (Fig. 2B, gel 2) although no loss in total activity occurred. These results are similar to those re-

TABLE I

PURIFICATION OF HYDROGENASE FROM *A. EUTROPHUS* H 16

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)	Yield (%)
Crude extract	3410	2730	0.80	1.0	100
Cetavlon treatment	2410	2700	1.12	1.4	99
Ammonium sulfate fractionation	1050	2860	2.72	3.4	105
DEAE-cellulose, pH 7.0	158	1770	11.20	14	65
DEAE-cellulose, pH 8.2	74	1530	20.68	26	56
Sephadex G-200	18.5	765	41.35	52	28
Hydroxyapatite	10.1	550	54.46	68	20

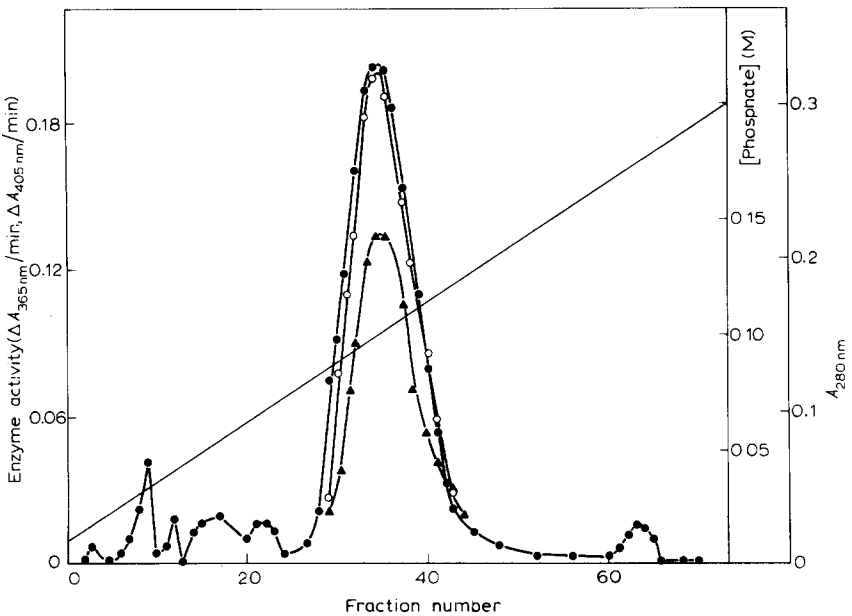

Fig. 1. The elution profile of hydrogenase from the hydroxyapatite column. 18.5 mg of protein was applied to the column (1.6 X 15 cm) and eluted with a linear gradient of 10—300 mM potassium phosphate, pH 7.0. Fractions of 2 ml were collected. Hydrogenase activity (o———o) was measured photometrically by following the reduction of NAD at 365 nm. Diaphorase activity (▲———▲) was measured photometrically by following the reduction of ferricyanide at 405 nm. ●———●, absorbance at 280 nm.

ported by Nakos and Mortenson [23], who suggested that the "multiple forms" of hydrogenase [24] in crude enzyme preparations of *C. pasteurianum* were artifacts, resulting from protein-protein interactions.

Molecular weight and isoelectric point

The estimation of the molecular weight by sucrose density gradient centrifugation yielded a value of 206 000. A Svedberg constant of 9.54 S was found for hydrogenase with alcohol dehydrogenase ($S_{20,w}$ = 7.61) as reference enzyme. Using gel filtration on a Sephadex G-200 column, a molecular weight of 204 500 was obtained when referred to ferritin, aldolase, alcohol dehydrogenase and chymotrypsinogen A. Investigations to determine subunit structure are in progress.

The isoelectric point of hydrogenase was found to be 4.85 when examined by electrofocusing in a LKB-column containing ampholytes pH 3.5—10.

Enzyme reactivation and stability

The oxidized hydrogenase, purified under aerobic conditions, was not reactive, but could easily be activated by the addition of either reducing agents or catalytic amounts of NAD(P)H when the enzyme solution was saturated with hydrogen. While incubation (up to 30 min at 30°C) or storage (several days at 4°C) of hydrogenase under H_2 in the absence of NAD(P)H or reducing agents did not restore activity the enzyme was activated also by the complete removal of oxygen by the addition of glucose and glucose oxidase.

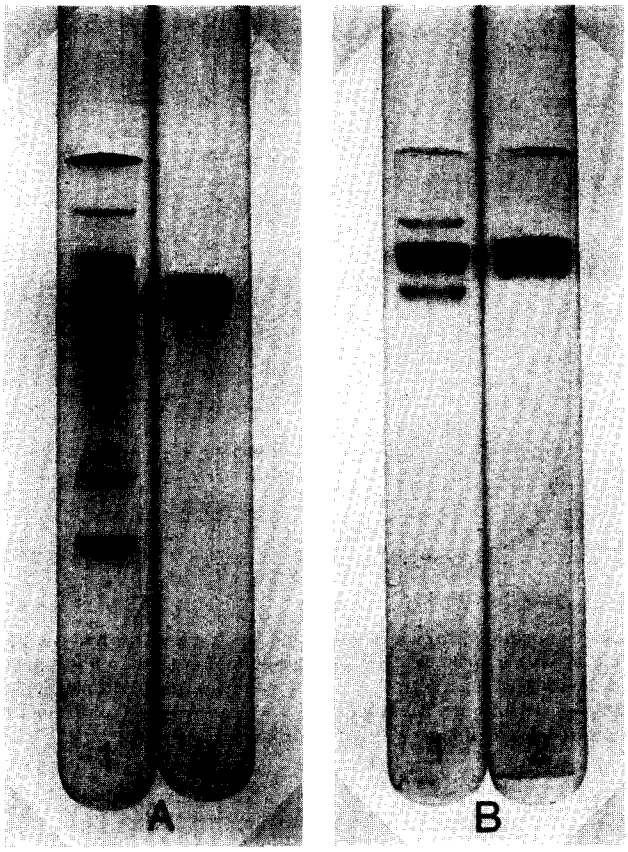

Fig. 2. Polyacrylamide gel electrophoresis of purified hydrogenase. (A) Protein staining. Gels containing 10% acrylamide (pH 9.5) were run at 2.5 mA per gel for 5 h. Protein was stained with Coomassie brilliant blue. 1, eluate from Sephadex G-200 column; 2, eluate from hydroxyapatite column. (B) Activity staining. Gels containing 12.5% acrylamide (pH 9.5) were run at 2.5 mA per gel for 8 h. The activity bands were located as described in Materials and Methods. 1, crude extract; 2, protein precipitate from ammonium sulfate treatment (40—60% saturation), redissolved in 20 mM potassium phosphate (pH 7.0).

Irradiation of hydrogenase with ultraviolet light (366 nm) for 2 h at 4°C under an atmosphere of H_2 or N_2 reactivated the enzyme too. In contrast to the irreversible activation of the hydrogenase from *Proteus vulgaris* by ultraviolet light [25], the observed reactivation is considered to be only a consequence of reduction of hydrogenase by ultraviolet light as indicated by the bleaching of the yellow enzyme solution and its diminished absorption (30—40%) in the 350—450 nm range of wavelengths.

In its oxidized inactive state, the hydrogenase was of high stability. There was no loss of activity when stored under air at —20°C for several months; a small decrease in activity (25% after 3 weeks) during storage at 4°C could be prevented by addition of 1.5 M $(NH_4)_2SO_4$. However, the reduced active form of hydrogenase was unusually unstable. Storage at 4°C under H_2 in the presence of 5 μM NADH resulted in complete loss of enzyme activity within 5

days. Presence of dithionite or mercaptoethanol (1 mM) in an enzyme solution stored under N_2 led to the same enzyme instability.

The observed instability in crude extracts is believed to be due to the presence of reduced pyridine nucleotides in those preparations.

Optimum conditions for enzyme activity

The pH optimum for catalytic activity was 8.0 and identical in Tris · HCl, triethanolamine and phosphate buffer. High ionic strength resulted in inhibition of enzyme activity. The most marked effects were produced by salts of sodium. 0.2 M phosphate buffer containing Na_2HPO_4 caused 70% inhibition of hydrogenase. Maximum activity was obtained using 0.05 M Tris · HCl buffer.

The optimal temperature was 33°C, and an activation energy of 14 kcal/mol was calculated.

An essential requirement for dissociable cofactors could not be demonstrated. Only 25 and 30% stimulation of hydrogenase activity, effected by FMN and ATP, respectively, was observed. Addition of ferrous or other divalent ions of heavy metals (Co^{2+}, Mn^{2+}, Ni^{2+}) did not increase hydrogenase activity as reported by Eberhardt [7].

Kinetics of NAD reduction

The reduction of NAD in the presence of saturating concentrations of H_2 did not require preliminary reactivation of the enzyme. During the lag phase, passed through before maximal reaction velocity was reached, a "self-activation" of enzyme was occurring. Probably a small number of active enzyme molecules generated the NADH required for activation. The length of the lag phase was dependent upon the reaction conditions. Addition of NADH to the reaction mixture shortened or removed the lag according to the concentration used, while the activation process was retarded by NAD (Fig. 3). The effect of NADH was greater the lower the NAD concentration. In the presence of 0.8 mM NAD, about 0.1 mM NADH was required to suppress the lag phase completely. The lag phase was also eliminated, if NADH was replaced by NADPH, if ultraviolet-irradiated or dithionite-reduced enzyme was used or if glucose (10 mM) and glucose oxidase (18 units) were added to the raction mixture 5 min before the reaction was started by NAD.

When measuring enzyme activities at varying substrate concentrations, the hydrogenase was reductively activated by incubation under H_2 in the presence of 5 μM NADH at 4°C for 6 h. This pretreatment resulted in linear kinetics from the beginning of the reaction and allowed accurate determination of K_m values. The H_2 saturation curve of hydrogenase was hyperbolic, but did not follow classical Michaelis-Menten kinetics. Because of the high substrate affinity at low H_2 concentrations and decreasing affinity with increasing H_2 concentrations, the curve resulting from a double-reciprocal plot was concave downward and the Hill coefficient was $n = 0.4$, thus indicating the presence of negative cooperativity. The apparent K_m value was 0.037 mM. The Michaelis constant for NAD was found to be 0.56 mM.

Specificity for hydrogen acceptors

Pfitzner et al. [15] described soluble hydrogenase as an enzyme which is

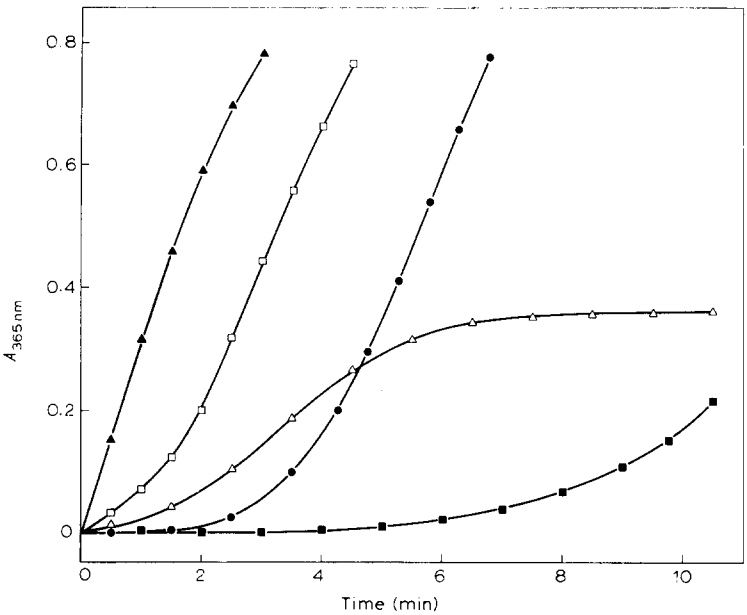

Fig. 3. Effect of NAD and NADH on lag phase of NAD reduction. The reaction mixture (3 ml) contained H_2-saturated 50 mM Tris · HCl buffer (pH 8.0), 4.5 μg protein and the following concentrations of NAD and NADH: 0.1 mM NAD, no NADH (△———△); 0.8 mM NAD, no NADH (●———●); 5 mM NAD, no NADH (■———■); 0.8 mM NAD, 0.005 mM NADH (□———□); 0.8 mM NAD, 0.1 mM NADH (▲———▲). The reaction was started by the addition of NAD to the reaction mixture.

specific for NAD and not able to react directly with any other compound. This statement was confirmed concerning NADP and ferredoxin from *C. pasteurianum* as hydrogen acceptors. Hydrogenase did not reduce NADP and ferredoxin either in the absence or presence of NAD(H) as well as in its oxidized or reduced state. Even if the enzyme concentration was increased 20-fold and NADP concentrations up to 10 mM were used, the activity was absolutely zero. With respect to other compounds, however, the characterization of the enzyme to be strictly NAD specific was not confirmed. The complete reduction of a wide range of artificial and physiological hydrogen acceptors and electron carriers (Table II) and the consumption of corresponding amounts of H_2 could be demonstrated by manometric tests. In fact, menaquinone, phenazine methosulfate, DCPIP and ferricyanide reacted at 3-, 3.5-, 3.8- and 5-fold higher rates than NAD, respectively. These reactions occurred not only when catalytic amounts of NAD(H) were added to the reaction mixture, but also in the presence of 5—25 μM NADPH. It is unlikely that NADPH, present in such small amounts, can serve as electron donor, since regeneration of NADPH is not possible because NADP does not react with hydrogenase. One may thus conclude: Reduced pyridine nucleotides are necessary only for the reductive activation of hydrogenase. The enzyme, in its active state, has the ability to react directly also with compounds different from NAD. Neither NAD/NADH, previously considered to be essential for mediating electrons to secondary acceptors [15, 26], nor other carrier systems are required for the reduction of substrates. This

TABLE II

REDUCTION OF HYDROGEN ACCEPTORS

Hydrogenase activity was measured manometrically by following the uptake of H_2 at 30°C. 100% activity corresponds to the uptake of 50 μmol H_2/min per mg protein. Assay conditions are described in Materials and Methods.

Hydrogen acceptor	Activity (%)	Hydrogen acceptor	Activity (%)
Methyl viologen	70	Menaquinone	307
Ferredoxin	0	Ubiquinone	85
Benzyl viologen	34	Methylene Blue	125
NADP	0	Phenazine methosulfate	354
NAD	100	DCPIP	385
Janus Green	108	Cytochrome *c*	35
FAD	77	Ferricyanide	503
FMN	85	O_2	2

hypothesis was confirmed by the following experiments: Oxidized inactive hydrogenase was activated by methods completely independent of the influence of pyridine nucleotides, i.e. ultraviolet irradiation, addition of dithionite or O_2 removal by the glucose/glucose oxidase system. Even thus pretreated enzyme preparations were shown to be able to reduce all the hydrogen acceptors mentioned above.

Apart from the ability to activate and transfer molecular hydrogen to a large number of acceptors, purified hydrogenase appears also to exhibit diaphorase and NAD(P) oxidase activity. Oxidants such as ferricyanide, methylene blue, DCPIP, cytochrome *c* and even oxygen were reduced with NADH (K_m = 0.08 mM) and NADPH (K_m = 6.4 mM) as hydrogen donors. After the elution of protein from DEAE-cellulose, Sephadex G-200 and hydroxyapatite (Fig. 1) the profiles of hydrogenase and diaphorase activity coincided with one another. The ratio of NAD-reducing hydrogenase activity to ferricyanide-reducing diaphorase activity remained constant throughout the above mentioned purification steps. Comparing the rates of reduction of hydrogen acceptors with either H_2 or NADH as hydrogen donor it was found that these rates were almost identical. The K_m value for ferricyanide was determined to be 0.2 mM in both cases.

Reversibility of enzyme function

H_2 evolution from NADH, dithionite-reduced methyl viologen and benzyl viologen could be demonstrated manometrically. Under N_2 and in the presence of 2.4 mM NADH and 5.5 mM dithionite 1.2 μmol of H_2 were evolved per min per mg protein (Table III). This rate corresponded to 2.4% of the reverse reaction rate. The gas produced was identified as molecular hydrogen by gas chromatography. The highest rate of H_2 production (48 μmol H_2 evolved/min per mg protein) was obtained when reduced methyl viologen was used as hydrogen donor. Evolution of H_2 from reduced ferredoxin from *C. pasteurianum* was not catalyzed by hydrogenase.

TABLE III

H_2 EVOLUTION FROM NADH, METHYL VIOLOGEN AND BENZYL VIOLOGEN

Assay conditions are described in Materials and Methods. Ferredoxin, methyl viologen and benzyl viologen were reduced using dithionite.

Hydrogen-donor	Specific activity (μmol H_2 evolved *per min per mg protein*)	Relative activity * (%)
Reduced methyl biologen	48	96
Reduced benzyl viologen	20	40
NADH plus 5.5 mM dithionite	1.20	2.40
NADH without dithionite	0.16	0.32
Dithionite (5.5 mM)	0.09	0.18
Ferredoxin	0	0

* 100% activity: 50 μmol H_2 uptake/min per mg protein in the presence of NAD as hydrogen acceptor.

Spectral properties

The absorption spectrum of hydrogenase, shown in Fig. 4, was typical for proteins containing non-heme iron and similar to those, described for other pure hydrogenases [11—13].

No significant peak was identifiable, but there was considerable absorption

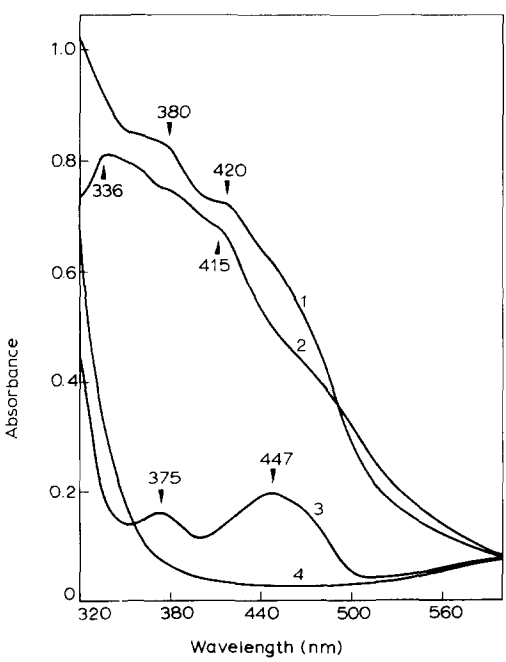

Fig. 4. Absorption spectra of hydrogenase and trichloroacetic acid extract. The spectra were taken in a Zeiss DMR 21 spectrophotometer. Protein concentration of the hydrogenase solution was 2.1 mg/ml in 50 mM potassium phosphate (pH 7.0). The trichloroacetic acid extract was prepared as described in Materials and Methods. Curve 1, native hydrogenase under air; curve 2, apoprotein of hydrogenase (native enzyme minus trichloroacetic acid extract); curve 3, trichloroacetic acid extract; curve 4, dithionite-reduced trichloroacetic acid extract.

in the 300—500 nm range with shoulders at 380 and 420 nm. The maximum of the protein absorption peak was at 276 nm. The ratio of absorbance at 276 nm to that at 380 nm was 3.1. On reduction of hydrogenase by incubation under H_2 in the presence of 5 μM NADH for 6 h at 4°C, the maxima at 380 and 420 nm disappeared; the decrease in absorption at 380 nm was 42%. For comparison, it may be mentioned, that the hydrogenases from *C. pasteurianum* [23] and *Chromatium* [13] showed only a slight decrease in the visible absorption peaks on reduction, while LeGall et al. [11] reported, that the decline in absorption of hydrogenase from *D. vulgaris* corresponded to 30% at 408 nm and to 36% at 450 nm, when the enzyme was reduced with H_2.

The existence of bound flavin was evidenced by the following: (1) The native enzyme was fluorescent with a characteristic emission maximum at 526 nm (Fig. 5B). The fluorescent chromophore could be released by precipitation of the apoprotein with 20% trichloroacetic acid. The extract, neutralized with K_2HPO_4, showed a fluorescence excitation spectrum with maxima at 460, 383 and 285 nm (Fig. 5A) and compared to the untreated hydrogenase a 12-fold increased fluorescence at 526 nm (emission spectrum). (2) The trichloroacetic acid extract exhibited a recognizable flavin absorption spectrum with distinct peaks at 447 and 375 nm (Fig. 4). On reduction of the free chromophore by dithionite, fluorescence and absorption disappeared completely. The release of the flavin component caused no considerable change in absorption properties of hydrogenase. The shoulder at 420 nm shifted to 415 nm, the shoulder at

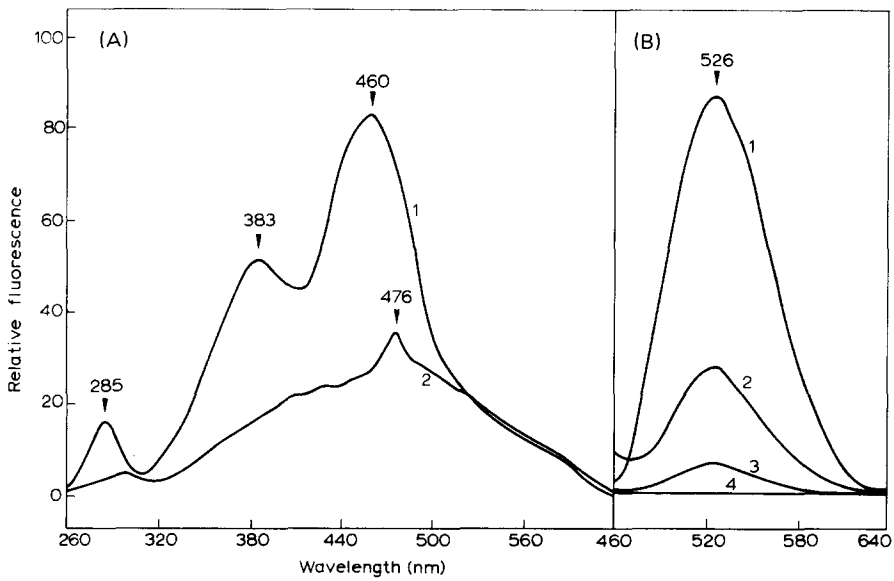

Fig. 5. Fluorescence spectra of hydrogenase and trichloroacetic acid extract. The spectra were taken in a Hitachi fluorescence spectrophotometer 204. (A) Excitation spectra. Curve 1, trichloroacetic acid extract; curve 2, native hydrogenase (1.2 mg/ml). (B) Emission spectra, excited at 430 nm. Curve 1, trichloroacetic acid extract from hydrogenase solution that contained 1.2 mg protein/ml; curve 2, native hydrogenase (4.8 mg/ml); curve 3, native hydrogenase (1.2 mg/ml); curve 4, dithionite-reduced trichloroacetic acid extract.

380 nm disappeared and a new maximum arose at 336 nm. The remaining absorption in the 300—500 nm range was supposedly due to iron components.

Discussion

Compared with known and described hydrogenases of other bacteria, the soluble hydrogenase from *A. eutrophus* H 16 has some outstanding properties. One of these is its abnormal stability behavior. The enzyme is stable when stored under air, but completely unstable under reducing conditions (i.e. in the presence of H_2, NADH or reducing agents). Other hydrogenases show just the opposite stability properties. Nakos and Mortenson [27] reported, that the enzyme from *C. pasteurianum* is completely inactivated after exposure to air for 60 min. Stability and activity of the hydrogenase from a different *A. eutrophus* strain (ATCC 17 697) depends upon a H_2 atmosphere and the presence of a reducing agent [28]. Reducing conditions are also essential for the retention of activity of hydrogenase from *Desulfovibrio* species [29—31]. A number of hydrogenases have also been described that are only slightly (*N. opaca* 1 b) [16] or not sensitive to oxygen (*Chromatium*, *P. ruhlandii*) [13,32], however, it has not been reported that just H_2 and reducing conditions have a destabilizing effect on hydrogenase. One possible explanation for this phenomenon is that in the presence of oxygen the oxidation of non-functional SH groups occurs with the formation of (a) disulfide bridge(s); such an oxidation may stabilize the protein structure, protect the enzyme against irreversible denaturation and does not cause enzyme inactivation. This interpretation is supported by the finding that inactivity of hydrogenase, caused by enzyme preparation under air, can be reversed already by the complete removal of O_2. Enzyme inactivity would thus have to be considered to be due to oxygenation of hydrogenase rather than to oxidation by O_2. The enzyme is also reactivated by reduction with reducing agents, but simultaneous splitting of the disulfide bond(s) might lead to the unfolding of the protein. Possible mechanisms, by which oxygen exerts its inhibition to hydrogenase were also discussed by Fisher et al. [33] and Mortenson and Chen [3].

Results concerning the determination of molecular weight of hydrogenases from hydrogen bacteria have not been previously reported. So the comparison of hydrogenases with known molecular weight is of interest. The molecular weight of 205 000 estimated for the soluble hydrogenase from *A. eutrophus* H 16 differs greatly from a value of 60 000, determined for a type of hydrogenase, represented by *D. vulgaris* [11] and *C. pasteurianum* [3,23]; it differs also from a molecular weight of 98 000, recently found for the particulate enzyme of *Chromatium* [13].

Packer and Vishniac [26] reported, that the reduction of dyes, other artificial and physiological electron carriers and hydrogen acceptors by the soluble hydrogenase from *P. ruhlandii* took place only in the presence of catalytic amounts of NAD. The authors concluded, that NAD is the only compound able to react directly with the enzyme, while other substrates are reduced in two or more reaction steps. This hypothesis was confirmed by Pfitzner et al. [15] who characterized the soluble hydrogenase of *A. eutrophus* H 16 as being NAD specific. Our results show, however, that NAD does not function as a specific and

obligatory primary acceptor. When transformed into its active state, the enzyme has the ability to react directly and in the absence of NAD(H) with a large number of artificial and physiological compounds (i.e. FMN, FAD, ubiquinone and cytochrome *c*). This finding is of physiological significance as it suggests that the soluble hydrogenase, apart from its primary function of supplying reducing power (NADH) for CO_2 fixation, is also able to channel electrons directly into the respiratory chain at sites with different redox potentials.

Most of the high molecular weight iron-sulfur proteins are conjugated with flavin, molybdenum or both, which act as additional electron transferring components [34]. Although the hydrogenases of *C. pasteurianum* [23], *D. vulgaris* [11] and *Chromatium* [13] have been identified as iron-sulfur proteins, there have been no reports on bound flavin. The partially purified hydrogenases of *P. ruhlandii* [14] and *A. eutrophus* H 16 [15] have also been described as having no flavin. The absorption spectrum of the pure hydrogenase from *A. eutrophus* H 16, showing no recognizable flavin peak, seemed in agreement with the above work. However, little acceptor specificity and presence of diaphorase activity, shown to be characteristics of flavoproteins [35], suggested the involvement of a flavin component in enzyme catalysis. Indeed, a flavin component could be isolated by treatment with trichloroacetic acid and characterized by its fluorescence and absorption properties. The relatively featureless absorption spectrum of the native enzyme lacking the typical flavin profile and pronounced maxima in general, can easily be explained: The flavin absorption was completely masked by simultaneous iron absorption in the same range of wavelengths. After the flavin was released from the apoprotein the total absorption was diminished only to a small extent, suggesting that the observed absorption of the native enzyme between 300 and 500 nm is due mainly to non-heme iron. Similar observations have been made with several iron-sulfur proteins containing flavin [36—38]. Höpner und Trautwein [38] described an absorption profile of formate dehydrogenase from *Pseudomonas oxalaticus* very similar to that of hydrogenase. The formate dehydrogenase was found to contain only one FMN but 5—8 iron atoms.

The rates of the reduction of all suited hydrogen acceptors and the K_m values (determined for ferricyanide as hydrogen acceptor) were almost identical to either H_2 or NADH as hydrogen donor. This indicates that reduction of acceptors, whether the reduction is associated with preliminary hydrogenase-dependent H_2 activation or not, is catalyzed by the same enzyme and follows the same reaction mechanism. The question that remains is, whether this enzyme, responsible for the reduction of acceptors, is identical to hydrogenase, or whether there is, in spite of the apparent homogeneity of the enzyme, another enzyme, a diaphorase, which was not yet removed from enzyme preparation. As it was shown, that hydrogenase reacts directly with substrates, independent of NAD/NADH, it is not possible that NADH is first formed from NAD and H_2 subsequently to be used in the reduction of acceptors by a hydrogenase-independent diaphorase. Thus two lines of evidence have led us to conclude that hydrogenase and diaphorase are one and the same enzyme: (1) the involvement of only a single enzyme in the reduction of acceptors by H_2, (2) identical K_m values for ferricyanide and very similar reduction rates for various acceptors whether H_2 or NADH were used as hydrogen donor. In addition, the identity of

hydrogenase and diaphorase suggests, that the flavin, found in the enzyme preparation is indeed a component of hydrogenase and not part of a contaminating protein. The flavin supposedly functions as an enzyme-bound intermediate carrier, accepting hydrogen and electrons from H_2 or NADH and transferring them to the acceptors. The almost identical reaction rates obtained with both hydrogen donors indicate that this transfer reaction is the rate-limiting reaction rather than the activation of molecular hydrogen or the binding of NADH.

Future investigations will be concerned with discerning subunit structure, identifying the flavin component, and characterization of hydrogenase as an iron-containing enzyme.

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