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Purification and Characterization of Two Forms of Hydrogenase Isoenzyme 1 from *Escherichia coli*†

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A hydrogenase associated with dihydrogen uptake (HUP hydrogenase) was purified from an *Escherichia coli* mutant (strain SE1100) defective in utilization of molybdate and thus fermentative dihydrogen production. This protein had two subunits with apparent molecular weights of 59,000 and 28,000 (form 1). An immunologically cross-reactive hydrogenase was also purified from *E. coli* K10 grown in glucose-minimal medium and harvested at the mid-exponential phase of growth. Upon purification to homogeneity, this hydrogenase contained only one subunit with an apparent molecular weight of 59,000 (form 2). The two forms of the HUP hydrogenase exhibited similar kinetic characteristics. The electrophoretic properties of the enzyme and its response to pH suggest that this HUP hydrogenase is the HYD1 isoenzyme. The HYD1 isoenzyme was the only hydrogenase detectable during the stationary phase of growth in *E. coli* grown in Mo-deficient medium.

Under anaerobic culture conditions, *Escherichia coli* synthesizes three membrane-associated hydrogenase isoenzymes, which catalyze either uptake or evolution of dihydrogen (4, 27, 28). Hydrogenase 1 (HYD1) and an active fragment of hydrogenase 2 (HYD2) have been purified to homogeneity and biochemically characterized (4, 28). These two enzymes, although very similar in biochemical properties, did not cross-react immunologically. Using this immunological difference, Sawers et al. (27) investigated the potential physiological role of the two enzymes by monitoring the induction and repression of the enzymes in cells cultured under different growth conditions. They postulated that HYD2 was involved in H_2 uptake (HUP). Since the conditions that favored the production of HYD3 also stimulated the formate hydrogenlyase (FHL) activity, it was presumed that the HYD3 is responsible for dihydrogen production in whole cells (27). However, HYD1 responded to both dihydrogen evolution and uptake conditions, indicating a potential role in both evolution and uptake of dihydrogen.

We studied the physiological role of a specific hydrogenase isoenzyme by purifying the enzyme from appropriate mutant strains that produced only dihydrogen evolution or uptake activity. We have isolated and described a mutant strain of *E. coli*, SE1100 (*molR*), that lacks hydrogen evolution activity when grown in a medium devoid of molybdate (21). This mutant strain is normal for HUP activity. A hydrogenase associated with this HUP activity was purified to homogeneity from cells grown in Mo-deficient medium. Upon purification, this hydrogenase yielded a protein with two polypeptides. By using modified growth and purification procedures, the same hydrogenase, with only the large subunit, was also isolated from *E. coli* K-12 strain K10 (wild type for H_2 metabolism). In this report, we describe the biochemical properties of the two forms of the HUP hydrogenase from *E. coli*.

MATERIALS AND METHODS

Culture of bacterial cells. *E. coli* K-12 strain SE1100 [Δ(*lacU*)*169 rpsL* Φ(*molR-lacZ*$^+$)] was cultured in a Mo-deficient medium with the following composition: Na_2HPO_4, 14.9 g; KH_2PO_4, 6.1 g; NaCl, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 1.0 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $NiCl_2 \cdot 6H_2O$, 1.18 mg; yeast extract, 10.0 g; glucose, 10.0 g; and H_2O, 1.0 liter. A culture grown in the same medium was used as the inoculum at a final concentration of 1.0%. Cells were grown at 37°C under an atmosphere of N_2 and harvested after 16 h.

E. coli K-12 strain K10 (Hfr P02A *relA1 pit-10 tonA22* T2^r λ$^+$ *spoT*) was cultured at room temperature in minimal medium (21) supplemented with glucose (3%) and Casamino Acids (0.1%; Difco Laboratories) (P. Patel, Ph.D. thesis, University of Florida, Gainesville, 1985). An overnight culture grown anaerobically in the same medium was used as the inoculum at a final concentration of 10%. The cells were harvested immediately after detection of H_2 gas produced by the organism.

Purification of hydrogenase from strain SE1100. Frozen cell paste (100 g [wet weight]) was suspended in 1 liter of phosphate buffer (0.1 M, pH 7.0) containing EDTA (10 mM), reduced glutathione (1 mM), and the protease inhibitors benzamidine and phenylmethylsulfonyl fluoride (1 mM each). The cells were lysed by treatment with 3.5 mg of lysozyme per ml and 10 mM EDTA. The suspension was incubated at 37°C with constant shaking (200 rpm) under a gas phase of H_2. After 1 h, $MgCl_2$ (10 mM) and DNase and RNase (100 mg each) were added to reduce the viscosity, and the suspension was incubated for 1 h at 37°C with shaking. The cell suspension was sonicated for 3 min in three cycles of 1 min each with intermittent cooling in the presence of sodium dithionite (10 mM). From this step on, all buffer solutions contained dithionite (10 mM) and all operations were carried out at 4°C unless otherwise indicated. Unlysed cells and cell debris were removed by centrifugation at 600 × *g* for 5 min. The supernatant was centrifuged at 15,000 × *g* for 30 min, and the pellet was suspended in phosphate buffer

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(0.1 M, pH 7.0) containing dithionite (10 mM), reduced glutathione (1 mM), and NaCl (150 mM) to a protein concentration of 5 mg/ml; hydrogenase was purified from this fraction.

Membranes were solubilized by the procedure of Bordier (6). Precondensed Triton X-114 solution (15%) was slowly added to the sample with constant stirring to a final concentration of 2%. This mixture was stirred for 30 min at 4°C and then centrifuged at 15,000 × *g* for 45 min. The supernatant was overlayed on a 50-ml cushion containing 0.25 M sucrose in phosphate buffer (0.1 M, pH 7.0) and incubated at 30°C for 30 min. During this period, as the temperature of the solution increased, Triton X-114 micelles enclosing hydrophobic proteins including hydrogenase formed; they were collected by centrifugation at 3,000 × *g* for 15 min at 25°C. After centrifugation, three distinct layers were observed: lower brownish Triton X-114 micelles and middle sucrose and upper aqueous layers. The top aqueous and middle sucrose layers lacking hydrogenase activity were discarded. The bottom detergent layer (ca. 75 ml), which contained hydrogenase activity, was diluted to 500 ml with phosphate buffer (0.1 M, pH 7.0) and incubated at 4°C to dissolve the micelles. Polyethylene glycol (PEG) 6000 was added to this sample to a final concentration of 35% saturation by addition of 100% saturated PEG in the same buffer. The PEG-containing solution was incubated overnight at 4°C with constant mixing in a closed container to prevent O_2 diffusion into the solution. The sample was centrifuged at 15,000 × *g* for 30 min, and the pellet containing hydrogenase was redissolved in 300 ml of phosphate buffer (0.1 M, pH 7.0). Solid ammonium sulfate crystals were added to this solution to a final concentration of 20% saturation. The PEG and ammonium sulfate precipitation steps removed most of the Triton X-114 associated with the sample and some of the lipoproteins. The hydrogenase, which precipitated at this concentration, was collected by centrifugation at 15,000 × *g* for 45 min and suspended in 100 ml of Tris hydrochloride buffer (10 mM, pH 8.0) with 0.01% sodium deoxycholate and dialyzed overnight against two changes of the same buffer (3.5 liters each). We found that the hydrogenase–Triton X-114 complex did not bind efficiently to DEAE-cellulose. However, the hydrogenase-deoxycholate complex did bind to this weak anion exchanger. This necessitated exchange of the Triton X-114 with deoxycholate (26).

An octyl-Sepharose CL-4B column (50 by 150 mm; Pharmacia) was equilibrated with the Tris-deoxycholate dialysis buffer to saturate the octyl-Sepharose beads with deoxycholate. The dialyzed hydrogenase was loaded on the column and washed with the same low-salt buffer. Hydrogenase, which did not bind to the matrix at this low molarity of salt, was collected as a deoxycholate complex. Since Triton X-114 absorbs strongly in the UV region, this exchange was followed by a decrease in A_{280}. The eluant was passed through a DEAE-cellulose column (TSK, DEAE-5PW, LKB Glaspac column connected to an LKB high-performance liquid chromatograph) preequilibrated with the Tris-deoxycholate buffer. The column was washed with a 50 to 150 mM NaCl linear gradient, and hydrogenase eluted at 90 mM NaCl as one peak. All fractions containing maximum hydrogenase activity were pooled and dialyzed overnight against phosphate buffer (20 mM, pH 7.0) containing 100 mM ammonium sulfate. After dialysis, the protein was further purified by hydrophobic interaction chromatography in an octyl-Sepharose CL-4B column (2.5 by 10 cm; Pharmacia) and eluted with a linear gradient, initially with decreasing ammonium sulfate and increasing octylglucopyranoside (0.3% in 10 mM phosphate buffer, pH 7.0). The detergent was changed to octylglucopyranoside because of the ease with which this detergent can be removed from protein by dialysis. Fractions with hydrogenase activity were pooled and dialyzed overnight against 1-methylpiperazine buffer (0.025 M, pH 5.7) containing 0.01% octylglucopyranoside. After dialysis, the protein solution was applied to a chromatofocusing column (Mono P; 5 by 20 mm; Pharmacia-LKB) preequilibrated with the dialysis buffer and eluted by a pH gradient generated by polybuffer 74 (pH 4.0; Pharmacia-LKB), at 1/10 concentration containing 0.01% octylglucopyranoside and 1 mM dithiothreitol. In this step, the buffer was devoid of dithionite since it precipitated at this low pH, and dithiothreitol was substituted. Fractions of 0.5 ml were collected in tubes containing 0.25 ml of 1.0 M phosphate buffer (pH 7.0) with 10 mM dithionite so as to rapidly raise the pH of the eluant to a neutral value. The fractions with high hydrogenase activity were tested for purity by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (18). Peak fractions that contained pure protein were pooled and concentrated to a small volume and stored in phosphate buffer (0.05 M, pH 7.0) with dithionite (10 mM) at −85°C under dihydrogen.

Purification of hydrogenase from *E. coli* K10. Cells (100 g) were lysed by the lysozyme-EDTA procedure as described earlier for strain SE1100. After lysis and removal of unbroken cells and cell debris, membrane vesicles were obtained after centrifugation at 100,000 × *g* for 1 h at 4°C. The pellet was suspended in 0.1 M phosphate buffer, pH 7.0. The membranes were solubilized with the nonionic detergent Triton X-100 at a final concentration of 1.0% for 1 h at 4°C. After centrifugation at 100,000 × *g* for 1 h at 4°C, the Triton X-100-solubilized membrane fraction was treated with PEG 8000 (12) (this batch of PEG 8000 was later renamed by Sigma Chemical Co. as PEG 6000). The pellet was suspended in 500 ml of phosphate buffer (0.1 M, pH 7.0), and the hydrogenase was precipitated with ammonium sulfate. The hydrogenase-containing fraction, obtained between 25 to 60% $(NH_4)_2SO_4$ saturation, was suspended in 200 ml of Tricine buffer (0.01 M, pH 8.0), containing NaCl (130 mM) and Triton X-100 (1.0%) and dialyzed against 6 liters of the same buffer for 6 h. The dialyzed extract was loaded on a DEAE-cellulose column (2.8 by 90 cm) equilibrated with the same buffer. Hydrogenase was eluted with a linear gradient of 150 to 225 mM NaCl in a volume of 2 liters. Several peaks of activity were detected; the fractions containing the highest activity were pooled, and the free Triton X-100 was removed by dialysis in 12 liters of phosphate buffer (10 mM, pH 7.0) containing 100 mM $(NH_4)_2SO_4$. After dialysis for about 16 h, the remaining traces of the detergent were removed by adding Bio-Beads SM-2 (Bio-Rad Laboratories) to the dialysate and incubating the mixture for 2 h with gentle mixing (25). This step was followed by chromatography in an octyl-Sepharose column (2.8 by 40 cm), and the hydrogenase was eluted by a detergent gradient (0.5% sodium deoxycholate–0.05% Triton X-100). The hydrogenase eluted as a single peak at 0.225% deoxycholate and 0.0225% Triton X-100. Fractions containing hydrogenase activity were dialyzed overnight in histidine hydrochloride buffer (25 mM, pH 5.5) containing Triton X-100 (0.3%) and then subjected to chromatofocusing in a Poly Buffer Exchanger (Pharmacia-LKB) column (1.5 by 45 cm). Fractions containing the highest hydrogenase activity were pooled and dialyzed against 6 liters of phosphate buffer (10 mM, pH 7.0) containing NaCl (50 mM) and Triton X-100 (0.3%). Hydrogenase was purified to homogeneity with a second anion-exchange

chromatography step at pH 7.0. A DEAE-cellulose column (1.2 by 30 cm) equilibrated with phosphate buffer (10 mM, pH 7.0) and containing NaCl (50 mM) was used at this step, and hydrogenase was eluted as a single protein with a linear gradient of 50 to 125 mM NaCl in phosphate buffer (10 mM, pH 7.0). Fractions with maximum hydrogenase activity were analyzed for purity, using 12% SDS-PAGE gels.

Enzyme assay. Hydrogenase activity was determined by measuring HUP, dihydrogen evolution, and tritium-exchange reactions (20, 21). HUP activity was determined spectrophotometrically by dye reduction, using an SLM-Aminco DW-2C spectrophotometer. The reaction was carried out anaerobically in a total volume of 3 ml containing 100 mM phosphate buffer (pH 7.0) and electron acceptors at concentrations described for each experiment in a test tube (12 by 75 mm) sealed with a serum stopper. The gas phase in the tubes was replaced with dihydrogen. The reaction was initiated by adding hydrogenase (2 μg in a volume of 50 μl), which was maintained under dihydrogen, at 0°C. The tube contents were mixed, and reduction of the electron acceptors was measured at the appropriate wavelengths. The electron acceptors used and their extinction coefficients were as follows: methyl viologen (MV), 12,000 M^{-1} cm^{-1} at 600 nm; benzyl viologen (BV), 7,780 M^{-1} cm^{-1} at 550 nm; neutral red, 7,600 M^{-1} cm^{-1} at 450 nm; methylene blue, 7,000 M^{-1} cm^{-1} at 601 nm; and potassium ferricyanide, 13,200 M^{-1} cm^{-1} at 405 nm.

Dihydrogen evolution was measured polarographically, using a dihydrogen electrode (32) or a gas chromatograph (Varian model 920) fitted with a molecular sieve 0.5-nm column as described previously (20). The reaction mixture in a total volume of 1 ml contained phosphate buffer (50 mM, pH 7.0), dithionite (10 mM), MV (5 mM), and hydrogenase (2 μg).

The tritium-exchange reaction was carried out in test tubes (12 by 75 mm) containing phosphate buffer (50 mM, pH 7.0), dithionite (1 mM), and hydrogenase (2 μg) in a final volume of 0.1 ml. The tubes were sealed with serum stoppers and filled with helium after degassing. Dihydrogen gas (800 μl) and tritium gas (25 μl) (11.2 mCi/mmol; Dupont, NEN Research Products) were added to each tube. After 1 h of incubation at the desired temperature, the serum stopper was removed and the gas was vented. After 10 min, 0.05 ml of the assay mixture was added to 2.5 ml of scintillation fluid, and the amount of ^3H_2O produced was determined in a scintillation counter.

Analytical methods. Total nonheme iron and acid-labile sulfur contents of the proteins were determined by using published procedures (17, 22). The effect of pH on hydrogenase activity was determined in three-component, constant-ionic-strength buffer systems (10), ranging from 4 to 10. Protein was estimated by the BCA method (30) or Coomassie blue procedure (7). Nondenaturing PAGE was carried out in 7.5% polyacrylamide gel, in either tubes or slabs (8, 23), with 0.1% Triton X-100 in both the gel and the buffers. Hydrogenase was visualized by using H_2-dependent reduction of BV by immersing the gel in a mixture containing phosphate buffer (0.1 M, pH 7.0), cysteine (1 mM), BV (1 mg/ml), and triphenyl tetrazolium chloride (1 mg/ml). The gas phase was replaced with H_2. SDS-PAGE was carried out in 12.0% polyacrylamide as described by Laemmli (18). The N-terminal amino acid sequence was determined by the Protein Chemistry Core Facility, Interdisciplinary Center for Biotechnology Research, University of Florida, using an Applied Biosystems model 470A Gas Phase Protein Sequenator.

TABLE 1. Isolation of HUP hydrogenase from *E. coli* SE1100

Fraction	Total protein (mg)	Total activity[a]	Sp act[b]	Yield (%)	Purification (fold)
Extract	4,462	4,908	1.1	100	1.0
Triton X-114	528	2,217	4.2	45.2	3.8
PEG pellet	450	2,205	4.9	44.6	4.4
20% $(NH_4)_2SO_4$ precipitate	290	2,001	6.9	40.8	6.2
Octyl-Sepharose (low ionic strength)	262	1,860	7.1	37.5	6.4
DEAE-cellulose	25	1,495	59.8	30.4	54.4
Octyl-Sepharose (hydrophobic chromatography)	16.3	997	61.2	20.1	55.7
Chromatofocusing	0.12	173	1,445.0	3.5	1,313.0

[a] Expressed as micromoles of ^3H_2O produced per hour.
[b] Expressed as micromoles of ^3H_2O produced per hour per milligram of protein.

Molecular weight determination. The native molecular weights of the proteins were determined by pore-gradient gel electrophoresis (11, 15) and by gel filtration (9) in both the presence and absence of Triton X-100 (0.1%). Subunit molecular weight was determined by SDS-PAGE (18), and the gels were stained with silver stain (SE1100; Bio-Rad) or Coomassie blue (K10).

Monoclonal antibody preparation. Hydrogenase purified from strain SE1100 was used as antigen in the production of antihydrogenase antibodies by the Hybridoma Core Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida. BALB/c mice were immunized with 50 μg of protein in phosphate-buffered saline mixed with an equal volume of Ribi adjuvant system (trehalose dimycolate and monophosphoryl lipid A; Ribi Immunochem Research Inc., Hamilton, Mont.). After 2 weeks, the mice were immunized again with the same antigen-adjuvant mixture at a concentration of 25 μg of protein per mouse. After construction and identification, several hybridoma clones producing antihydrogenase antibodies were selected and tested for their ability to react in enzyme-linked immunosorbent assay and Western immunoblot procedures. Supernatants of one clone each which reacted with either large or small subunit in a Western blot were selected and pooled for use in the experiments. The same clones were also used to prepare ascites fluid containing antihydrogenase antibodies.

RESULTS

Purification of hydrogenase from strain SE1100. The steps involved in the purification of hydrogenases to homogeneity from *E. coli* SE1100 and K10 are presented in Tables 1 and 2, respectively. The yield of hydrogenase isolated from strain SE1100 was 173 U from an initial amount of 4,908 U. This yield of 3.5% represents the amount of homogeneous protein obtained after the first chromatofocusing step. Repeated dialysis and chromatofocusing increased the yield of pure hydrogenase to about 10%. This membrane-associated enzyme was not solubilized fully by any of the detergents examined {3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate, laurylsarcosinate, deoxycholate, Nonidet, Tween 80, and Triton X-100}. Triton X-114 was used in the experiments involving strain SE1100, since it presented the advantage of excluding most of the hydrophilic proteins in a single initial step by trapping the hydrogenase by a temperature-induced phase transition. This procedure also elimi-

TABLE 2. Isolation of HUP hydrogenase from *E. coli* K10

Fraction	Total protein (mg)	Total activity[a]	Sp act[a]	Yield (%)	Purification (fold)
Membranes	16,369	68,095	4.2	100	1.0
Triton X-100	8,977	37,613	4.2	55	1.0
PEG pellet	2,921	18,519	6.3	26	1.5
25% $(NH_4)_2SO_4$ supernatant	2,906	17,377	6.0	25	1.4
25–60% $(NH_4)_2SO_4$ precipitate	650	18,668	28.7	27	6.9
DEAE-cellulose (pH 8.0)	15.4	1,999	129.5	4.9	31.0
Octyl-Sepharose	10.6	2,392	225.7	3.5	54.0
Chromatofocusing	3.4	2,866	843.0	4.2	204.0
DEAE-cellulose (pH 7.0)	0.73	1,198	1,642.0	1.8	394.0

[a] Expressed as in Table 1.

nated the need for preparing pure membrane vesicles, since only highly hydrophobic proteins were trapped as part of the Triton X-114 vesicles. One disadvantage of this method was the difficulty encountered in removing the Triton from the hydrogenase.

Although a significant amount of Triton X-114 was removed in the two precipitation steps (PEG and ammonium sulfate), the sample still contained considerable amounts of Triton X-114, as indicated by the high viscosity of the solution. Extensive dialysis did not appreciably remove the detergent. After the Triton X-114 was exchanged with deoxycholate, the hydrogenase was eluted as a sharp peak from the DEAE-cellulose column at a concentration of about 90 mM NaCl. When pooled, fractions containing the highest activity yielded a 54-fold increase in the specific activity, with 30% recovery.

The hydrogenase was purified to homogeneity by hydrophobic interaction chromatography followed by chromatofocusing. Octyl-Sepharose chromatography did not increase the specific activity of the enzyme; however, it was necessary for purification of the protein to homogeneity in the next step. This step also served to exchange the detergent from deoxycholate to octylglucopyranoside. Proteins bound with deoxycholate failed to bind to the Mono P column used for chromatofocusing. Also, octylglucoside can be readily removed from the protein by dialysis to obtain a detergent-free protein for biochemical analysis. The final chromatofocusing step yielded a homogeneous hydrogenase preparation, as demonstrated by SDS-PAGE analysis (Fig. 1). At this final stage, the specific activity of the enzyme was 1,445 U.

Purification of hydrogenase from strain K10. When solubilized with Triton X-100, the membranes obtained from strain K10 released about 55% of the total hydrogenase activity. The fractions with the highest hydrogenase activity from the DEAE-cellulose chromatography step amounted to only about 10% of the total activity that was passed through the DEAE-cellulose column, yielding a fivefold purification from the previous step. During the first three columns in the purification steps, the total hydrogenase activity stayed constant, with an increase in specific activity. The hydrogenase obtained after the chromatofocusing step still contained contaminating proteins and required an additional DEAE-cellulose chromatographic step at pH 7.0. Hydrogenase, which eluted at 90 mM NaCl, was homogeneous as determined by SDS-PAGE (Fig. 1) and was enriched by a factor of 394. The specific activity of this preparation was 1,642 U and represented 1.8% of the starting hydrogenase activity.

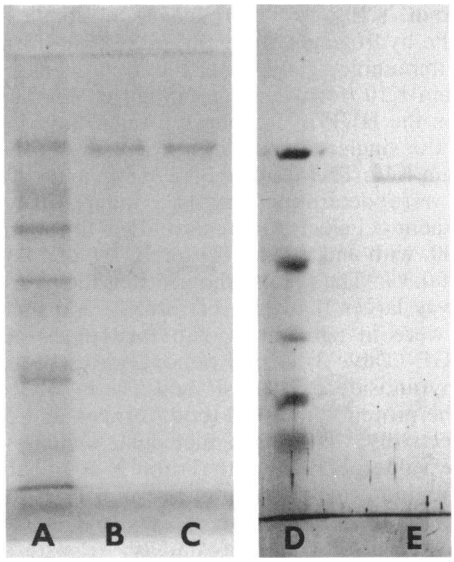

FIG. 1. SDS-PAGE analysis of the hydrogenase purified from *E. coli* SE1100 and K10. Lanes: A, molecular size markers (top to bottom, 66, 45, 36, 29, 24, 20.1, and 14.2 kilodaltons); B and C, hydrogenase from strain SE1100, two different samples; D, molecular size markers (top to bottom, 66, 45, 36, 29, and 24 kilodaltons); E, hydrogenase from strain K10 (2 μg of protein).

Physical properties. Upon electrophoresis in nondenaturing (native) polyacrylamide gels, the hydrogenase purified from strains K10 and SE1100 produced one band when assayed for activity or protein. Under denaturing PAGE (0.1% SDS), the hydrogenase purified from strain SE1100 resolved into two polypeptides with apparent molecular weights of 59,000 and 28,000 (Fig. 1; Table 3). Under the same analytical conditions, only one protein band with a molecular weight of 59,000 was observed in the hydrogenase

TABLE 3. Physical properties of the two forms of HUP hydrogenase

Source of hydrogenase	Native mol wt (10^3)				Subunit mol wt (10^3)[a]	Nonheme iron[b]	Acid-labile sulfur[b]	pH optimum	Half-life in air (h)	Activation energy (kJ mol^{-1})	
	Pore-gradient electrophoresis		Gel filtration								
	−Triton X-100	+Triton X-100	−Triton X-100	+Triton X-100						Below 20°C	Above 20°C
SE1100 (form 1)	175	90	180	125	59, 28	5.9 ± 0.3	5.8 ± 0.2	Broad (6–8)	6.0	161	19
K10 (form 2)	115	58	85	58	59	4.0 ± 0.5	4.7 ± 0.5	7.0	10.0	82	15

[a] Determined by SDS-PAGE.
[b] Atoms per molecule of native hydrogenase.

from strain K10. Antihydrogenase antibodies prepared against the hydrogenase from strain SE1100 (form 1) cross-reacted immunologically with the hydrogenase prepared from strain K10 (form 2), suggesting that the large subunit present in the HUP hydrogenase from strain SE1100 is the same as the single polypeptide present in the hydrogenase from strain K10. The native molecular weights of the hydrogenases were determined by two independent methods: pore-gradient gel electrophoresis and gel filtration in Sephadex G-200, with and without Triton X-100 at a final concentration of 0.3%. The results showed that the hydrogenase of form 1 was larger than that of form 2, and the molecular weights were in agreement with the values obtained by SDS-PAGE (Table 3). Upon removal of the detergent (octylglucopyranoside by dialysis and Triton with Bio-Beads SM-2), the protein dimerized (both forms 1 and 2; $\alpha_2\beta_2$ and α_2, respectively), yielding a molecular weight which was twice the value obtained with Triton X-100 (Table 3). The order of amino acids in the amino-terminal part of the large subunit from form 1 enzyme is Ser-Thr-Gln-Tyr-Glu-Thr-Gln-Gly-Tyr-Thr-Ile-Asn-Asn-Ala-Gly. . ., with serine representing the amino-terminal end. The order of amino acids in the amino-terminal end of the small subunit of form 1 enzyme obtained after SDS-PAGE is Leu-Glu-Asn-Lys-Pro-Arg-Ile-Pro-Val-Val. . ., and the N-terminal amino acid is leucine.

Analysis of nonheme iron content of the purified hydrogenase revealed values of 5.9 ± 0.3 and 4.0 ± 0.2 atoms per molecule for form 1 and form 2, respectively. The acid-labile sulfide was determined to be 5.8 ± 0.2 for form 1 and 4.7 ± 0.5 for form 2 of the HUP hydrogenase. These values represent the amount present in atoms per molecule of native hydrogenase (in the presence of detergent) on the basis of the protein values obtained with either Coomassie blue or BCA reagent. The purified protein was O_2 sensitive, and the activity declined in air, with half-lives of about 6 and 10 h for form 1 and form 2 enzymes, respectively. The original activity was maintained for over 3 weeks at 4°C under an H_2 atmosphere with added dithionite (1 mM). Storing the protein immediately after purification at −85°C prolonged enzyme activity.

The temperature profile of the enzyme was determined as $^3H_2$ exchange activity. The Arrhenius plot showed a sharp break at 20°C. At temperatures below 20°C, the activation energy for form 2 enzyme was about half the value obtained with form 1 enzyme. Both hydrogenase forms had similar activation energies at temperatures above 20°C (15 to 20 kJ mol^{-1}). The experiment was also performed in the absence of detergents to rule out the possibility that the increase in the activation energy at lower temperatures was a result of detergent micelle formation at these temperatures. No significant differences were observed under these conditions. Both forms of hydrogenases had broad pH optima (pH 6 to 8) for the tritium-exchange reaction. At pH 10.0, both forms had less than 30% of the activity detected at pH 7.0.

Kinetic properties. The two forms of the hydrogenase differed in the ability to utilize MV as an electron acceptor (Table 4). The form 2 enzyme had a higher apparent K_m for oxidized MV and a lower V_{max} for reduced MV than did the form 1 enzyme. The form 1 enzyme had a higher turnover number (molecular activity) for reduced MV-dependent H_2 evolution. The two forms had similar apparent K_ms for BV and H_2. The HUP hydrogenase reduced various artificial electron acceptors; ferricyanide and methylene blue were the most efficient, whereas the viologen dyes were only about 20% as effective as ferricyanide (Table 5). The two forms of hydrogenase reduced these electron acceptors at comparable levels.

TABLE 4. Kinetic properties of the two forms of hydrogenase

Substrate	Determination[a]			
	Form 1		Form 2	
	Apparent K_m	V_{max}	Apparent K_m	V_{max}
MV oxidized	16.1	13.8 (1,242)	26.7	24.3 (1,409)
BV oxidized	5.2	38.6 (3,474)	7.7	49.4 (2,865)
MV reduced	2.0	76.0 (6,840)	1.5	35.1 (2,035)
BV reduced	2.1	8.3 (747)	4.0	12.5 (725)
Molecular hydrogen	3.1	1,460 (131,400)	2.0	1,962 (107,245)

[a] V_{max} values are expressed as follows: for MV and BV oxidized, micromoles of dye reduced per minute per milligram of protein; for MV and BV reduced, micromoles of hydrogen evolved per minute per milligram of protein; for molecular hydrogen, micromoles of ^3H_2O produced per minute per milligram of protein. Values in parentheses represent the molecular activity of each form of hydrogenase.

Identification of the hydrogenase isoenzyme. Ballantine and Boxer (3) reported that the HYD1 and HYD2 isoenzymes could be separated in a native PAGE system run at neutral pH. At alkaline pH, only HYD1 was detected and HYD2 was inactivated irreversibly. These results, which were confirmed with HYD1 and an active fragment of HYD2, indicated that the sensitivity to alkaline pH can be used to distinguish between the two hydrogenase isoenzymes. The HUP-hydrogenase isolated from strain SE1100 exhibited low sensitivity to alkaline pH, and the activity of form 1 could be fully restored by readjusting the pH to 7.0 (Table 6). The form 2 enzyme was more sensitive than the form 1 enzyme to incubation at pH 10.0 (loss of about 25% of activity). The tritium-exchange activities of both forms were slightly more sensitive to alkaline pH than the dihydrogen-dependent reduction of BV. At pH 10.0, both enzymes had about 20 to 30% of the activity obtained at pH 7.0. These results indicate that the hydrogenase purified from strain SE1100 is the HYD1 isoenzyme.

DISCUSSION

Under appropriate growth conditions, *E. coli* can produce or consume H_2. These reactions, catalyzed by a complex set of enzymes, require the production of three different isoenzymes (27). Depending on the direction of the enzyme reaction in vivo, hydrogenases are referred to as either

TABLE 5. Effects of different electron acceptors on hydrogenase activity

Electron acceptor (1 mM)	E_0' (mV)	Sp act[a]	
		Form 1	Form 2
MV	−440	33.6	22.3
BV	−360	27.9	33.2
Neutral red	−325	22.9	18.4
Methylene blue	+11	139.1	157.0
Potassium ferricyanide	+360	160.3	193.0

[a] Expressed as micromoles of substrate reduced per minute per milligram of protein.

TABLE 6. Effects of alkaline pH on the two forms of HUP hydrogenase from *E. coli*[a]

Incubation pH	Assay pH	Sp act			
		Form 1		Form 2	
		$^3H_2$ exchange	BV	$^3H_2$ exchange	BV
7.0	7.0	1,032	38	1,586	37
10.0	7.0	790	36	989	28
10.0	10.0	331	11	365	8

[a] Hydrogenase (2 μg) was incubated at pH 7.0 or 10.0 at 4°C as described previously (4, 28) and then assayed at the indicated pH. The specific activity of hydrogenase is expressed as micromoles of ^3H_2O produced per hour per milligram of protein and micromoles of BV reduced per minute per milligram of protein.

uptake hydrogenase or reversible hydrogenase (2). Boxer and co-workers (4, 28) purified HYD1 and an active fragment of HYD2 and reported that these two isoenzymes are immunologically distinct. Other descriptions of hydrogenase purification from *E. coli*, to different levels of purity, can be found in the literature (1, 5, 13, 14, 31). Hydrogenases that had been purified to homogeneity had apparent molecular weights of 56,000 (1), 58,000 (14), and 200,000 (28). The hydrogenase described by Adams and Hall (1) was similar to the form 2 hydrogenase isolated from strain K10 (apparent molecular weight of 59,000), but the protein isolated by Adams and Hall (1) did not dimerize in the absence of detergents. This may have been a consequence of protease use during membrane solubilization. This hydrogenase (kindly supplied by D. O. Hall) and the form 2 hydrogenase from strain K10 cross-reacted immunologically with the form 1 hydrogenase purified from strain SE1100. Hydrogenase purified by Sawers and Boxer (28) is similar to the hydrogenase purified from strain SE1100 except for a slight difference in the apparent molecular weight of the dimer (200,000 versus 180,000, respectively).

Although purified hydrogenase from strain K10 consisted of only the large subunit, a protein with the apparent molecular weight of the small subunit copurified (along with few other minor contaminating proteins) until the last chromatofocusing step. In these experiments, hydrogenase was purified from actively growing cultures at the mid-exponential phase of growth. When strain K10 was grown to stationary phase in the same medium (24 h), the small subunit was not separable from the large subunit under the same protein purification regimen. The hydrogenase purified by Adams and Hall (1), which also had only the large subunit, was obtained from aerobically (commercially) grown cells. Since active hydrogenase is not detected in aerobic cultures of *E. coli*, it is likely that the culture used by Adams and Hall (1) became oxygen limited during growth, which led to induction of hydrogenase. The ability to obtain the hydrogenase with only the large subunit from these cells and the cells of strain K10 used in the study described here suggests that this characteristic is related to the culture conditions and the time of harvest of the cells. It is possible that the small subunit is an electron transport protein which is biochemically modified with culture age and that the unmodified small subunit is easily removable. Sawers and Boxer (28) identified a precursor of the small subunit with an apparent molecular weight of 35,000. The observed difference of about 7,000 could be a leader peptide which is cleaved from the subunit during incorporation into the membrane. The 28,000-dalton peptide present in the hydrogenase from strain SE1100 may represent the molecular weight of the subunit in vivo because the protein was trapped as Triton X-114 micelles immediately after preparation of extract which contained protease inhibitors. Sawers and Boxer (28) reported that the 35,000-dalton protein yielded 31,000- and 29,000-dalton peptides during the hydroxyapatite chromatography step. Hydroxyapatite caused a loss of activity in our experiments and was not used.

It is interesting that the N-terminal sequence of 10 amino acids from the small subunit of form 1 enzyme was found to be located 45 amino acid residues inside from the N-terminal part of the DNA sequence-derived protein (*hyaA* gene; 24). It is likely that the small subunit exists in two different forms, one containing the complete amino acid sequence and the other lacking the N-terminal 45 amino acids, accounting for a molecular weight difference of 4,997. The hydrogenase produced at the early stages of growth (form 2 from strain K10) probably contained the readily removable higher-molecular-weight small subunit, and the HYD1 produced during the stationary phase of growth (form 1 from strain SE1100) contained predominantly the smaller subunit which copurifies with the large subunit. This difference in the size of the protein may be introduced by posttranslational modification.

The N-terminal sequence of the large subunit from form 1 enzyme (15 residues) was found to be identical irrespective of the method of determination (direct chemical analysis or derived from DNA sequence; 24). These results also provide direct evidence that the DNA sequence reported by Menon et al. (24) codes for the HYD1 isoenzyme, and these genes were mapped at 22.0 min in the *E. coli* chromosome (K. T. Shanmugam, unpublished data).

The nonheme iron and inorganic sulfur contents of the form 1 hydrogenase are in agreement with the values reported by Sawers and Boxer (28). The presence of only two-thirds the number of nonheme iron and sulfur residues in the form 2 enzyme (Table 3) indicates that the larger subunit contains twice as many of these elements as does the smaller subunit.

Our previous experiments, confirmed by two independent methods, showed that HYD1 is associated with HUP activity. When a *hup* mutation, which eliminates the production of H_2-dependent BV reduction, was transduced into strain SE1100, which lacks the FHL system, the double mutant failed to produce both HYD1 and HYD2 activities (20, 21). An *fhlA* mutant, strain SE1174, which lacks the different enzymes associated with the FHL complex, still produced the HYD1 hydrogenase, as determined by both biochemical and immunological methods (data not presented). These results are in agreement with the postulated (based on the results obtained with *Salmonella typhimurium* strains [29]) physiological role of HYD1 in the oxidation of H_2 in the cell.

We propose that the HYD1 is a component of a metabolic pathway in which formate serves as the electron donor and fumarate serves as the physiological electron acceptor. This would explain the reported stimulatory effect of formate on the production of HYD1 (27) and the need for Fnr protein (27), an anaerobic regulatory protein (19). In this pathway, electrons flow through hydrogenase isoenzyme 1 but are not released as H_2 (Fig. 2). In this physiological role, HYD1 serves as an intermediate electron transport protein. This metabolic pathway, which would still require formate dehydrogenase associated with H_2 evolution, would be independent of the FHL system. HYD1 would be produced at the mid-exponential phase of growth, when the energy demand in the cell is high and a sufficient amount of formate is present in the medium. Production of HYD1 is not H_2

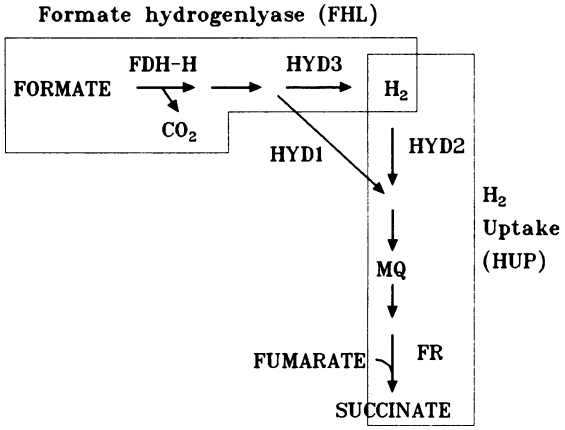

FIG. 2. Model depicting the physiological roles of hydrogenase isoenzymes in *E. coli*. FDH-H, Formate dehydrogenase associated with H_2 evolution; HYD1, HYD2, and HYD3, the three hydrogenase isoenzymes; MQ, menaquinone; FR, fumarate reductase. The gaps between the arrows represent unidentified electron carriers.

dependent, since strain SE1100 is Fhl^- in Mo-deficient medium (21).

Boxer and colleagues (4, 27, 29) reported that the physiological role of HYD2 is to oxidize H_2 and couple it to reduction of fumarate, with concomitant production of energy (16). Strain SE1100 and its parent strain BW545 produced both HYD1 and HYD2 activities during the late exponential to early stationary phase of growth when grown in LB-glucose medium, when the H_2 concentration in the medium would be saturating as a result fermentation (data not presented). On the basis of these results, a role for HYD1 in H_2 oxidation could not be ruled out, especially during the late exponential to early stationary phase of growth.

The size and nature of the small subunit, which serves as a hydrogenase-associated specific electron carrier, probably determines whether HYD1 serves as a bridge between the FHL and HUP pathways or functions as a HUP hydrogenase. In this connection, it is interesting that the purified form 1 enzyme evolved dihydrogen (from reduced MV) at twice the maximal rate of the form 2 enzyme, which lacks the small subunit (Table 4). The form 2 enzyme also showed a slight preference in the H_2-dependent reduction of more electropositive electron carriers such as ferricyanide and methylene blue (Table 5). Both forms of hydrogenase reduced these electron carriers more readily, although at slightly different rates, in contrast to the reported inability of HYD1 to reduce ferricyanide and methylene blue (28). However, the actual physiological role of the hydrogenase needs to be determined by using mutant strains that are defective in the different isoenzymes.

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