BBA 66269

PURIFICATION AND PROPERTIES OF HYDROGENASE, AN IRON SULFUR PROTEIN, FROM *CLOSTRIDIUM PASTEURIANUM* W5

GEORGE NAKOS AND LEONARD MORTENSON
Department of Biological Sciences, Purdue University, Lafayette, Ind. 47907 (U.S.A.)

SUMMARY

1. Hydrogenase (H_2:ferredoxin oxidoreductase, EC 1.12.1.1) from *Clostridium pasteurianum* W5 has been purified to a state that shows one protein band with one coincident activity peak on disc electrophoresis on polyacrylamide gel and a single peak in the analytical ultracentrifuge.

2. The purified enzyme has a molecular weight of 60 000 and an isoelectric point of 5.0, and it contains 4.0 iron and 4.0 acid-labile sulfide groups per molecule.

INTRODUCTION

Hydrogenase (H_2:ferredoxin oxidoreductase, EC 1.12.1.1) is an enzyme involved in the H_2 metabolism of certain microorganisms. Whole cells or crude extracts of some of these organisms evolve H_2 in the presence of suitable electron donors (dithionite) and electron carriers (methyl viologen)[1]. They also take up H_2 gas in the presence of electron acceptors (methylene blue).

Purification procedures for hydrogenase have been described for the bacteria, *Clostridium butylicum*[1], *Desulfovibrio desulfuricans*[2–4] and *Proteus vulgaris*[5]. A partial purification of hydrogenase from *Clostridium pasteurianum* has also been reported[6] but no details of its purity and properties were given.

C. pasteurianum is a free-living nitrogen-fixing microorganism which possesses an extremely active hydrogenase. The hydrogenase of this organism has been shown to function in its nitrogen-fixing system (a reductive process) when H_2 is the electron donor. Clostridial-type ferredoxin accepts the electrons from hydrogenase and transfers them to nitrogenase[7]. Ferredoxin can also transfer electrons from dithionite to hydrogenase in the H_2 evolution assay[8].

This paper describes the purification and some properties of hydrogenase from *C. pasteurianum*.

MATERIALS AND METHODS

Chemicals

Chemicals used in this work were obtained: from Canalco Inc., aniline blue

black (RDS-1); from General Biochemicals, protamine sulfate (salmine, Lot 89179, control: 039413); from Eastman Organic Chemicals, acrylamide and *N,N*-methylbisacrylamide; from Fisher Scientific Company, dithionite and bromophenol blue; from Mann Research Laboratories, methyl viologen; from Merck and Co. Inc., methylene blue; from Pharmacia, Sephadex G-100, G-200 and blue dextran 2000; from Sargent Co, *N,N,N',N'*-tetramethylenediamine; from Sigma Chemical Co., aldolase (rabbit muscle, mol. wt. 140 000), α-chymotrypsinogen A (bovine pancreas, mol. wt. 25 700), cytochrome *c* (horse heart, mol. wt. 11 700), deoxyribonuclease (beef pancreas, noncrystalline), γ-globulins (bovine, Cohn Fraction II, mol. wt. 160 000), ovalbumin (Grade V, mol. wt. 43 000), ribonuclease (bovine pancreas, 5 times crystallized), and transferrin (Siderophilin, mol. wt. 85 000); from Whatman, DE32; from Worthington Biochemical Corp., lysozyme. The rest of the chemicals used were obtained commercially and were of the highest purity obtainable.

Hydrogenase assays

H_2 evolution. The oxidation by hydrogenase of dithionite-reduced methyl viologen[9] was monitored manometrically at 25° by measuring the amount of H_2 evolved. The main compartment of each Warburg flask contained 83 μmoles of degassed Tris–HCl (pH 8) buffer and 1.0 μmole methyl viologen in water. The center well contained 0.1 ml of 40% KOH. The flasks were evacuated and flushed with H_2 gas about 10 times before the addition to the main compartment (by means of a syringe with a bent needle) of 10 μmoles of a freshly prepared 0.1 M dithionite solution (degassed water was added by syringe to dry dithionite in a tube under H_2). Finally a 0.02-ml hydrogenase fraction was injected into the side arm and the reaction was started by tipping the fraction into the main compartment. The total volume of the reaction mixture was 2.0 ml.

H_2 uptake. The reduction of methylene blue by hydrogenase with H_2 as substrate was followed manometrically by measuring the amount of H_2 consumed. After electrophoresis of various hydrogenase samples gels were tested for hydrogenase activity by the method of ACKRELL *et al.*[10].

A unit of hydrogenase was defined by the standard biochemical unit designation as the amount required for the evolution or uptake of 1.0 μmole of H_2 gas per min.

Hydrogenase purification

Crude extract. All purification steps were performed anaerobically under H_2 and transfers of protein samples were made with hypodermic syringes through serum stoppers. Cells of *C. pasteurianum* were grown with N_2 as the sole source of nitrogen and cell-free extracts were prepared from dried cells[11].

Protamine sulfate treatment. Protamine sulfate (40.0 mg/ml water) was added dropwise from a separatory funnel to the stirred crude extract in the proportion of 3–5 mg/100 mg of extracted protein. The precipitate was removed by centrifugation at 10 000 × *g* and discarded.

Heat treatment. The supernatant solution from the protamine sulfate treatment was heated to 55–60° under H_2 in a water bath and kept at this temperature for 10 min, and then cooled in an ice–water bath for 10 min. The insoluble proteins were removed by centrifugation at 10 000 × *g* for 20 min and discarded.

DEAE-cellulose step. The supernatant solution from the heat-treatment step containing 15–20 mg of protein per ml was loaded on an anaerobically packed and preequilibrated DE32 column (10.0 cm × 5.0 cm) at a flow rate of about 100 ml/h. A linear KCl gradient[12] was employed to elute the hydrogenase fraction from the DEAE-cellulose column.

First Sephadex G-100 step. The contents of the tubes from the DEAE-cellulose step that contained hydrogenase activity were pooled and concentrated to 70.0–100.0 mg/ml in a Diaflo ultrafilter, Amicon Corp. The concentrated hydrogenase solution was further fractionated by passing it through two anaerobic columns (50.0 cm × 5.0 cm) of Sephadex G-100 that were preequilibrated by elution with degassed 0.05 M Tris–HCl (pH 8) buffer at a flow rate of 25.0 ml/h.

Second Sephadex G-100 step. The protein solution from the second band of both columns was concentrated and applied to two new Sephadex G-100 columns (100.0 cm × 3.0 cm) that were packed and eluted as the first set of Sephadex G-100 columns but were run with a flow rate of 10–12 ml/h. Fractions of 3.0 ml were collected anaerobically.

Analytical methods

Protein was determined by the method of LOWRY *et al.*[13]. For the purified enzyme a correction factor, 0.85, should be used to equate colorimetric protein determinations[13] based on serum albumin to dry weight values. Polyacrylamide disc gel electrophoresis[14,15] and the analytical ultracentrifugation (Model E) were employed as criteria of purity. Gels were scanned in a microdensitometer (Joyce, Loebl & Co. Ltd., England). The method of SCHALES AND SCHALES[16] was used for the Cl determination. The molecular weight was estimated by gel filtration[17]. Total iron was determined by the *o*-phenanthroline method as described by LOVENBERG *et al.*[18]. Acid-labile sulfide was measured by a modification[19] of the method of FOGO AND POPOWSKY[20]. Molybdenum was determined by dithiol reagent[21] as modified by BULEN AND LECOMTE[22]. The buffer systems of RICHARDS *et al.*[23] were used for the isoelectric point determination.

RESULTS AND DISCUSSION

Purification

A summary of the hydrogenase purification procedure is given in Table I. The crude extract was treated with 5% protamine sulfate by weight of extract protein. This step removed nucleic acids, nucleoproteins but not more than 5–10% of the total protein. The heat treatment step removed 40–50% of the remaining protein but it should not be above 60°. In the DEAE-cellulose step about 50–60% of the protein in the heat-treated supernatant solution passes through the column and has no hydrogenase activity. The first band to be eluted with the KCl gradient was yellow-brown in color and had no activity. At about 0.15 M Cl^- (Fig. 1) hydrogenase was eluted as a dark yellow band (Fig. 1). A 5–10-fold purified hydrogenase was collected under H_2 in about twenty 10-ml fractions. The active fractions were pooled, concentrated and put on two anaerobic Sephadex G-100 columns which had been equilibrated with 0.05 M Tris–HCl (pH 8) containing trace amounts of dithionite. During elution two protein bands appeared (Fig. 2), the first contained most of the

TABLE I

THE HYDROGENASE PURIFICATION SCHEME

Step	*Vol. (ml)*	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Activity (units)*	*Recovery (%)*	
					Protein	*Activity*
Crude extract	1640	38 400	1.30	50 000	100.0	100.0
5% protamine sulfate	1620	26 000	1.80	47 000	68.0	94.0
Heat (55°)	1540	21 000	2.00	43 500	57.0	87.0
DE32	200	3 400	8.80	30 000	9.0	60.0
First Sephadex G-100	185	925	30.0	27 800	2.4	55.0
Second Sephadex G-100	55	380	50.0*	19 000	1.0	38.0

* Note that this would correspond to an activity based on prior units[6] and the methylene blue assay of about $2.1 \cdot 10^6$ μl H_2 uptake per mg protein nitrogen per h.

protein but no hydrogenase activity (by all assay methods). The bottom section of the second band was yellow and it blended into a green upper section. Most of the activity was found in the fractions from the yellow section.

The protein from the second band was concentrated and fractionated on a second set of longer Sephadex G-100 columns. Two colored protein bands were resolved (Fig. 3). The first was golden yellow (mol. wt. 60 000) and the second green (mol. wt. 48 000–50 000). The solution in Tubes 5–9 showed one protein peak when examined in the analytical ultracentrifuge (Fig. 4) and a single protein band when analyzed by electrophoresis on polyacrylamide gels (Fig. 5). Hydrogenase from *Desulfovibrio desulfuricans*[4] has been reported to show two peaks in the analytical ultracentrifuge. Fractions 10–14 have low hydrogenase activity and two protein

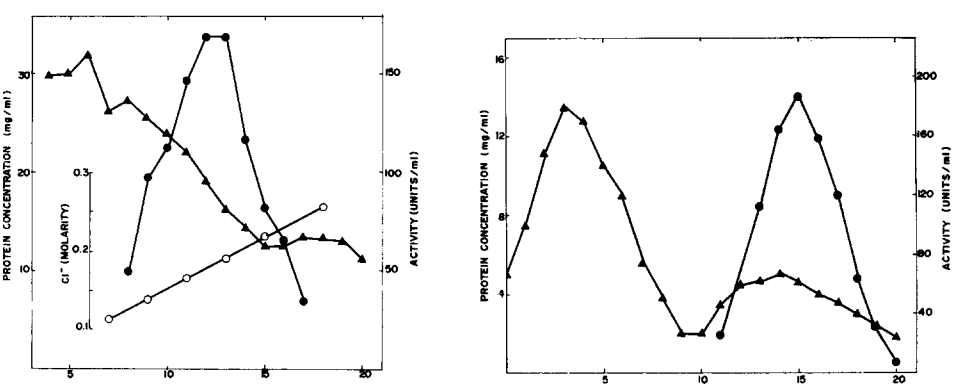


Fig. 1. Protein concentration and hydrogenase activity profiles from a DE32 column (10.0 cm × 5.0 cm). About 820 ml of heat-treated protein solution (15–20 mg/ml) was applied on the column and eluted as described in MATERIALS AND METHODS. ▲—▲, protein concentration; ●—●, activity from the H_2 evolution assay; ○—○, Cl^- gradient.

Fig. 2. Protein concentration and hydrogenase activity profiles from the first Sephadex G-100 column (50.0 cm × 5.0 cm). 1–2 g of protein (70–100 mg/ml) was passed onto and through the column at a flow rate of 20–25 ml/h. 10-ml fractions were collected up to Tube 10 (first band) and 5.0 ml thereafter. ▲—▲, protein concentration; ●—●, activity from the H_2 evolution assay.

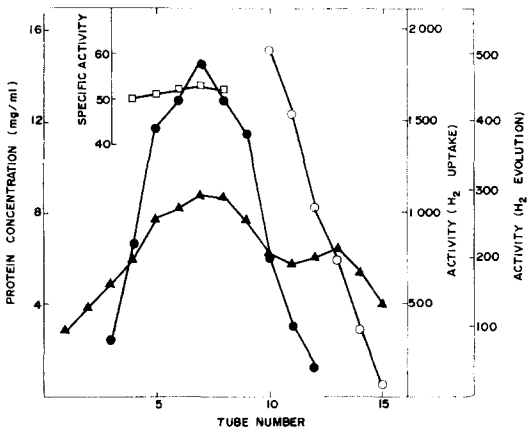
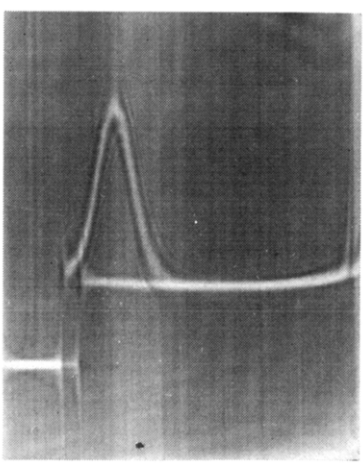


Fig. 3. The elution pattern of hydrogenase from the second Sephadex G-100 column (100.0 cm × 5.0 cm). Conditions of chromatography are given in the text. ▲—▲, the protein concentration; ●—●, activity from the H_2 evolution assay; ○—○, activity from the H_2 uptake assay; □—□, specific activity from the H_2 evolution assay. Activity in units/ml.

bands when examined by electrophoresis. Under some circumstances two hydrogenase activity peaks were seen when the contents of Tubes 11–14 were assayed[10]. The possibility that the second protein band might be a hydrogenase isoenzyme[10] with low activity in the "methyl viologen assay" and with the ability to oxidize H_2 gas in the presence of methylene blue was excluded since assay of the second protein band gave no corresponding activity peak with either hydrogenase assay (Fig. 3).



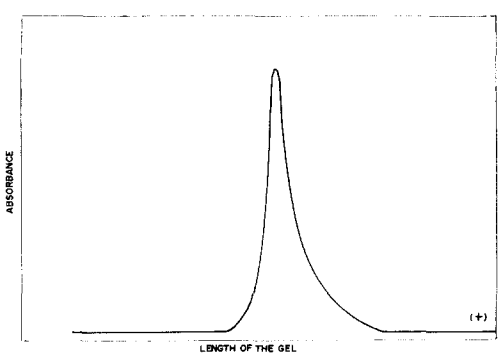
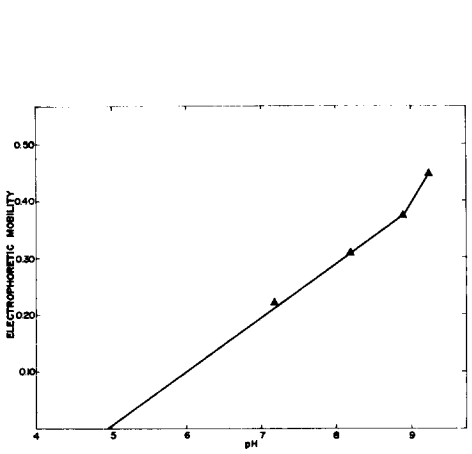


Fig. 4. Sedimentation pattern of hydrogenase. The photograph was taken 45 min after reaching full speed (50 740 rev./min) in a Beckman analytical ultracentrifuge (Model E). Conditions of the run: Rotor, AN-D; cell, Double sector; optics, Schlieren system; photographic angle, 50°; protein concentration, 6–7 mg/ml.

Fig. 5. Microdensitometer tracing of purified hydrogenase. A 5.0% acrylamide gel was used and the electrophoresis was carried out in borate–Tris–EDTA buffer (pH 8.3) at room temperature for 45 min at 2.5 mA per column.



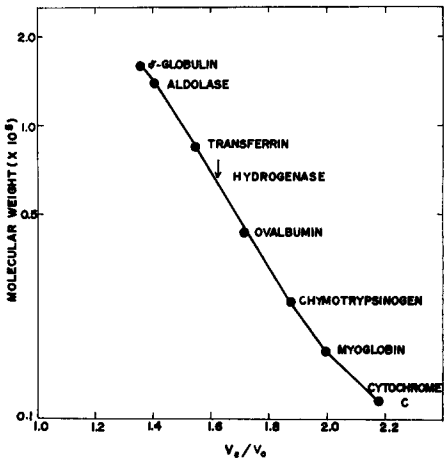


Fig. 6. Isoelectric point determination of pure hydrogenase. For the pH values tested ,gels were placed with 10% acrylamide *plus* 0.13% bisacrylamide. Electrophoresis was carried out at room temperature in the buffer systems of RICHARDS *et al.*[23]. Hydrogenase was identified before staining by the methyl viologen test (ACKRELL *et al.*[10]) and by the position of its protein band after staining followed by destaining of the gel. The activity and protein bands coincided.

Fig. 7. Molecular weight estimation of hydrogenase. The protein markers and hydrogenase (7–10 mg of each protein in 0.5 ml of 0.05 M Tris–HCl pH 8) were applied one at a time on a Sephadex G-200 column (120.0 cm × 1.5 cm). The application was in order of decreasing molecular weight. The column was eluted with 0.05 M Tris–HCl (pH 8) at a flow rate of 4.28 ml/h. Fractions of 2.14 ml were collected. V_e represents the elution volume of each protein. V_0 represents the elution volume of blue dextran.

Properties of hydrogenase

The catalysis of H_2 evolution by hydrogenase with reduced methyl viologen as electron donor was linear for the first 4–6 min and this assay was used routinely during the purification of the enzyme. Use of freshly prepared dithionite in deoxygenated buffer and removal of any traces of O_2 from the reaction vessel are the most important precautionary criteria of the assay. The optimum pH for the "methyl viologen assay" was approx. 7.8. Little H_2 was evolved below pH 6.6. This optimum results from the ineffective reduction of methyl viologen by dithionite under acid conditions rather than from "inactivation" of the enzyme by higher H^+ concentration since the optimum pH for the methylene blue assay is 6.8.

TABLE II

IRON AND ACID-LABILE SULFIDE CONTENT OF HYDROGENASE (MOL. WT. 60 000)

Sample No.	*Protein concn. (nmoles/ml)*	*Iron concn. (nmoles/ml)*	*Sulfide concn.* (nmoles/ml)*	*Fe/molecule*	*S^{2-}/molecule*
1	97.0	370.0	380.0	3.82	3.9
2	107.0	445.0	430.0	4.15	4.0
3	111.0	455.0	—	4.10	—

* Ferredoxin was used as standard and it had the equivalent of seven acid-labile groups per molecule.

Even though heat treatment was used as an early purification step, purified hydrogenase was considerably inactivated when heated for 5 min above 60° and completely inactivated at temperatures above 75°. Hydrogenase is completely inactivated after exposure to air (O_2) for 50–60 min but it can be kept under hydrogen at 0–5° for 2–3 months without loss of activity. The isoelectric point of hydrogenase is around pH 5.0 (Fig. 6).

The molecular weight estimated by gel filtration was found to be 60 000 (Fig. 7). This is in close agreement with the molecular weight of 50 000–55 000 reported for crude preparations of hydrogenase from the same organism[24] but much higher than the molecular weight of 9000 found for the partially purified hydrogenase from *Desulfovibrio desulfuricans*[4].

Analysis for iron and inorganic sulfide (Table II) shows that hydrogenase contains 4.0 iron and 4.0 sulfide atoms per molecule. Iron was found to be associated with hydrogenase from *Clostridium butylicum*[1] and *Desulfovibrio desulfuricans*[4]. The complete inactivity of purified hydrogenase from *Desulfovibrio desulfuricans*[3] and the absolute requirement for iron to reactivate the enzyme might result from the purification procedure employed[3] especially from the isoelectric precipitation step at pH 3.9 which could lead to inactivation of the enzyme because of a loss of iron and inorganic sulfide.

Small amounts of molybdenum were detected in the hydrogenase fractions from the DEAE-cellulose step. In the following Sephadex G-100 filtration most of the molybdenum (75 nmoles Mo per ml) was found in the first protein band which has no hydrogenase activity. In the fractions from the second Sephadex G-100 column trace amounts of molybdenum were found in the green protein band but not in the golden-yellow active hydrogenase band. This argues that the suggestion of SHUG *et al.*[6] that molybdenum is part of hydrogenase is in error.

Hydrogenase is an iron-sulfur protein of the "ferredoxin" high-molecular-weight class. It contains four iron atoms and four "acid labile" sulfide groups per dimer of mol. wt. 60 000. Each subunit of mol. wt. 30 000 (manuscript in preparation) might contain two iron atoms and two "acid labile sulfide" groups and whether or not it would fall into the "plant ferredoxin" class remains to be seen.

ACKNOWLEDGMENTS

This work was supported by Grant GB-22620. The authors wish to thank Mr. T. C. Huang for conducting the analytical ultracentrifuge experiment. The technical assistance of Mrs. C. Polis, Mrs. D. Sheets and Mr. C. Noonan is appreciated.

REFERENCES

1 H. D. PECK AND H. J. GEST, *J. Bacteriol.*, 73 (1957) 569.
2 J. C. SADANA AND V. JAGANNATHAN, *Biochim. Biophys. Acta*, 19 (1956) 440.
3 J. C. SADANA AND A. V. MOREY, *Biochim. Biophys. Acta*, 50 (1961) 153.
4 E. RIKLIS AND D. J. RITTENBERG, *J. Biol. Chem.*, 236 (1961) 2526.
5 C. SCHENGRURD AND A. I. KRASNA, *Biochim. Biophys. Acta*, 185 (1969) 332.
6 A. L. SHUG, P. W. WILSON, D. E. GREEN AND H. R. MAHLER, *J. Am. Chem. Soc.*, 76 (1954) 3355.
7 L. E. MORTENSON, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 272.

8 R. C. VALENTINE, L. E. MORTENSON AND J. E. CARNAHAN, *J. Biol. Chem.*, 238 (1963) 1141.
9 H. D. PECK AND H. GEST, *J. Bacteriol.*, 71 (1956) 70.
10 B. A. C. ACKRELL, R. N. ASATO AND H. F. MOWER, *J. Bacteriol.*, 92 (1966) 828.
11 L. E. MORTENSON, *Biochim. Biophys. Acta*, 81 (1964) 473.
12 T. K. LALSHMAN AND S. LIEBERMANN, *Arch. Biochem. Biophys.*, 53 (1954) 258.
13 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
14 B. J. DAVIS, *N.Y. Acad. Ann.*, 121 (1964) 404.
15 A. C. PEACOCK, S. L. BONTING AND K. G. QUEEN, *Science*, 147 (1965) 1451.
16 O. SCHALES AND S. S. SCHALES, *J. Biol. Chem.*, 140 (1941) 879.
17 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.
18 W. LOVENBERG ,B. B. BUCHANAN AND J. C. RABINOWITZ, *J. Biol. Chem.*, 238 (1963) 3899.
19 P. E. BRUMBY, R. W. MILLER AND V. MASSEY, *J. Biol. Chem.*, 240 (1965) 2222.
20 J. K. FOGO AND M. POPOWSKY, *Anal. Chem.*, 21 (1949) 732.
21 L. J. CLARK AND J. H. AXLEY, *Anal. Chem.*, 27 (1955) 2000.
22 N. A. BULEN AND J. M. LECOMTE, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 979.
23 E. G. RICHARDS, J. A. COLL AND M. A. GRATZER, *Anal. Biochem.*, 12 (1965) 452.
24 A. D. KIDMAN, R. YANAGIHARA AND R. N. ASATO, *Biochim. Biophys. Acta*, 191 (1969) 170.