Biochimica et Biophysica Acta, 570 (1979) 43—55

BBA 68805

COMPARISON OF THE MEMBRANE-BOUND AND DETERGENT-SOLUBILISED HYDROGENASE FROM *PARACOCCUS DENITRIFICANS*

ISOLATION OF THE HYDROGENASE

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(Received February 23rd, 1979)

Key words: Solubilization; Hydrogenase isolation; (Paracoccus denitrificans membrane)

Summary

The hydrogenase from *Paracoccus denitrificans* is an integral membrane protein and has been solubilised by Triton X-100. The membrane-bound and detergent-solubilised forms of the enzyme have been compared. Both forms of the enzyme show a pH optimum for reduction of benzyl viologen at pH 8.5—9.0 and are both inhibited by concentrations of NaCl greater than 30 mM. An Arrhenius plot of the activity of hydrogenase in the membrane shows no 'break'. The form of the Arrhenius plot and the activation energy are not significantly changed on solubilisation of the enzyme. The K_m and V values for benzyl viologen, methyl viologen and H_2 are unaltered when the enzyme is extracted from the membrane. Therefore, solubilisation of hydrogenase from the membrane by Triton X-100 is unlikely to disrupt the native conformation of the enzyme. The detergent-solubilised hydrogenase has subsequently been purified using ammonium sulphate precipitation, sucrose density gradient centrifugation and chromatography on hydroxyapatite. The overall yield of activity is 23%, with a final purification of over 100-fold.

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Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; MES, 4-morpholinoethanesulphonic acid.

Introduction

Hydrogenase enzymes which catalyse the uptake and/or release of H_2 (Eqn. 1) were first described in 1931 [1]

$$H_2 \rightleftharpoons 2H^+ + 2e^- \quad (1)$$

but it is only in the last decade that information has become available on hydrogenases at the molecular level (see Ref. 2 for review). The purification of soluble hydrogenases from a variety of micro-organisms has been reported, including *Clostridium pasteurianum* [3,4], *Desulphovibrio vulgaris* [5,6], *Desulphovibrio gigas* [7] and *Anabaena cylindrica* [8].

Many organisms have, in addition to a soluble hydrogenase, a membrane-bound hydrogenase, e.g., *Alicaligenes eutrophus* (strain H16) [9] and *Thiocapsa roseopersicina* [10]. Other organisms, e.g., *Paracoccus denitrificans* [9,11] have only one, membrane-bound, hydrogenase. *P. denitrificans* can grow autotrophically on H_2, CO_2 and O_2 [12]. Under these growth conditions, hydrogenase catalyses the uptake of H_2 gas, which is the sole source of reducing power for the organism. Hydrogenase is therefore a key enzyme for survival of autotrophically grown *P. denitrificans*. It has been demonstrated that hydrogenase is an intrinsic membrane protein in *P. denitrificans* [11,13] and it forms an integral part of the electron transport chain [11].

Purification or isolation of membrane-bound hydrogenases from a small number of organisms has been reported although some such purifications have involved removal of the enzymes from the membrane by proteolytic cleavage [14,15]. The hydrogenase from *Proteus mirabilis* has been solubilised with organic solvents [16], whilst the membrane-bound hydrogenases from *Chromatium vinosum* [17,18] and *A. eutrophus* (strain H16) [19] have been purified after solubilisation with detergents.

The present study was undertaken to determine the effect of the non-ionic detergent, Triton X-100, on the enzymic characteristics of hydrogenase from *P. denitrificans* and the subsequent purification of the detergent-solubilised enzyme is described.

Materials and Methods

Bacterial culture and preparation of sub-cellular fractions

P. denitrificans (DSM strain 381, a gift from Professor H.G. Schlegel, Gottingen, F.R.G.) was grown autotrophically, harvested and membrane particles were prepared by sonication as described previously [11].

Solubilisation of hydrogenase

Routinely, hydrogenase was solubilised by incubating membranes (15—20 mg protein/ml) in 10 mM Tris-HCl, pH 8.0, under H_2 in the presence of 1 mM phenyl methyl sulphonyl fluoride with 1 mg Triton X-100 per mg membrane protein, for 20 min at 30° C with gentle shaking. The mixture was then centrifuged (140 000 × *g*; 90 min) at 20° C. The supernatant, which is called the Triton X-100 extract, was stored frozen (—20° C) under H_2 until used.

Enzymic assays

Hydrogenase activity was measured spectrophotometrically, by following the reduction of methyl viologen or benzyl viologen by H_2 as previously described [11] except that a reaction volume of 2 ml was used. Viologen dyes were at a final concentration of 5 mM in Tris-HCl, pH 8.0 and activity was measured at 30° C, except in the following cases:

(a) For determination of the activation energy, buffer (10 mM Tris-HCl, pH 8.0) (1.7 ml) saturated with H_2 at 25° C, was injected into an assay tube containing hydrogenase (0.2 ml) in an atmosphere of N_2. After incubation (5 min) at the assay temperature, reaction was started by injection of 0.1 ml of benzyl viologen (100 mM) presaturated with N_2, to fill completely the assay tube. Temperature was maintained (±0.1°C) with a Huber Mini-stat refrigerated circulating water bath.

(b) To determine the effect of pH on hydrogenase activity, 25 mM MES/25 mM HEPES/25 mM glycine/25 mM Tris was used as buffer and the pH was adjusted at 30° C with either HCl or NaOH.

(c) To measure the effect of varying the concentration of H_2, the sample of hydrogenase (0.2 ml) was incubated under N_2 (10 min) and then varying quantities of N_2-saturated benzyl viologen (5.6 mM in Tris-HCl, pH 8.0) were added. The reaction was then started by injection of sufficient H_2-saturated benzyl viologen (5.6 mM in Tris-HCl, pH 8.0) to complete the reaction volume (2.0 ml).

(d) For determination of the K_m for benzyl viologen and methyl viologen, reactions were started by injection of H_2-saturated viologen dyes at varying concentrations from 10 μM to 25 mM in 10 mM Tris-HCl, pH 8.0.

In all cases, control assays were done using only N_2-saturated benzyl viologen or methyl viologen, as appropriate.

Purification of hydrogenase

After solubilisation of hydrogenase with Triton X-100, the Triton X-100 extract was gassed with H_2 for 10 min at room temperature. Ammonium sulphate was added to 35% saturation and the material was stirred at room temperature under a gentle stream of H_2, for 45 min. The sample was then centrifuged (20 000 × *g* for 30 min) at room temperature and over 90% of hydrogenase activity was recovered as precipitate, which formed a brown pad of material, floating at the top of the tube. A white precipitate sedimenting to the bottom of the tube did not contain hydrogenase activity. The brown precipitate from ammonium sulphate precipitation was resuspended in 50 mM Tris-HCl, pH 8.0, to a final protein concentration of approx. 20 mg/ml and was gassed with H_2. Aliquots (1 ml) were applied to sucrose density gradients (10—40% sucrose (w/v) in 50 mM Tris-HCl, pH 8.0) prepared from sucrose solutions pregassed with H_2, and centrifuged for 16 h at 35 000 × *g* in a Beckman SW41 rotor in either a Beckman L2 65B or a Kontron ultracentrifuge at 20° C. After centrifugation, gradients were fractionated into 23 equal fractions and hydrogenase activity, protein content and absorbance at 420 nm measured in each fraction of a representative gradient from each set. The pool of hydrogenase activity from sucrose density gradient centrifugation was then adjusted to pH 6.9 with 0.1 M HCl and to a final phosphate concentration of 5 mM with equal

volumes of KH_2PO_4 and K_2HPO_4, each stored as 250 mM stock solutions, Triton X-100 was added to a final concentration of 0.1%. These adjustments resulted in a final Tris concentration of 25 mM. The material was then gassed with H_2 and applied to a column of spheroidal hydroxyapatite (1.5 × 8 cm) pre-equilibrated at 20°C with 2.5 mM KH_2PO_4/2.5 mM K_2HPO_4/25 mM Tris-HCl/0.1% Triton X-100, pH 6.9. Prior to equilibration with this buffer the column had been saturated with 10 ml of human serum to saturate all non-specific binding sites and washed with 250 mM potassium phosphate buffer, pH 6.9, (approx. 10 column volumes) until the absorbance at 280 nm of the eluate was zero. Hydrogenase activity was eluted from hydroxyapatite by a gradient of phosphate consisting of 50 ml of 5 mM potassium phosphate and 50 ml of 100 mM potassium phosphate in 25 mM Tris-HCl/0.1% Triton X-100, pH 6.9. Fractions of 1.5 ml were collected and hydrogenase activity, protein and absorbance at 420 nm measured. A pool of hydrogenase activity was made.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was carried out using 7.7% gels by the method of Weber and Osborn [20]. Gels were stained with Coomassie blue and scanned at 550 nm in a Beckman Acta 3 spectrophotometer with scanning attachment.

Other methods

Protein was determined by the method of Lowry et al. [21] using bovine serum albumin as standard.

Materials

Spheroidal hydroxyapatite was from British Drug Houses, Ltd., Poole, Dorset, U.K. Benzyl viologen and methyl viologen were from Serva, Heidelberg, F.R.G. Triton X-100, protein standards for gel electrophoresis, Tris, MES, HEPES and iodoacetamide were from Sigma Chem. Co., St. Louis, MO, U.S.A. Sucrose was purchased from Mallinkrodt, St. Louis, MO, U.S.A. Dithiothreitol was from Calbiochem, La Jolla, CA, U.S.A.

All other reagents were from Rhône-Poulenc Industries, Paris, France.

Results and Discussion

A. Comparison of Membrane-bound and solubilised hydrogenase

Solubilisation of hydrogenase by Triton X-100 in an atmosphere of hydrogen gas causes activation of the hydrogenase. Typically, 110—125% of the membrane-bound activity is recovered in the solubilised extract which contains 35—45% of the membrane protein (Table I). The increase in activity of hydrogenase when thus solubilised is likely to be due to the atmosphere of H_2 rather than to solubilisation by detergent, per se, since no activation has been observed previously when hydrogenase was solubilised in the absence of H_2 [11].

(i) Effects of pH. Both the solubilised and membrane-bound forms of hydrogenase show a pH optimum between 8.5 and 9.0 (Fig. 1), for reduction of benzyl viologen by H_2. The pH versus activity curve of the solubilised form is

TABLE I

KINETIC PARAMETERS OF HYDROGENASE

K_m and V values for benzyl viologen and methyl viologen were determined from Lineweaver-Burk plots of hydrogenase activities of samples of membranes (250—750 μg) or of Triton X-100 extracts (100—400 μg protein). Assay conditions were as described in Methods. V values are expressed as nmol viologen dye reduced/min per mg protein. K_m and V values for H_2 were similarly measured by following the reduction of benzyl viologen (5.6 mM) as described in Methods. Results are shown as the mean ± S.D. of three separate experiments or as the average of two separate experiments.

Substrate	Membranes		Triton X-100 extract	
	K_m (mM)	V (nmol dye reduced/ min per mg protein)	K_m (mM)	V (nmol dye reduced/ min per mg protein)
Benzyl viologen	0.29 ± 0.03	122 ± 12	0.25 ± 0.02	370 ± 24
Methyl viologen	2.94 ± 0.15	80 ± 11	2.80 ± 0.12	290 ± 19
H_2	0.026	118	0.020	356

broader and displaced slightly towards more acid pH values. These differences may be due to changes in the buffering capacity of the microenvironment of hydrogenase on solubilisation, rather than to a change in the mode of interaction of hydrogenase with benzyl viologen, since the K_m values for benzyl viologen and H_2 do not change on solubilisation.

It has been reported that the pH optimum of the membrane-bound hydrogenase from *A. eutrophus* (strain H16) decreases by 1.5 pH units on solubilisation [19] when activity is followed by reduction of methylene blue by H_2. However, that the standard electrode potential of methylene blue is pH-dependent may contribute to the observed pH shift. Differences in the methods of determining hydrogenase activity have been shown to influence the apparent pH optima obtained [22]. The pH optimum of hydrogenase from *Chromatium* is around 6 for hydrogen/deuterium exchange activity and biphasic with a

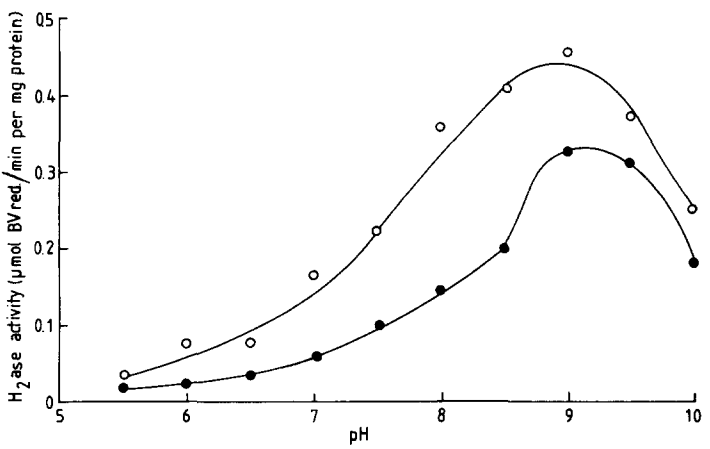


Fig. 1. Effect of pH on hydrogenase (H_2ase) activity. Membranes (200—400 μg protein) or a Triton X-100 extract (100—200 μg protein) were incubated (30°C, 10 min) at different values of pH in 25 mM MES/25 mM HEPES/25 mM Tris/25 mM glycine under H_2. Hydrogenase activity (nmol benzyl viologen (BV) reduced/min per mg protein) was measured in the same buffer. ●———●, membranes; ○———○, Triton X-100 extract.

second peak at pH 8.5 for methylene blue reduction [17]. *D. vulgaris* shows a plateau of activity for cytochrome *c* reduction between pH 7 and 9, whereas the pH optimum for reduction of methyl viologen is narrow and centred around pH 9 [6]. Therefore the differing effects on solubilisation of the hydrogenase from the two aerobic hydrogen bacteria, *P. denitrificans* and *A. eutrophus* (strain H16), may not be due to inherent differences in the enzymes but to differences in the assay systems used to determine activity.

(ii) Effect of ionic strength. Both the membrane-bound and solubilised forms of hydrogenase from *P. denitrificans* are similarly inhibited in the presence of NaCl between 30 mM and 200 mM (Fig. 2). The solubilised enzyme has also been found to be inhibited by 30% in the presence of 200 mM Tris-HCl. Other hydrogenases are also inhibited by high ionic strength, for example the soluble hydrogenase from *A. eutrophus* (strain H16) [23] and from *D. vulgaris* which is inhibited 80% by 200 mM Tris-HCl [6].

(iii) Kinetic parameters. The kinetic behaviour of hydrogenase in the membrane and in the Triton X-100 extract has been compared (Table I). The affinity of hydrogenase for benzyl viologen, methyl viologen and H_2 is not significantly altered in so far as affinity is reflected by the K_m value of the enzyme. For both forms of hydrogenase, the K_m value for benzyl viologen is 10-fold lower than the K_m for methyl viologen, whereas the V values of the solubilised and membrane-bound forms of hydrogenase are of the same order of magnitude for both viologen dyes (Table I). The earlier observation that hydrogenase reduces 5 mM benzyl viologen more rapidly than 5 mM methyl viologen [11] is therefore likely to be due to a higher affinity of the enzyme for benzyl viologen. This effect may be a result of more extensive interaction between hydrogenase and benzyl viologen via the two extra aromatic rings of this viologen dye.

The K_m for H_2 for the membrane-bound and solubilised hydrogenases, are 20 and 26 μM, respectively. Similar values have been found for *C. pasteurianum* hydrogenase [24] and for the cytoplasmic hydrogenase from *A. eutrophus* (strain H16) [23] for which an apparent K_m of 37 μM has been determined.

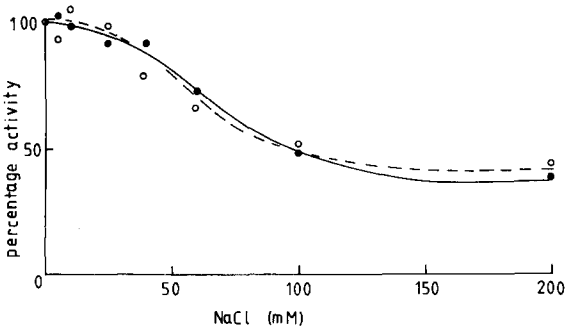


Fig. 2. Effect of ionic strength on hydrogenase activity. Membranes (200—400 μg protein) or a Triton X-100 extract (100—200 μg protein) were incubated under H_2 (30° C, 10 min) in the presence of different concentrations of NaCl. Hydrogenase activity was then measured at the concentrations of NaCl shown. Results are expressed as percentage of activities found in the absence of added NaCl, i.e. 104 nmol benzyl viologen reduced/min per mg protein for the membranes and 325 nmol benzyl viologen reduced/min per mg protein for the detergent extract. ●———●, membranes; ○- - - - -○, Triton X-100 extract.

The solubility of H_2 at 25°C is 0.8 mM [25]. The low K_m of hydrogenase for H_2 compared with the solubility of the gas is likely to be important physiologically for the hydrogenase of *P. denitrificans* and *A. eutrophus* which use H_2 as sole source of reducing power. Thus at low local concentrations of H_2, hydrogenase may remain saturated with its substrate, H_2, which is vital for the survival of these bacteria when restricted to autotrophic growth.

The increase in V for all substrates tested on solubilisation of hydrogenase by Triton X-100 (Table I) is due mainly to partial purification of the enzyme but may also reflect better accessibility of hydrogenase for its substrate.

(iv) Temperature dependence. An Arrhenius plot of the reduction of benzyl viologen by H_2 catalysed by the hydrogenase of *P. denitrificans* gives a straight line for both the membrane-bound and solubilised enzymes (Fig. 3). For many membrane-bound enzymes, such a plot shows a discontinuity at a specific temperature, e.g., 5′-nucleotidase from rat liver plasma membrane [26]. These 'breaks' have been interpreted as a reflection of a change in the physical state of phospholipids closely associated with the enzyme, although the molecular basis remains unclear [27]. Not all membrane-bound enzymes show discontinuities in Arrhenius plots, however [28].

It has been observed that the form of the Arrhenius plot of the activity of hepatic microsomal mono-oxygenase [29] and 5′-nucleotidase [30], both integral membrane proteins, are influenced greatly on solubilisation of the enzymes by detergent. This is not so for hydrogenase for which the form of the Arrhenius plot is unchanged on solubilisation. This may be related to the observation that hydrogenase has been shown to have negligible phospholipid associated with the enzyme on solubilisation and it also appears to bind very little Triton X-100 [31].

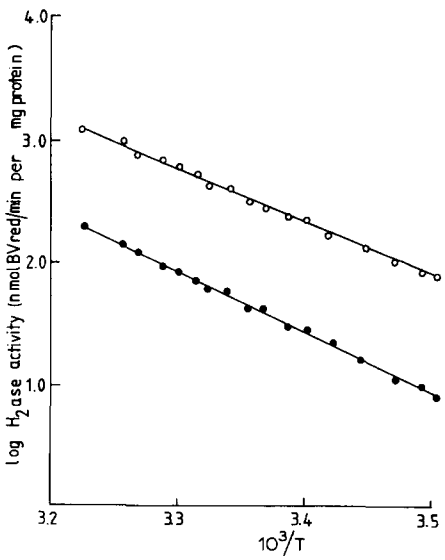


Fig. 3. Arrhenius plots of hydrogenase (H_2ase) activity. Hydrogenase activities of membranes (200—400 μg protein) and a Triton X-100 extract (100—200 μg protein) were measured by following the reduction of benzyl viologen (BV) at various temperatures. ●———●, membranes; ○———○, Triton X-100 extract.

The activation energies of the membrane-bound and solubilised forms of hydrogenase of *P. denitrificans* are similar, being 11.0 and 9.8 kcal/mol, respectively. Activation energies of the same order of magnitude have been found for the soluble hydrogenase from *Hydrogenomonas* H16 (*A. eutrophus* H16) for the reduction of NAD^+ (10.4 kcal/mol) [32] and for *C. pasteurianum* hydrogenase catalysing reduction of either ferredoxin or methyl viologen (14.6 kcal/mol) [33].

(v) Inhibitors. The effects of potential inhibitors of hydrogenase [2] have been investigated. For both membrane-bound and solubilised hydrogenase from *P. denitrificans*, transition metals are potent inhibitors (Table II). Heavy metals have also been demonstrated to inhibit hydrogenase from *Chromatium* [7].

Sulphydryl reagents inhibit *P. denitrificans* hydrogenase in the solubilised extract but are less effective inhibitors of the membrane-bound hydrogenase (Table II). This difference may be due to a greater accessibility of reactive sulphydryl groups after solubilisation. Inhibition by sulphydryl reagents is a general characteristic of hydrogenases [2]. For example, the hydrogenase from *C. pasteurianum* [34] is inhibited 40% by an 8-fold molar excess of mersalyl, an arsenical sulphydryl reagent. Iodoacetamide and *para*-chloromercuribenzoate (PCMB) strongly inhibit hydrogenase from *D. vulgaris* [7]. However, the hydrogenase from *Chromatium* is insensitive to inhibition by either PCMB or mersalyl [17].

The effect of heavy metals has also been suggested to be due to interaction with sulphydryl groups in enzymes [2]. This may not be the case for *P. denitrificans* hydrogenase, since the effects of heavy metals do not change on solubilisation of the enzyme whereas there is a marked difference in the effects of sulphydryl reagents.

TABLE II

EFFECTS OF INHIBITORS

Hydrogenase activity was measured in the presence of inhibitors at the concentrations indicated, by following the reduction of benzyl viologen at 30°C, as described in Methods. Samples of membranes (300 μg protein) or Triton X-100 extract (150 μg protein) were incubated under H_2 for 10 min in the presence of inhibitor prior to assay. Zero percent inhibition corresponds to 115 nmol benzyl viologen reduced/min per mg protein for the membrane preparation and 340 nmol benzyl viologen reduced/min per mg protein for the Triton X-100 extract.

Inhibitor (mM)		Membranes (% inhibition)	Triton X-100 extract (% inhibition)
Sulphydryl reagents			
N-ethylmaleimide	(1)	5	37
Iodoacetamide	(10)	5	36
PCMS *	(4)	43	80
Dithiothreitol	(0.2)	0	24
Transition metals			
$FeCl_3$	(1)	100	100
$CuSO_4$	(1)	95	95
$HgCl_5$	(1)	27	29

* PCMS, *para*-chloromercuribenzene sulphonic acid.

B. Purification of detergent-solubilised hydrogenase

In ealier attempts to purify hydrogenase from *P. denitrificans* problems arose due to loss of activity [11]. It has been discovered, however, that while hydrogenase loses activity rapidly at 4° C, it is more stable at room temperature [13]. Therefore a procedure was developed such that hydrogenase could be purified at 20° C within two days. Lengthy dialysis and concentration steps were avoided. It has been observed that recovery of activity was improved if samples were gassed with H_2 prior to carrying out purification steps. A summary of the purification procedure is shown in Table III.

The enzyme was solubilised by Triton X-100 (1 mg/mg protein). Hydrogenase, which was purified 2.5—3-fold in the solubilised extract, was then precipitated by ammonium sulphate (35% saturation). Use of a preliminary precipitation at 10—15% saturation with ammonium sulphate was found to remove less than 5% of the total protein and was of no advantage in purifying hydrogenase.

Sucrose density centrifugation has already been shown to be a useful purification step for hydrogenase from *P. denitrificans* [11], and it has been demonstrated that the enzyme remains soluble on centrifugation of the detergent-solubilised extract through a sucrose gradient containing no detergent [31]. The profile obtained on sucrose density gradient centrifugation of the resuspended ammonium sulphate precipitate is shown in Fig. 4. The absorbance at 420 nm is shown as cytochromes were found to represent major contaminants of the hydrogenase.

The hydrogenase pool, after sucrose density gradient centrifugation, was applied to a column of hydroxyapatite and eluted with a linear gradient of potassium phosphate. Hydrogenase activity was eluted after the peak of the cytochrome contamination, as indicated by the absorbance at 420 nm (Fig. 5). Fractions containing hydrogenase activity from hydroxyapatite chromatography were pooled and the mean specific activity was found to be 5.1 μmol

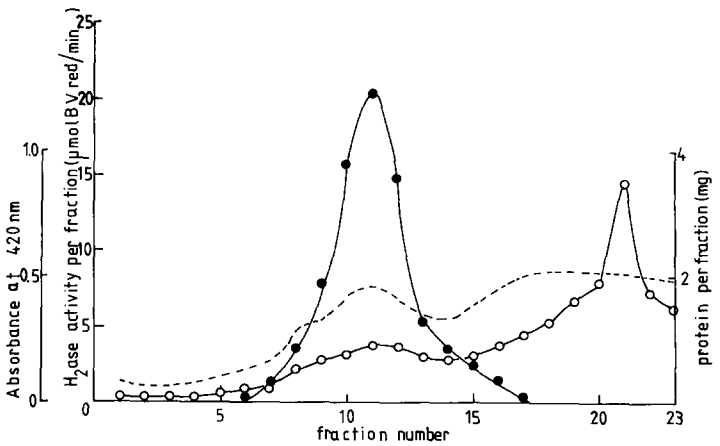


Fig. 4. Sucrose density gradient centrifugation of hydrogenase (H_2ase). Gradients (10—40% w/v) sucrose in 50 mM Tris-HCl, pH 8.0) were centrifuged (16 h, 35 000 rev./min) in a Beckman SW41 rotor at 20° C in a Beckman L2 65B ultracentrifuge. Hydrogenase activity (●———●), protein (○———○) and absorbance at 420 nm (- - - - -) were measured in each fraction. BV, benzyl viologen.

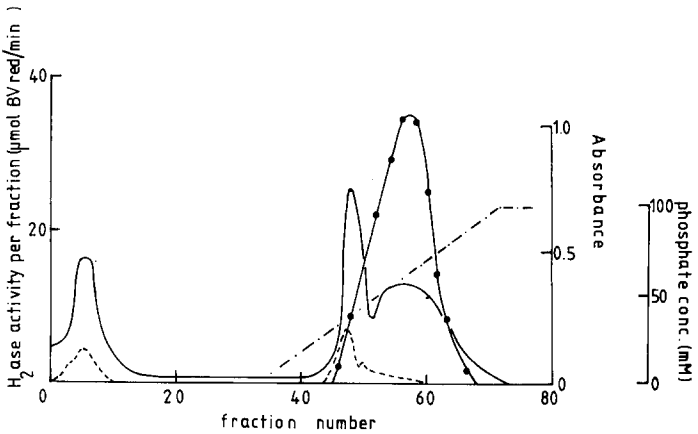


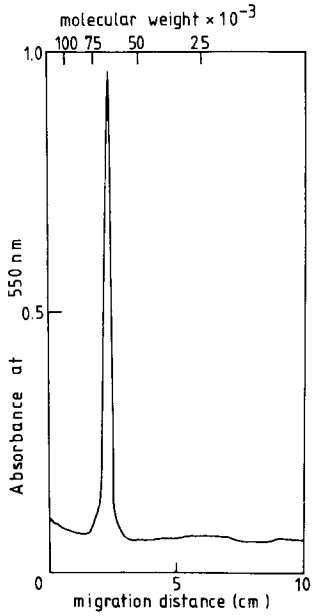
Fig. 5. Elution of hydrogenase (H_2ase) from hydroxyapatite. A sample of the hydrogenase pool from sucrose density gradient centrifugation (15 mg protein) was applied to a column of spheroidal hydroxyapatite (1.5 × 12 cm). The column was washed with 25 mM Tris-HCl/0.1% (w/v) Triton X-100/5 mM potassium phosphate, pH 6.9 (approx. 2 column volumes) and hydrogenase was eluted with a linear gradient of 5—100 mM potassium phosphate. Fractions (1.5 ml) were collected. Hydrogenase activity (●———●), absorbance at 280 nm (———) versus 0.1% Triton X-100 and absorbance at 420 nm (- - - - - -) were measured. Phosphate concentration (· — · — ·). BV, benzyl viologen

benzyl viologen reduced per min per mg protein, indicating over 100-fold purification of hydrogenase (Table III).

A sample of hydrogenase was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Hydrogenase appeared as a single band of apparent molecular weight 63 000 (Fig. 6). This is in agreement with previous estimates of the subunit molecular weight of the hydrogenase from *P. denitrificans* [31].

Since hydrogenase appears as a single band after gel electrophoresis in the presence of sodium dodecyl sulphate, this indicates that the enzyme is apparently homogeneous and, in any case, is likely to be greater than 95% pure. The enzyme has been purified over 100-fold compared with the cell homogenate and thus hydrogenase represents around 1% of the total cell protein. Hydrogenase has been purified 45—50-fold compared with the membrane preparation (Table III). Therefore, hydrogenase is a major protein of the membrane of *P. denitrificans* and it may be estimated to constitute 2—2.5% of the membrane protein of the bacterium when grown autotrophically on H_2, O_2 and CO_2.

An optical spectrum of hydrogenase has also been obtained (Fig. 7). Although the hydrogenase appears homogeneous on gel electrophoresis in sodium dodecyl sulphate, the enzyme preparation still may have traces of cytochrome-type contamination. This is indicated by the increase in absorbance at 420 nm on reduction with dithionite. Otherwise, the hydrogenase from *P. denitrificans* has an optical spectrum similar to those of other hydrogenases, e.g., those of *D. gigas* [7] and *C. pasteurianum* [3]. There does not appear to be a flavin component as described for the soluble hydrogenase from *A. eutrophus* (strain H16) [23,35].



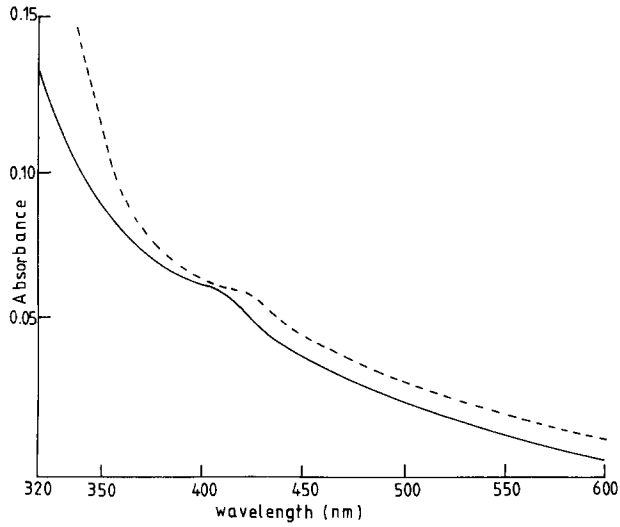


Fig. 6. Polyacrylamide gel electrophoresis of hydrogenase in the presence of sodium dodecyl sulphate. Gels (7.7% (w/v) acrylamide) of the hydrogenase pool from hydroxyapatite were stained with Coomassie brilliant blue and then scanned at 550 nm in a Beckman Acta III spectrophotometer. The molecular weight scale was constructed from the migration of standard proteins (phosphorylase a, 92 000; bovine serum albumin 68 000; catalase 60 000; IgG heavy chain 52 000; ovalbumin 42 000; IgG light chain 25 000).

Fig. 7. Absorption spectrum of hydrogenase eluted from hydroxyapatite. The spectra were recorded in a Beckman Acta III spectrophotometer. Protein concentration of the hydrogenase solution was 50 μg/ml. Hydrogenase under air, as isolated (———); hydrogenase reduced by addition of dithionite (- - - - -).

TABLE III

PURIFICATION OF HYDROGENASE

Step	Total protein (mg)	Specific activity (nmol BV * reduced/mg protein per min)	Total activity (μmol BV * reduced/min)	Purification (-fold)	Yield (%)
Homogenate	5577	47	260.3	1	100
Membrane preparation	2056	108	222.8	2.3	85.6
Solubilisation by Triton X-100	756.9	338	256.1	7.2	98.4
Precipitation (0—35% $(NH_4)_2SO_4$)	285.1	785	223.2	16.7	96.5
Sucrose density gradient centrigation	32.2	2420	78.0	51.5	29.9
Hydroxyapatite	11.5	5130	59.0	109.2	22.7

* BV, benzyl viologen.

Conclusions

When the hydrogenase of *P. denitrificans* is solubilised from the membrane by Triton X-100, its enzymic characteristics are essentially unchanged although it is more readily inhibited by sulphydryl reagents.

The solubilised hydrogenase from *P. denitrificans* has been purified and shown to have an apparent molecular weight, after gel electrophoresis in the presence of sodium dodecyl sulphate, of 63 000. There is no evidence for a smaller chain such as has been observed for the membrane-bound hydrogenase from *A. eutrophus* (strain H16), which consists of two polypeptide chains of apparent molecular weight 67 000 and 31 000 [36]. *A. eutrophus*, like *P. denitrificans*, belongs to the class of aerobic hydrogen bacteria. The hydrogenase from *P. denitrificans* has been shown to be distinct from the membrane-bound hydrogenase from *A. eutrophus* since an antiserum raised against the purified membrane protein from *A. eutrophus* (strain H16) does not cross-react with hydrogenase from *P. denitrificans*.

Although *P. denitrificans* and *A. eutrophus* are related bacteria, they differ in that *A. eutrophus* has both a soluble and a membrane-bound enzyme whereas *P. denitrificans* has only one membrane-bound hydrogenase. The structural differences between the membrane-bound enzymes may be due to differences in the physiological role played by these hydrogenases.

Acknowledgements

We are grateful to Dr. R.B. Sim for helpful advice and discussion and to Dr. B. Schink for a gift of antiserum against *A. eutrophus* (strain H16) membrane hydrogenase. Drs. G.S. Schoenmaker, B. Schink and Professor H.G. Schlegel kindly provided results prior to publication. We thank Mrs. Glenys Kelley for excellent technical assistance. E.S. was in receipt of a Royal Society European Exchange Fellowship.

This research was supported in part by grants from the Centre National de la Recherche Scientifique (ATP Photosynthèse) and the EEC Solar Energy Research and Development Programme (Contract No. 538-78 ESF).

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