Biochimica et Biophysica Acta, 766 (1984) 51–61
Elsevier

BBA 41517

THE PURIFICATION OF HYDROGENASE II (UPTAKE HYDROGENASE) FROM THE ANAEROBIC N_2-FIXING BACTERIUM *CLOSTRIDIUM PASTEURIANUM*

MICHAEL W.W. ADAMS and LEONARD E. MORTENSON

Corporate Research Science Laboratories, Exxon Research and Engineering Company, Clinton Township, Rte 22 East, Annandale, NJ 08801 (U.S.A.)

(Received December 13th, 1983)

Key words: Hydrogen uptake; Nitrogen fixation; Hydrogenase II purification; (C. pasteurianum)

The H_2 uptake activity (units/mg protein) of *Clostridium pasteurianum* cells with methylene blue as the electron acceptor increases with cell density independent of the growth conditions. The H_2 evolution activity (units/mg protein) of the same cells with reduced methyl viologen as the electron donor remains fairly constant under all growth conditions tested. Cells grown under N_2-fixing conditions have the highest H_2 uptake activity and were used for the purification of hydrogenase II (uptake hydrogenase). Attempts to separate hydrogenase II from hydrogenase I (bidirectional hydrogenase) by a previously published method were unreliable. We report here a new large-scale purification procedure which employs a rapid membrane filtration system to fractionate cell-free extracts. Hydrogenases I and II were easily filtered into the low-molecular-weight fraction (M_r less than 100 000), and from this, hydrogenase II was further purified to a homogeneous state. Hydrogenase II is a monomeric iron-sulfur protein of molecular weight 53 000 containing eight iron atoms and eight acid-labile sulfur atoms per molecule. Hydrogenase II catalyzes both H_2 oxidation and H_2 evolution at rates of 3000 and 5.9 μmol H_2 consumed or evolved/min per mg protein, respectively. The purification procedure for hydrogenase II using the filtration system described greatly facilitates the large-scale purification of hydrogenase I and other enzymes from cell-free extracts of *C. pasteurianum*.

Introduction

The enzyme hydrogenase (EC classes 1.12 and 1.18) catalyzes the reversible activation of molecular H_2 and was first discovered by Stephenson and Stickland [1]. In recent years, hydrogenases have been the subject of much research (see Refs. 2, 3). All are iron-sulfur proteins, but they differ considerably in their molecular and catalytic properties (for review, see Ref. 4).

The first hydrogenase to be characterized to any extent was the H_2-evolving enzyme from the anaerobic N_2-fixing bacterium *Clostridium pasteurianum* [5,6]. Reduced ferredoxin is a natural electron donor to this soluble hydrogenase (ferredoxin: H^+ oxidoreductase, EC 1.18.3.1) which catalyzes both H_2 production and H_2 oxidation in vitro (i.e., it is bidirectional) and is a monomer of molecular weight 60 000, containing 12 iron and 12 acid-labile sulfur atoms per molecule. Chen and Blanchard [7] reported the partial purification of a second hydrogenase from *C. pasteurianum*. In contrast to the well-characterized bidirectional hydrogenase, this enzyme appeared to catalyze only H_2 oxidation and was termed the unidirectional or uptake hydrogenase. They also reported that the H_2 uptake activity of N_2-grown cells was about twice that of NH_3-grown cells and that the uptake hydrogenase seemed to be located outside the cell membrane, since it was released upon protoplast formation.

In order to obtain more information on the

uptake hydrogenase and specifically to examine the differences bteween the pure bidirectional and uptake hydrogenases (henceforth termed hydrogenase I and II, respectively), we initiated a study of hydrogenase II. We determined the H_2 uptake activity of *C. pasteurianum* cells grown in batch culture under various conditions to maximize the cellular content of hydrogenase II for purification purposes, but our initial attempts to purify hydrogenase II further using the published procedure were unreliable. We decribe here a new large-scale reproducible purification procedure for hydrogenase II, which also allows a more rapid and larger scale purification of hydrogenase I, the nitrogenase components (MoFe and Fe proteins) and ferredoxin, all from the same batch of *C. pasteurianium* cells.

Materials and Methods

Chemicals. All chemicals were of the highest purity available from commercial sources. DEAE-Sepharose CL-6B, Octyl-Sepharose CL-6B and Sephacryl S-200 were from Pharmacia, Hydroxyapatite (High Resolution) from Calbiochem-Behring and DEAE-cellulose (DE-52) from Whatman. Lysozyme, DNAase, pancreatin and the standard proteins for molecular weight determination were obtained from Sigma Chemical Company.

Enzyme assays.

Uptake hydrogenase activity was routinely determined spectrophotometrically by measuring the reduction of methylene blue with H_2. The cuvettes (1 cm path-length, type C-030-0302; Perkin-Elmer) fitted with serum stoppers were repeatedly degassed and flushed with H_2, as were all reactant solutions. The reaction mixture, containing 50 mM Tris-HCl buffer (pH 8.0) and methylene blue (0.05 mM) in a final volume of 2.5 ml, was added by syringe and incubated at 30°C. The reaction was initiated by adding the hydrogenase and was measured by the decrease in adsorbance at 590 nm. The adsorption coefficient of methylene blue was 30900 $M^{-1} \cdot cm^{-1}$ at this wavelength, and this value was used in all calculations. One unit of hydrogenase activity catalyzes the reduction of 1 μmol methylene blue/min under these conditions; this is equivalent to 1 μmol H_2 oxidized/min. In the assay of pure hydrogenase I, bovine serum albumin (1 mg/ml) was included in the assay mixture, but this was not necessary with crude preparations or with pure hydrogenase II, which were unaffected by bovine serum albumin. Hydrogenase activity was also measured by H_2 evolution with methyl viologen (1 mM) as the electron carrier and sodium dithionite (20 mM) as the electron donor in 50 mM Tris-HCl (pH 8.0). H_2 was quantitated using a Varian Vista 6500 gas chromatograph (see Ref. 4). H_2 production was followed over a 5-min period and the results are expressed as μmol H_2 evolved/min.

Electrophoresis and molecular weight determination. Analytical slab gel electrophoresis was performed by the method of Davis [8] using a 4.7% stacking gel (w/v, acrylamide; pH 6.8) and a 10% analyzer gel (w/v acrylamide; pH 8.8). The gels ($16 \times 13 \times 0.075$ cm) were run in an SE 630 vertical slab electrophoretic unit (Hoefer Scientific Instruments). To detect protein, the gels were stained with Coomassie Blue for 30 min at 25°C and were destained in a Slab Diffusion Destainer (Hoefer Scientific Instruments). Hydrogenase activity was located on the gels by the method of Adams and Hall [9]. Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to Weber et al. [10] using the same slab gel apparatus. The standard marker proteins used and their molecular weights were: bovine serum albumin (67 000), catalase (57 500), ovalbumin (45 000), aldolase (40 000) and chymotrypsinogen A (25 000). The molecular weight of native hydrogenase was established by gel filtration on a column (1.5×90 cm) of Sephacryl S-200 equilibrated at 10 ml/h with 50 mM Tris-HCl (pH 8.0)/50 mM NaCl. The column was calibrated with bovine serum albumin (67 000), chymotrypsinogen A (25 000), trypsin (23 300) and cytochrome *c* (12 000).

Other methods. The iron and acid-labile sulfide content of hydrogenase was estimated using *o*-phenanthroline [11] and methylene blue formation [12], respectively. Ferredoxin from *C. pasteurianum* was used as a reference protein in both assays and was purified by the method of Mortenson [13]. Nitrogenase was assayed in cell-free extracts by acetylene reduction [14]. Protein was estimated by the method of Lowry et al. [24] using bovine serum albumin as the standard.

Growth of C. pasteurianum. For the purification of hydrogenase II, *C. pasteurianum* W5 was grown under N_2-fixing conditions in a 160-liter batch culture using the medium previously described [15]. The inoculum (5%) was from a chemostat culture grown on N_2 and controlled by limiting sucrose. To study the effect of growth conditions on hydrogenase content, 160-liter batch cultures were also grown: (a) with NH_3 as the N-source ($NH_4^+CH_3COO^-$, 25 mM) under Ar; (b) with limiting NH_3 ($NH_4^+CH_3COO^-$, 3.5 mM) under N_2 to allow derepression of nitrogenase; and (c) under sucrose-limiting conditions. In the latter case with NH_3 as the nitrogen source, the initial sucrose concentration was 14 mM and when cell growth stopped, sucrose was added to a final concentration of 30 mM. Under N_2-fixing conditions, the sucrose concentrations used were 18 mM and 40 mM, respectively. Cell growth was measured by determining the optical absorbance at 550 nm and nitrogenase activity was estimated by assaying whole-cell acetylene reduction (see Ref. 16).

Preparation of cell extracts during growth studies. To determine the hydrogenase activities of cells during growth in batch culture, samples (10 ml) were removed by syringe and injected into centrifuge tubes (No. 03121, Sorvall) fitted with serum stoppers (No. 8573-D42, Arthur H. Thomas Co.) which had been previously degassed and flushed with Ar. The cells were collected by centrifugation at $15000 \times g$ for 5 min. The supernatant was removed by syringe and, where indicated, was analyzed for sucrose [17] and ammonia [18] content by these published methods. The sedimented cells were resuspended in 1 ml of 50 mM Tris-HCl buffer (pH 8.0) containing sodium dithionite (1 mM), lysozyme (1 mg/ml) and DNAase (0.1 mg/ml). After degassing and flushing with Ar, the suspensions were incubated at 30°C for 1 h and then stored at 4°C until examined. Cell lysis was confirmed by microscopic examination. The hydrogenase activities of the cell-free extracts were determined as described above.

Whole-cell washes and protoplast formation. Protoplasts of *C. pasteurianum* were prepared from fresh cells by lysozyme treatment either in Tris/sucrose buffer [7] or phosphate/lactose/$MgCl_2$ buffer [19] by the methods described in these references. All manipulations were carried out anaerobically, and all buffers contained sodium dithionite (1 mM). The release of hydrogenase activity from the periplasmic space was also attempted by replacing lysozyme with pancreatin (1–4 mg/ml), sodium deoxycholate (0.5–2.0%, w/v), NaCl (2 M) or EDTA (1–20 mM) in both of the above buffer systems and the cells were treated as for the preparation of protoplasts. In all cases, intact cells or protoplasts were removed by centrifugation at $20000 \times g$ for 10 min and the supernatant solutions were assayed for H_2 evolution and H_2 uptake activities.

Purification of hydrogenase II. For the purification of hydrogenase II and all other proteins, all steps were performed under anaerobic conditions at room temperature. All buffers were repeatedly degassed and flushed with Ar, pre-purified with heated BASF catalyst to remove traces of O_2, and were maintained under positive pressure. To protect against trace O_2 contamination, sodium dithionite (1 mM) was added to all buffers unless stated otherwise.

Frozen cells (2 kg) were thawed in 4 liters of 100 mM Tris-HCl (pH 8.5) containing lysozyme (1 mg/ml) and DNAase (0.1 mg/ml) with repeated degassing and flushing with H_2. The cells lysed during incubation of the cell suspension at 25°C for 2 h with stirring under H_2 and a cell-free extract was obtained by centrifugatoin at $53000 \times g$ for 20 min in a type 19 rotor in a Beckman L8-80 ultracentrifuge. For ease of handling, the supernatant solution (about 5 liters) was split into two equal volumes and each was thoroughly degassed and flushed with H_2.

Pellicon filtration. A Millipore Pellicon Cassette System (Millipore Corp., Bedford, MA) was used to separate the cell-free extract into two fractions which nominally contained proteins with molecular weights greater than, and less than, 100000. The Pellicon unit consisted of a high-volume cell containing 25 ft^2 of polysulfonate (PTHK) filters and a high-volume pump (9 l/min). A completely anaerobic system was assembled and used to wash the cell-free extract and to collect the Pellicon filtrate (containing proteins with molecular weights less than 100000). The filtrate containing the hydrogenase was concentrated by batch absorption onto DEAE-cellulose (DE-52). The complete system is illustrated in Fig. 1, where A and B are 4 l

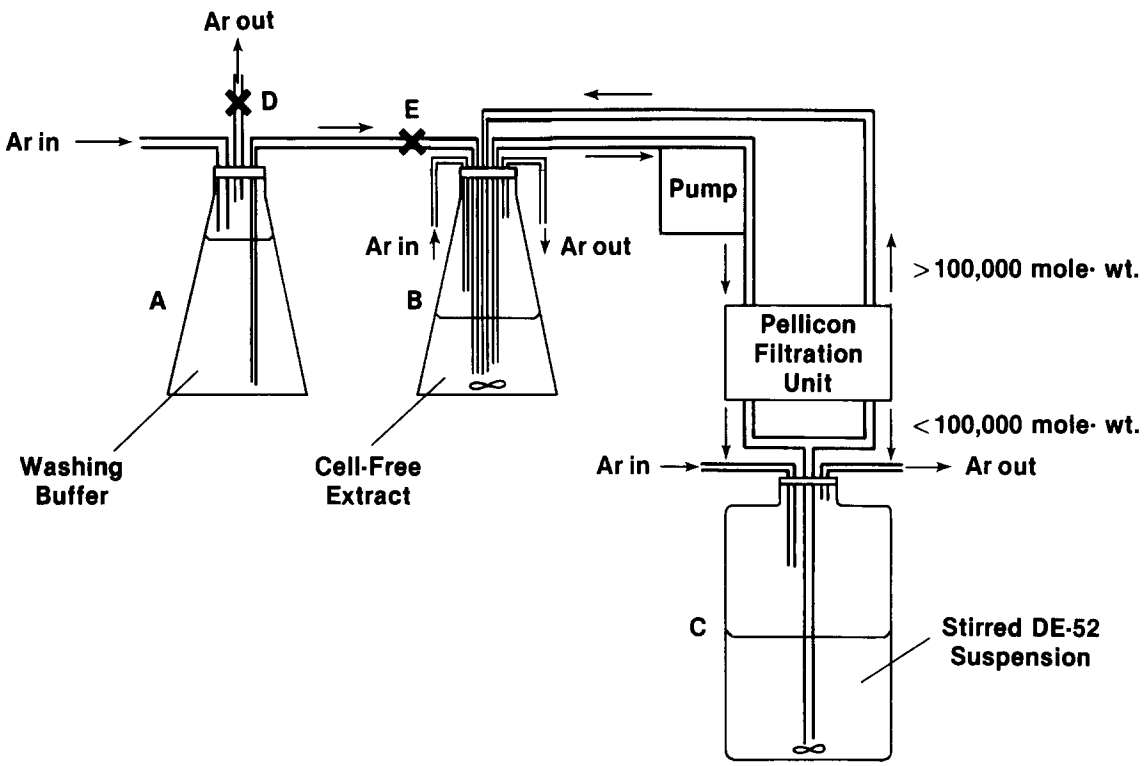

Fig. 1. Operation of the Pellicon filtration system under anaerobic conditions (see text for details).

flasks and C is a 12 l flask containing initially 1.2 l of a slurry of DE-52 (800 ml settled bed volume) equilibrated with 50 mM Tris-HCl (pH 8.0). All connecting lines are of Teflon or Tygon tubing, and all flasks are continually flushed with Ar. Initially, A and B contain 20 mM Tris-HCl (pH 8.0) that is used to rinse thoroughly the Pellicon and all connecting tubing. All transfers are made by forcing solutions from one flask to another with Ar pressure. The cell-free extract (in B) is first reduced to 1.5 l by filtration (clamp D open, clamp E closed) with a filtrate flow rate of 200–300 ml/min into the stirred DE-52 suspension. The pump is switched off, clamp D is closed, clamp E opened, and 1.5 l of washing buffer (20 mM Tris-HCl (pH 8.0)) is transferred from A to B. With clamp D open and clamp E closed, the solution containing proteins of $M_r > 100000$ is again reduced to about 1.5 l to remove proteins of $M_r < 100000$. This procedure is repeated (replenishing A with fresh buffer) until approx. 11-l of filtrate are collected. The DE-52 in the suspension in flask C has absorbed the hydrogenase activity and, after the suspension settles, it is used in the next step. The second portion of the crude cell-free extract is treated similarly. The remaining cell-free extract with proteins of $M_r > 100000$ is used for the purification of nitrogenase and other proteins (see Results and Discussion).

DE-52 chromatography. The supernatant solutions from the settled DE-52 beds (see above) were discarded and two empty columns were filled with the slurry containing hydrogenase absorbed to DE-52. The columns were packed to about 7.5 × 20 cm and washed with 50 mM Tris-HCl (pH 8.0) at a flow-rate of 500 ml/h. A dark band containing hydrogenase was eluted with buffer containing 0.13 M NaCl (1 l) and collected in 100-ml fractions. Those fractions from the two columns with an H_2 uptake/H_2 evolution ratio greater than 6 (see Results and Discussion) were combined, diluted to 4 l with 10 mM Tris-HCl (pH 8.0) and used for the further purification of hydrogenase II (see below). For the purification of hydrogenase I,

the same buffer containing 0.28 M NaCl (1 l) was passed through the columns and 100-ml fractions were collected. Those fractions containing H_2 evolution activity were combined and added to the fractions from the 0.13 M NaCl wash with an H_2 uptake/H_2 evolution ratio of 6 or less. The combined fractions from both columns were further purified by the method of Chen and Mortenson [5] omitting the first three steps of their purification procedure.

DEAE-Sepharose CL-6B chromatography. The diluted hydrogenase II fractions were loaded onto a column of DEAE-Sepharose CL-6B (7.5 × 15 cm) pre-equilibrated with 50 mM Tris-HCl (pH 8.0) at 450 ml/h. The column was washed with 1 column volume of the same buffer, and the absorbed proteins were eluted in 40 ml fractions with a 1.6 l linear gradient from 0 to 0.20 M KCl in the same buffer. Hydrogenase II eluted at about 0.11 M KCl.

Ammonium sulfate fractionation. Fractions from the DEAE-Sepharose column with a specific activity in the H_2 uptake assay greater than 60 units/mg were combined and 90% ammonium sulfate in 100 mM Tris-HCl (pH 8.0) was slowly added with stirring to a final concentration of 55%. The solution was stirred for a further 30 min and then centrifuged at 53000 × *g* for 20 min.

Octyl-Sepharose CL-6B chromatography. The 55% ammonium sulfate supernatant solution was applied directly to a column (2.5 × 50 cm) of Octyl-Sepharose CL-6B pre-equilibrated with 55% ammonium sulfate in 100 mM Tris-HCl (pH 8.0). After washing the column with the same buffer, the protein was eluted with a 1.6 l linear gradient from 55% to 0% ammonium sulfate in the same buffer at a flow-rate of 120 ml/h. The hydrogenase eluted as a dark green band at about 25% ammonium sulfate and was collected in 20-ml fractions. Fractions with a specific activity greater than 700 units/mg were combined and concentrated to about 20 ml in an Amicon ultrafilter fitted with a PM-30 membrane. The sample was diluted to about 200 ml with 20 mM potassium phosphate (pH 7.2)/0.1 M KCl, concentrated to 20 ml and then diluted to 800 ml with the same buffer.

Hydroxyapatite chromatography. The diluted hydrogenase solution was loaded onto a column of High Resolution Hydroxyapatite (2.5 × 45 cm) pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.2)/0.1 M KCl. The column was washed with the same buffer, and the hydrogenase was eluted at 110 ml/h with potassium phospahte in a 1.6 l linear gradient from 20 to 200 mM at pH 7.2 containing 0.1 M KCl. Fractions of 10 ml were collected with hydrogenase eluting as a dark green band at about 90 mM phosphate. Those active fractions judged homogeneous by polyacrylamide gel electrophoresis were combined and concentrated by ultrafiltration to about 4 ml, diluted to 200 ml with 50 mM Tris-HCl (pH 8.0) and then reconcentrated to about 5 mg/ml. Hydrogenase II was then rapidly frozen in liquid N_2 in the form of beads which were stored under liquid N_2 until required.

Results and Discussion

Effect of growth conditions on hydrogenase content

The specific activity of H_2 evolution (hydrogenase I) in cell extracts of *C. pasteurianum* grown with N_2 as the nitrogen source was fairly constant throughout the growth cycle (Fig. 2), whereas the specific activity of H_2 uptake (hydrogenase II) increased. The same result was obtained when cells were grown with NH_3 as the nitrogen source (Fig. 2), i.e., H_2 evolution/mg protein remained constant, whereas H_2 uptake per mg protein increased. Under N_2-fixing conditions, the H_2 uptake activity at the end of exponential growth was slightly higher (11–13 units/mg) than with NH_3 as the nitrogen source (8–10 units/mg), but the H_2 evolution activities were the same (2–4 μmol H_2 evolved/min per mg). This increase in H_2 uptake activity during growth did not correlate with the sucrose concentration, which decreased with increasing cell density (Fig. 2). H_2 uptake/mg protein of cells grown under sucrose-limiting conditions and in the presence of excess sucrose (see Materials and Methods) still increased with cell density and was slightly higher in N_2-grown cells. The H_2 evolution activity/mg protein remained constant under all conditions (data not shown). The derepression of nitrogenase activity in cells growing on limiting NH_3 also had little affect on either of the hydrogenase activities (Fig. 3). The rate of increase of the activity was slightly higher

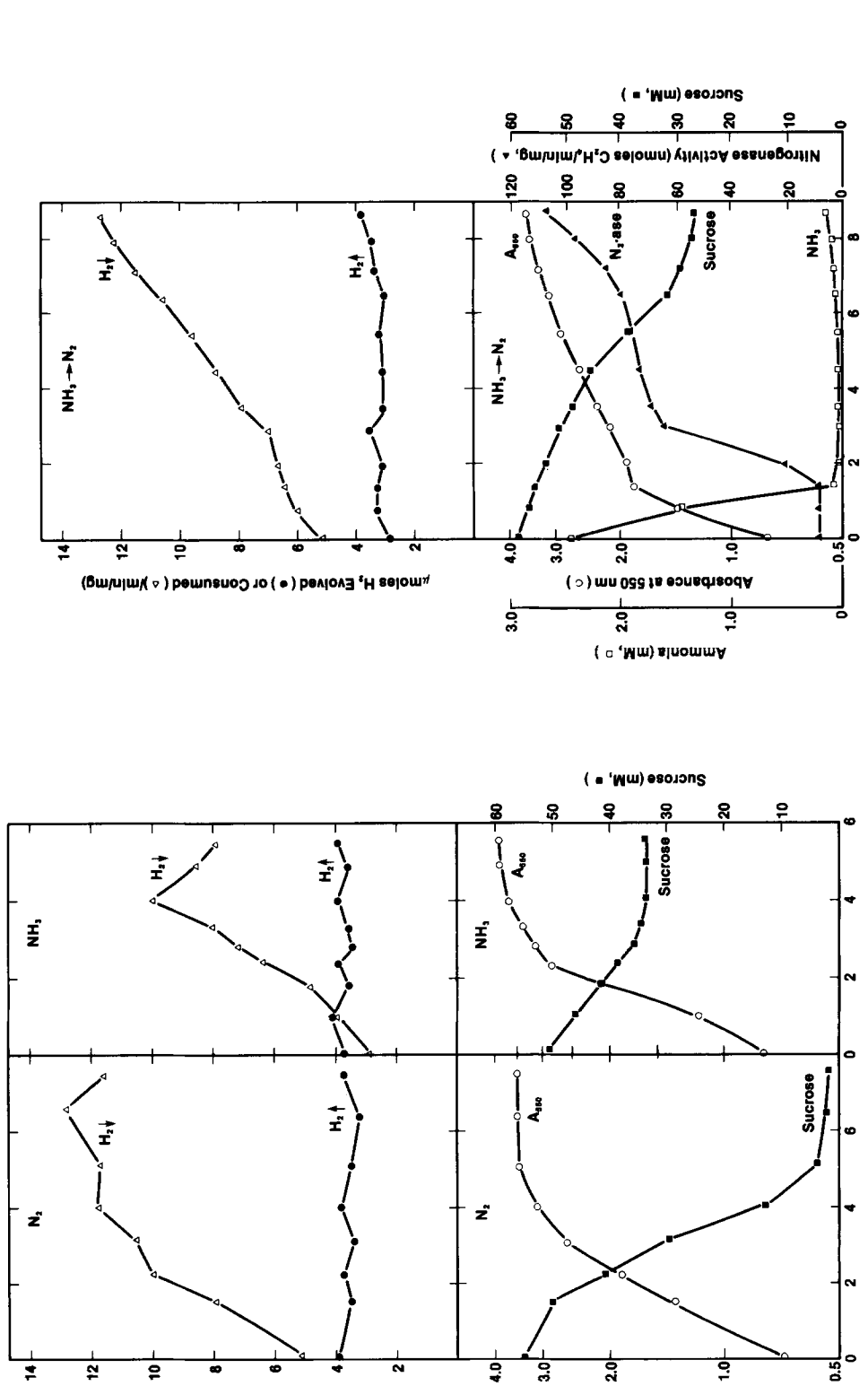

Fig. 2. H_2 uptake and H_2 evolution activities during growth on N_2 and NH_3. The growth conditions and assays were as described in the Materials and Methods section. N_2 and NH_3 indicate the nitrogen source used. $H_2\downarrow$ and $H_2\uparrow$ refer to H_2 uptake and H_2 evolution, respectively.

Fig. 3. H_2 uptake and H_2 evolution activities during derepression of nitrogenase. The growth conditions and assays were as described in the Materials and Methods section. $H_2\downarrow$, $H_2\uparrow$ and N_2-ase indicate H_2 uptake, H_2 evolution and nitrogenase activity, respectively.

after derepression of nitrogenase, indicating a sligthly higher concentration of H_2 uptake activity in N_2-fixing cells. This difference is less than that reported by Chen and co-workers [7,20], but the results do show that the highest H_2 uptake activity is found in N_2-fixing cells, harvested approximately 1 h after the end of exponential growth.

Hydrogenase II was reported to be unable to evolve H_2 from dithionite-reduced methyl viologen [7,20]. Since the H_2 evolution activity of cells remained fairly constant, independent of cell density and growth conditions, we assumed that N_2-fixing cells at the end of the log phase have the highest concentration of hydrogenase II.

Localization of hydrogenase

Attempts to release hydrogenase II preferentially from the 'periplasm' by protoplast formation or by treating intact cells with deoxycholate, high salt concentrations, pancreatin or EDTA were unsuccessful. Only deoxycholate (2%, w/v) released significant quantities of hydrogenase, but the ratio of H_2 uptake/H_2 evolution was the same as in cell-free extracts after cell breakage (see below). Thus, hydrogenase II, in the cells as we grew them, is an intracellular enzyme and for its purification, whole cells were routinely lysed by lysozyme in hypotonic buffer.

Fractionation of C. pasteurianum cell-free extracts

Previous papers detailing the fractionation of cell-free extracts of *C. pasteurianum* for the purification of hydrogenase I [5] and nitrogenase [21] relied upon an initial DEAE-cellulose column to achieve crude separation. Hydrogenase II was reported not to bind to the ion-exchanger (at pH 8.0) and was partially purified by further column chromatography [7]. We found this method to be irreproducible for hydrogenase II since: (a) a portion of the hydrogenase II did bind to the DEAE-cellulose; (b) the amount bound varied from preparation to preparation; and (c) *a* and *b* depended on the column size and the protein load. We also desired a fractionation procedure that would allow the purification of both hydrogenases (I and II), the nitrogenase proteins (MoFe and Fe proteins) and perhaps other enzymes from the same batch of cells. Hydrogenase I purification [5] involves a heat-treatment step which inactivates the nitrogenase system (Mortenson, L.E., unpublished data). We thus investigated the use of the Millipore Pellicon System to fractionate cell-free extracts, specifically as a first step in purifying hydrogenase II.

Separation of hydrogenases I and II

The filtrate from the Pellicon System operated as described (see Materials and Methods) nominally contains proteins with molecular weights less than 100 000. Extensive washing of the cell-free extract with 20 mM Tris-HCl (pH 8.0) allowed most of the hydrogenase activity to pass through the membrane, i.e., the filtrate contained 77% of the H_2 uptake activity and 74% of the H_2 evolution activity of the cell-free extract, but only 26% of the total protein (Table I). All the hydrogenase in the filtrate but only half the protein adsorbed onto the batch DE-52. The elution profile of the 0.13 M NaCl wash of the batch DE-52 packed into a column is given in Fig. 4. Since both hydrogenase I and hydrogenase II are very active in catalyzing H_2 uptake, whereas greater than 99% of the H_2 evolution activity represents only hydrogenase I, any fraction with a ratio of H_2 uptake/H_2 evolution greater than that in the cell-free extract is assumed to be enriched in hydrogenase II. For example, the first six fractions from the DE-52 column (Fig. 4) contain high H_2 uptake activity (17% of the total) but low H_2 evolution activity (4% of the total), and this corresponds to mainly hydrogenase II. The ratio of H_2 uptake/H_2 evolution is about 30 in fractions 2–6 combined compared to a ratio of about 7 in the original cell-free extract. The subsequent peak of hydrogenase activities shown in Fig. 4 is almost exclusively hydrogenase I, with an activity ratio of about 6 for fractions 7–10 combined (19% of the total). Fractions 2–6 were therefore used for the further purification of hydrogenase II (see below).

Treatment of the DE-52 column with 0.28 M NaCl released only hydrogenase I as judged by the activity ratio of about 6 (Table I). This accounts for 45% of the total H_2 evolution activity; and when combined with the hydrogenase I fractions (7–10 of Fig. 4) from the 0.13 M NaCl wash, gives a total recovery of H_2 evolution activity of 64%. The activity, 12 μmol H_2 evolved/min per mg, represents a 6-fold purification of hydrogenase I

TABLE I

H_2 UPTAKE AND H_2 EVOLUTION ACTIVITIES DURING FRACTIONATION OF CELL-FREE EXTRACTS

Step	H_2 uptake			H_2 evolution			Ratio H_2 uptake to H_2 evolution	Protein (mg)
	Activity (units)	Recovery (%)	Specific activity (units/mg)	Activity (μmol H_2/min)	Recovery (%)	Specific activity (μmol H_2/min per mg)		
Cell-free extract	1800000 [a]	100	14	269000	100	2.1	6.7	129000
Cell-free extract after filtration	420000	23	4.3	71860	26	0.7	5.8	96500
DE-52 batch supernatant	0	–	–	0	–	–	–	21700
0.13 M NaCl wash of DE-52								
Fractions 2–6	308200	17	38	10020	4	1.2	31	8100
Fractions 7–11	290800	16	86	51640	19	15	5.6	3380
0.28 M NaCl wash of DE-52	669300	37	61	120000	45	11	5.5	10900

[a] About 85% of this activity is a result of hydrogenase I. See text for details.

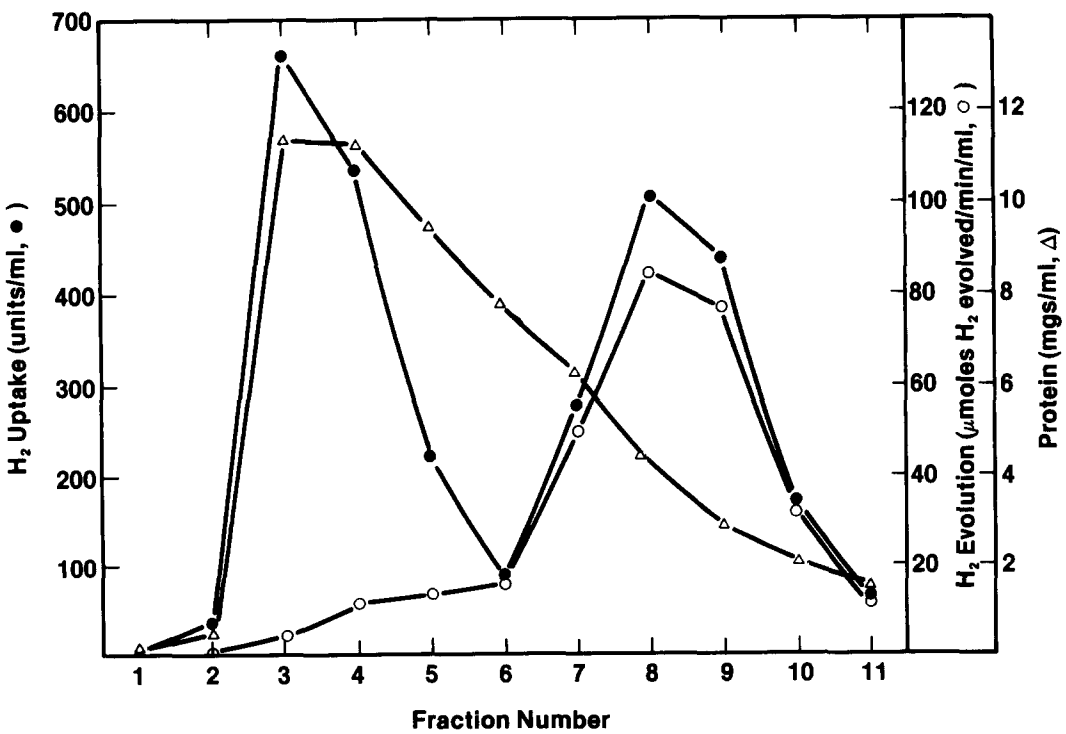

Fig. 4. Elution profile from the DE-52 column (0.13 M NaCl wash). H_2 evolution and H_2 uptake activities were determined as described in the Materials and Methods. The volume of each fraction is 100 ml.

over the cell-free extract. For comparison, in the published purification procedure for hydrogenase I [5], 63% of the activity was obtained with a specific activity of 9.4 μmol H_2 evolved/min per mg. Hydrogenase I was then further purified by the published method. Use of the Pellicon as described requires less than 90 min and shortens the purification time for hydrogenase I by 1 day. It also permits the reproducible purification of hydrogenase II, as described below.

Purification and properties of hydrogenase II

The hydrogenase in the combined fractions from the 0.13 M NaCl wash of the DE-52 column was purified by chromatography on columns of DEAE-Sepharose, Octyl-Sepharose and Hydroxyapatite. Typical results are given in Table II. The total number of units increased after ammonium sulfate fractionation (prior to Octyl-Sepharose chromatography), which is in agreement with the stimulation of the activity of the pure enzyme by high concentrations of phosphate and Tris-HCl buffers (Adams and Mortenson, unpublished data). Hydrogenase II was purified 250-fold over the cell-free extract on the basis of total H_2 uptake activity to an activity of 3000 units/mg. The enzyme did catalyze H_2 production from dithionite-reduced methyl viologen, but only at a rate of 5.9 μmol H_2 evolved/min per mg. This activity can be compared with pure hydrogenase I which, in the same assay, was 550 μmol H_2 evolved/min per mg. For pure hydrogenase II, the ratio of H_2 uptake/H_2 evolution under standard assay conditions is about 500. From Table II, it can be seen that the hydrogenase from the DEAE-Sepharose, Octyl-Sepharose and hydroxyapatite columns as well as the $(NH_4)_2SO_4$ fractionation, all have a ratio of H_2 uptake/H_2 evolution of about 500. If significant amounts of hydrogenase I were present, this ratio would certainly be expected to increase. We conclude, therefore, that hydrogenase II is essentially free of hydrogenase I contamination, even after DEAE-Sepharose chromatography and certainly after the final hydroxyapatite column. Schneider and co-workers [23] have recently shown that hydrogenase I and hydrogenase II can be separated from each other by affinity chromatography, but the two enzymes were not further purified so no conclusions regarding the relative activities of the two enzymes can be made. It can be seen, however, that even if they assayed all the hydrogenase II they purified, they would not readily detect the small amount of H_2-evolution activity in hydrogenase II.

The hydrogenase II activity eluting from the hydroxyapatite column gave only one protein band after gel electrophoresis under nondenaturing conditions, coincidental with one band of hydrogenase activity (H_2 uptake). A single protein band also was observed after electrophoresis in the presence of sodium dodecyl sulfate, which corresponded to an estimated molecular weight of 53000 ± 3000 (values from four determinations) when compared to marker proteins. The hydrogenase activity eluted from a calibrated column of Sephacryl S-200 with an apparent molecular weight

TABLE II

PURIFICATION OF HYDROGENASE II

The values in parentheses were calculated on the basis of the hydrogenase II content of the cell-free extract (270000 units, see text for details).

Step	H_2 uptake activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (-fold)	Ratio H_2 uptake to H_2 evolution
Cell-free extract	1773800	145400	12 (1.8)	1	6
DE-52 (0.13 M)	374000	8910	42	3 (23)	28
DEAE-Sepharose	227000	1350	169	14 (94)	494
55% $(NH_4)_2SO_4$	231000	730	316	26 (175)	505
Octyl-Sepharose	151000	96	1580	132 (880)	505
Hydroxyapatite	114000	38	2998	246 (1700)	508

of 50000 ± 4000 (values from three determinations). Hydrogenase II is thus a monomeric protein with a molecular weight of approx. 53000. Analysis of the enzyme for iron and acid-labile sulfide gave values of 8.05 ± 0.31 mol of iron and 8.11 ± 0.43 mol inorganic sulfur per mol protein (values from three different hydrogenase preparations). Hydrogenase II is therefore distinct from hydrogenase I, which is a monomeric protein of molecular weight 60000 containing 12 iron and 12 acid-labile sulfide atoms per molecule [5].

Cellular content of hydrogenase II

The fractionation of cell-free extracts with the Pellicon System is very reproducible, e.g., Tables I and II represent different preparations, and we routinely obtain yields of pure hydrogenase II of about 35 mg. The latter results were obtained with fast-growing cells harvested within 1 h after the end of exponential growth and stored frozen in liquid N_2 for 1 month or less. If (a) the chemostat contained aged or slow growing cells (as the inoculum), (b) cell lysis had begun before harvesting or (c) frozen cells older than 1 month were used, the yield of hydrogenase II (after the DEAE-Sepharose column) could be 25% or less of that indicated in Table II. This was in spite of the fact that the same cells contained 'normal' hydrogenase I activity. The reason(s) for the apparent loss of hydrogenase II activity is not known at present, but these observations help to explain the failure of some earlier attempts to purify the enzyme.

The question arises as to the true cellular content of hydrogenase II in 'healthy' cells. Pure hydrogenase I has a specific activity in the H_2 uptake assay of 4700 units/mg (Adams and Mortenson, unpublished data). Using the H_2 evolution activity data of Chen and Mortenson [5] to calculate the total amount of hydrogenase I in whole cells, one finds that hydrogenase I more than accounts for the total H_2 uptake activity observed (Table I). This can be explained by the fact that the H_2 evolution assay is not an accurate measure of total hydrogenase I in crude cell-free extracts because of the presence of ferredoxin (and perhaps other effector molecules) which stimulates hydrogenase I-catalyzed H_2 evolution from reduced methyl viologen [5]. However, one may conclude that the contribution of hydrogenase II to the total measured H_2 uptake activity of cells is low, and this is supported by the data shown in Table II. The fractions from the DEAE-Sepharose column contain little hydrogenase I and represent 13% of the total H_2 uptake units. Table I shows that 93% of the total H_2 uptake units can be accounted for in the various fractions. Therefore, with the assumption that there is no influence from effector molecules, hydrogenase II constitutes probably about 15% of the total H_2 uptake activity in N_2-grown cells. On this basis, the cell-free extract contained about 270000 units of hydrogenase II-catalyzed H_2 uptake activity and, from Table II, hydrogenase II was purified 1700-fold over the crude extract with a yield of activity of about 40%.

The presence of hydrogenase II, which preferentially catalyzes H_2 oxidation, in an organism that produces vast quantities of H_2 during normal fermentative metabolism is at present a mystery. It would seem unlikely that hydrogenase II functions intimately with the nitrogenase system to increase the efficiency of N_2 fixation, a situation analogous to that proposed in aerobic N_2-fixing bacteria (see Ref. 4) since (a) the H_2 uptake activity and the kinetics of this activity are similar in N_2-grown and NH_3-grown cells, (b) derepression of nitrogenase has little effect and (c) *C. pasteurianum* has no known electron transport coupled to ATP generation or any obvious need to reduce O_2. The large increase in H_2 uptake activity during logarithmic growth of *C. pasteurianum* is unusual, and a physiological function for this increase has not yet been discovered.

Purification of proteins other than hydrogenase

Both the MoFe protein and Fe protein of the nitrogenase system were detected only in those fractions with molecular weight greater than 100000, i.e., in the cell-free extract after removing proteins with $M_r < 100000$. This was expected for the MoFe protein (M_r 220000 [21]) but not for the Fe protein (M_r 59700 [22]). The specific activity of nitrogenase (nmol C_2H_4 produced/min per mg) of the cell-free extract increased 1.6-fold after filtration because contaminating protein was removed, but the total activity remained the same. This recovery of the extremely O_2-sensitive nitrogenase proteins [21] shows that the Pellicon

System can be kept completely anaerobic. The MoFe and Fe proteins were further purified to homogeneity by column chromatography (Bare and Mortenson, unpublished data).

Ferredoxin was removed from the cell-free extract in the $M_r < 100\,000$ fraction. This was surprising in view of the fact that it is a strongly acidic protein [13] and might be expected to bind to proteins in the $M_r > 100\,000$ fraction. Ferredoxin was removed from the DE-52 column with 0.5 M NaCl and was further purified by an established method [13]. Operation of the Pellicon System as described can also be used as a first step in the purification of formate dehydrogenase (Lui and Mortenson, unpublished data) and the molybdenum storage/binding protein (Hinton and Mortenson, unpublished data). The Millipore Pellicon System is thus an extremely useful method of fractionating cell-free extracts, since the nondenaturing conditions enable the purification from the same cell extracts of several enzymes in addition to the two hydrogenases.

Acknowledgement

The excellent technical assistance of Ms. Judy Kimak-Savas is gratefully acknowledged.

References

1 Stephenson, M. and Stickland, L.H. (1931) Biochem. J. 25, 205–241
2 Mortenson, L.E. and Chen, J.-S. (1974) in Microbial Iron Metabolism (Nielands, J.B., ed.), pp. 231–282, Academic Press, New York
3 Schlegel, H.G. and Schneider, K. (eds.) (1978) Hydrogenases, Their Catalytic Activity, Structure and Function, E. Goltze, Göttingen.
4 Adams, M.W.W., Mortenson, L.E. and Chen, J.-S. (1981) Biochim. Biophys. Acta 594, 105–176
5 Chen, J.-S. and Mortenson, L.E. (1974) Biochim. Biophys. Acta 371, 283–298
6 Chen, J.-S., Mortenson, L.E. and Palmer, G. (1976) in: Iron and Copper Proteins (Yasunobu, K.T., Mower, H.F. and Hoyaishi, O., eds.), pp. 68–82, Plenum Press, New York
7 Chen, J.-S. and Blanchard, D.K. (1978) Biochem. Biophys. Res. Commun. 84, 114–1150
8 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
9 Adams, M.W.W. and Hall, D.O. (1979) Biochem. J. 183, 11–22
10 Weber, K., Pringle, J.R. and Osborn, M. (1972) Methods Enzymol. 26, 2–27
11 Lovenberg, W., Buchanan, B.B. and Rabinowitz, J.C. (1963) J. Biol. Chem. 238, 3899–3913
12 Chen, J.-S. and Mortenson, L.E. (1977) Anal. Biochem. 79, 157–165
13 Mortenson, L.E. (1964) Biochim. Biophys. Acta 81, 71–77
14 Seto, B.L. and Mortenson, L.E. (1974) J. Bacteriol. 120, 822–830
15 Daesch, G. and Mortenson, L.E. (1972) J. Bacteriol. 110, 103–109
16 Upchurch, R.G. and Mortenson, L.E. (1980) J. Bacteriol. 143, 274–289
17 Ashwell, G. (1957) Methods Enzymol. 3, 73–107
18 Chaney, A.L. and Marbach, E.P. (1962) Clin. Chem. 8, 130–132
19 Clarke, D.J., Fuller, F.M. and Morris, J.G. (1979) Eur. J. Biochem. 98, 597–612
20 Chen, J.-S. (1978) in Hydrogenases, Their Catalytic Activity, Structure and Function (Schlegel, H.G. and Schneider, K., eds.), pp. 57–82, E. Goltze, Göttingen.
21 Zumft, W.G. and Mortenson, L.E. (1973) Eur. J. Biochem. 35, 401–409
22 Tanaka, M., Haniu, M., Yasunobu, K.T. and Mortenson, L.E. (1977) J. Biol. Chem. 252, 7081–7100
23 Schneider, K., Pinkwart, M. and Jochim, K. (1983) Biochem. J. 213, 391–398
24 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275