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Nickel-Containing Hydrogenase Isoenzymes from Anaerobically Grown *Escherichia coli* K-12

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Two membrane-bound hydrogenase isoenzymes present in *Escherichia coli* during anaerobic growth have been resolved. The isoenzymes are immunologically and electrophoretically distinct. The physically more abundant isoenzyme (hydrogenase 1) contains a subunit of M_r 64,000 and is not released from the membrane by exposure to either trypsin or pancreatin. The second isoenzyme (hydrogenase 2) apparently contributes the greater part of the membrane-bound hydrogen:benzyl viologen oxidoreductase activity and exists in two electrophoretic forms revealed by nondenaturing polyacrylamide gel analysis. This isoenzyme is irreversibly inactivated at alkaline pH and gives rise to an active, soluble derivative when the membrane-bound enzyme is exposed to either trypsin or pancreatin. Both hydrogenase isoenzymes contain nickel.

Hydrogen is metabolized by *Escherichia coli* in two distinct pathways, both of which involve hydrogenase activity (3). In the formate hydrogen lyase pathway, which oxidizes endogenously produced formate to carbon dioxide and hydrogen, hydrogenase passes electrons derived from formate oxidation via unidentified electron carriers to protons to yield molecular hydrogen. This pathway functions when the organism grows anaerobically in the absence of an exogenous electron acceptor (12). Alternatively, the organism can utilize hydrogen as an energy source to allow anaerobic growth on a nonfermentable carbon source that also functions as an electron acceptor, i.e., fumarate (21). In this case, hydrogenase oxidizes hydrogen to protons and passes electrons directly or indirectly in a proton-translocating, energy-conserving manner to the quinone pool (4, 15, 16, 21, 25).

Whether the above hydrogenase reactions are catalyzed by a single enzyme or by distinct isoenzymes has not been clarified. Several workers have cataloged multiple electrophoretic forms of hydrogenase in polyacrylamide gels, but neither the independence of the electrophoretic variants nor their physiological roles has been established (1, 25). Graham et al. from our laboratory have described a hydrogenase present in anaerobically grown *E. coli* (10). This enzyme appears similar to a hydrogenase isolated from the aerobically grown organism (2). Since in each of these studies a single hydrogenase species was cataloged, it appeared likely that multiple isoenzymes may not be present in *E. coli*. However, in this report we establish by a variety of criteria that a second hydrogenase isoenzyme is present in anaerobically grown *E. coli*. Both hydrogenase isoenzymes present during anaerobic growth contain nickel.

MATERIALS AND METHODS

Growth of *E. coli* and preparation of membrane vesicles. *E. coli* (strain P4X Hfr *metB1*; from E. Wollman) was routinely grown anaerobically in a medium based on that of Cohen and Rickenberg (6) containing glucose (0.4%, wt/vol), bacteriological peptone (0.5%, wt/vol), casein hydrolysate (0.1%, wt/vol), thiamine (0.001%, wt/vol), 1 mM $MgCl_2$, 1 μM $(NH_4)_6Mo_7O_{24}$, and 1 μM K_2SeO_3 adjusted to pH 6.4 (10). Bottles (500 ml) almost filled with the above medium were inoculated with 5 to 10 ml of overnight cultures, stoppered to promote anaerobic conditions, and incubated at 37°C for 4 to 5 h, when an increase in absorbancy at 600 nm of the medium of 0.4 to 0.5 was obtained.

The cultures were harvested by sedimentation at 7,000 × *g* for 15 min at 4°C. The cells were washed once in 50 mM potassium phosphate buffer, pH 6.8. The washed cells were either stored as pellets at −80°C for future use or used immediately. In addition during harvesting and breakage, buffers contained sodium dithionite (0.003%, wt/vol) to maintain anaerobiosis.

Cells labeled with $^{63}Ni^{2+}$ were prepared by growth in the presence of 0.14 μM $^{63}NiCl_2$ (specific radioactivity, 0.726 mCi/μmol; The Radiochemical Centre, Amersham, United Kingdom), equivalent to 0.1 μCi/ml of medium. Otherwise, growth and harvesting were as described above. Cells labeled with ^{35}S were obtained after growth essentially as described previously (10), with the addition of L-[^{35}S] methionine (Amersham; specific radioactivity, 1,400 Ci/mmol) to give 1 μCi/ml of medium.

The washed cell pellets were suspended in 50 mM Tris-hydrochloride (pH 7.5) containing 10 mM benzamidine-hydrochloride (5 to 10 ml of buffer per 500 ml of cell culture). Cell breakage was accomplished in a French pressure cell (30,000 lb in^{-2}) in the presence of traces of DNase (Sigma Chemical Co.). Unbroken cells were removed by centrifugation at 20,000 × *g* for 15 min, and the membrane fraction was prepared by further centrifugation of the supernatant at 130,000 × *g* for 2 h at 4°C. The membrane pellets were washed once in 50 mM Tris-hydrochloride (pH 7.5)–10 mM benzamidine-hydrochloride, suspended to a protein concentration of 20 to 30 mg/ml, and stored in working samples at −80°C.

Hydrogenase activity (assayed as described below) was measured in the membranes and the final supernatants; 50 to 70% of the activity recorded in the initial crude extract was recovered in the final fractions. Of the recovered activity, between 60 and 80% was located in the membrane pellets.

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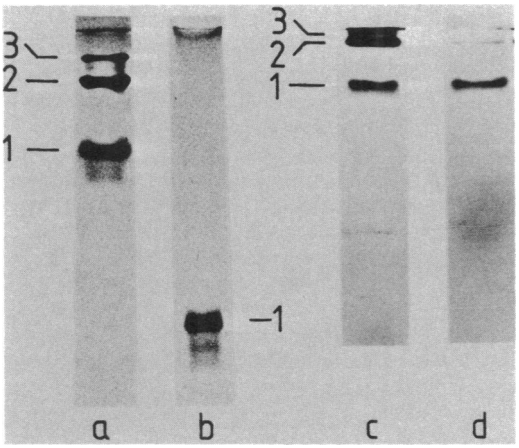

FIG. 1. Non-dissociation polyacrylamide gel analysis of membrane-bound hydrogenase activity. A 50-μg sample of Triton X-100–dispersed membrane protein was electrophoresed in a 7.5% (wt/vol) polyacrylamide gel in each case, and the gel was stained for hydrogenase activity. Panels: (a) electrophoresis performed with the pH 8.0 system; (b) electrophoresis performed with the pH 9.5 system; (c) sample preincubated at pH 7.0 as described in the legend to Fig. 4 before electrophoresis, otherwise as in a; (d) sample preincubated at pH 10.0, as described in the legend to Fig. 4 before electrophoresis, otherwise as in a.

The membranes exhibited specific hydrogenase activities of between 0.4 and 0.7 U per mg of protein.

Membrane vesicles were dispersed by suspension to a final protein concentration of 10 mg/ml in 50 mM Tris-hydrochloride (pH 7.5) containing 4% (wt/vol) Triton X-100 and incubation on ice for 15 min. A variable (50 to 300%) activation of the membrane hydrogen:benzyl viologen oxidoreductase activity was observed after this procedure. Triton X-100–insoluble material was removed by centrifugation at 145,000 × *g* for 20 min with an Airfuge (Beckman Instruments, Inc.). Under these conditions 50% of the membrane protein and up to 90% of the hydrogenase activity was recovered in the supernatant.

Assays. Hydrogenase activity was estimated spectrophotometrically by measuring H_2-dependent, oxidized benzyl viologen reduction at 600 nm (14). Anaerobic cuvettes were completely filled with H_2-saturated 100 mM potassium phosphate (pH 7.0), and oxidized benzyl viologen was added to a final concentration of 4 mM. The buffer in the cuvette was titrated with a solution of sodium dithionite to an absorbancy at 600 nm of between 0.3 and 0.7 before the addition of enzymes. An E_M value of 7,400 M^{-1} cm^{-1} at 600 nm was assumed for reduced benzyl viologen (14), and 1 U of hydrogenase activity was defined as 1 μmol of benzyl viologen reduced per min.

Hydrogenase activity on immunoplates or polyacrylamide gels was specifically located by immersion in 100 mM potassium phosphate (pH 7.0) containing 0.5 mM benzyl viologen and 1 mM triphenyl tetrazolium chloride and incubation under an H_2 atmosphere for up to 24 h. Activity was revealed as intense red zones. No such zones were observed after incubation under an N_2 atmosphere. Protein was determined by the method of Lowry et al. (20).

Electrophoretic procedures. Two different discontinuous buffering systems were employed for nondenaturing polyacrylamide gel electrophoresis. These were a high-pH, Tris-glycine buffer system (23) and a neutral-pH, Tris-barbitone buffer system (13). Electrophoresis was performed with either 5.5 or 7.5% (wt/vol) polyacrylamide separating gels with 2.5% (wt/vol) polyacrylamide stacking gels. Triton X-100 (0.1%, wt/vol) was incorporated throughout the system. Usually vertical slab gel electrophoresis was performed.

Elution of hydrogenase activity from polyacrylamide gel slices was accomplished by first macerating the gel slices by several passages through a 5-ml plastic syringe, followed by incubation, with shaking, for up to 16 h at room temperature in several volumes of 50 mM Tris (pH 7.5)–0.1% (wt/vol) Triton X-100. After the gel pieces were removed by filtration through glass wool, the eluted activity was concentrated 10-fold with Minicon B15 concentrators (Amicon B.H., Witten, West Germany). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (18).

Crossed immunoelectrophoresis (11) was performed in 1% (wt/vol) agarose gels (5 by 5 by 0.15 cm) containing 1% (wt/vol) Triton X-100 and 20 mM barbital-hydrochloride (pH 8.6). Samples of Triton X-100–dispersed membranes were electrophoresed in the first dimension at 4.5 mA per plate for 1.5 h in a water-cooled chamber. The agarose strip containing fractionated antigen was retained, the remainder was replaced with agarose containing antiserum (60 μl ml^{-1}), and then electrophoresis was performed in the second dimension at 2 mA per plate for 12 to 18 h. In some instances cylindrical polyacrylamide gels (2 mm by 10 cm) were employed for fractionating antigens in the first dimension (see Fig. 3). These were then embedded in the agarose gels (11 by 6 by 0.2 cm) cast on Gel Bond (Bio-Rad Laboratories) and electrophoresed in the second dimension at 3 mA per plate for 20 h. After electrophoresis, immunoplates were either stained for hydrogenase activity or stained for protein after three cycles of soaking in 0.1 M NaCl and air drying. For protein staining, plates were immersed in 0.5% (wt/vol) Coomassie brilliant blue in 10% (vol/vol) acetic acid–45% (vol/vol) methanol, for 5 min and destained in 10% (vol/vol) acetic acid–45% (vol/vol) methanol.

Trypsin treatment of membrane vesicles. Membrane vesicles (20 mg of protein per ml) were suspended in 50 mM Tricine (pH 7.8) in a final volume of 200 μl containing trypsin (Sigma) at 0.25 mg/ml and incubated for 1 h at 37°C. The reaction was stopped by the addition of soybean trypsin inhibitor (Sigma) to 0.5 mg/ml. A 100-μl sample was centrifuged (145,000 × *g* for 20 min), and the supernatant was removed for analysis. The remainder of the trypsin-treated membranes were dispersed with Triton X-100 after dilution to 10 mg/ml of protein in the same buffer.

Determination of the effect of pH on hydrogenase activity. A three-component, constant-ionic-strength buffer system consisting of 0.1 M *N*-(2-acetamido)-2-aminoethanesulfonic acid, 52 mM Tris, and 52 mM ethanolamine was used for incubations over the pH range 6 to 10 (7). Triton X-100–dispersed membranes were diluted 10-fold into buffer at the appropriate pH. The samples with initial hydrogenase activities of 0.82 U/ml were incubated for 4 h at room temperature. Duplicate samples were then removed and assayed for hydrogenase activity.

RESULTS

Electrophoretic analysis of membrane-bound hydrogenase activity. Electrophoresis of Triton X-100–dispersed membranes in a nondenaturing polyacrylamide gel system that resolves at pH 8.0 and subsequent staining for hydrogenase activity revealed three zones in the gel exhibiting hydrogenase activity (Fig. 1a). The R_fs for these bands were estimated from a 7.5% (wt/vol) polyacrylamide separating

gel as about 0.16, 0.07, and 0.04 and are referred to as bands 1, 2, and 3, respectively. Band 3 varied in intensity from gel to gel even for the same sample. Usually it was the slowest to develop and therefore appeared the most faint.

Similar analysis of the same Triton X-100–dispersed membrane sample with a gel electrophoresis system that resolves at pH 9.5 gave a single zone of hydrogenase activity of approximate R_f 0.36 in a 7.5% (wt/vol) polyacrylamide gel (Fig. 1b). A single band has been described previously by ourselves (11) and others (2). Clearly the number of hydrogenase species revealed by gel electrophoretic analyses is greatly influenced by the conditions employed.

Immunoelectrophoretic analysis of membrane-bound hydrogenase activity. We have previously identified an antigen possessing hydrogenase activity by crossed immunoelectrophoretic analysis of the Triton X-100–dispersed membranes employing polyspecific antiserum obtained by using membrane vesicles as the immunogen (11). Van der Plas et al. (24), describing a similar analysis, reported a major and a minor precipitin arc, both of which exhibited hydrogenase activity. When we subjected our immunoplates to prolonged activity staining in a container enclosing a hydrogen atmosphere, we observed two hydrogenase-containing arcs (Fig. 2a). We refer to these arcs as arcs 1 and 2, possessing greater and lesser electrophoretic mobility, respectively (Fig. 2a). We found considerable variation both in their relative rates of development in the activity stain and in their relative sizes when different batches of antiserum were employed. However, in all cases arc 1 corresponded to a major protein-staining arc, in contrast to arc 2, which was barely visible in protein-stained immunoplates. It appears, therefore, that the arc 1 antigen is much more abundant than that of arc 2. Arcs 1 and 2 always appeared as independent precipitin lines that overlapped one another. No indication of any fusion between the arcs was found. We conclude that these antigens are distinct hydrogenase isoenzymes.

As anticipated, when the activity-stained immunoplates were further stained for protein, many more precipitin arcs (>30) could be discerned that did not exhibit hydrogenase activity (Fig. 2b). The heavy staining of arcs 1 and 2 in Fig. 2b is due to the activity stain coloration, which is retained after the subsequent protein detection.

Search for further hydrogenase species. The supernatant soluble fraction as normally prepared may contain as much as 40% of the hydrogenase activity recovered after cell breakage and fractionation. Analysis of the supernatant fraction by crossed immunoelectrophoresis and gel electrophoresis, performed as described above in the presence of 1% (wt/vol) Triton X-100, failed to reveal any further hydrogenase species other than those described above. Since hydrogenase activity could not be removed from the membrane fraction by further washing, with the isolation buffer, in the presence of up to 0.4 M KCl or 10 mM EDTA, we conclude that the hydrogenase species that we have cataloged above are true membrane-bound enzymes and that the hydrogenase activity present in the supernatant fraction can be attributed to contamination by the membrane-bound activity. Up to 90% of the hydrogenase activity present after the addition of Triton X-100 (4%, wt/vol) can be dispersed from the membrane fraction so that the presence of a further major membrane-bound hydrogenase is unlikely. However, we have observed a variable (50 to 300%) increase in hydrogenase activity, on exposure of the membrane fraction to Triton X-100, which we attribute to latent activity present in the sealed membrane vesicles.

Relationship between hydrogenase species observed by polyacrylamide gel electrophoresis and immunoelectrophoresis. Excision of the zones in the polyacrylamide gels that possessed hydrogenase activity and immunoelectrophoretic analysis of the material eluted from the gel slices allowed several identities to be made. Identity was established by fusion of the precipitin arc of the eluted material with the corresponding arc (arc 1 or 2) on crossed immunoelectrophoresis. Material eluted from band 1 of the neutral gel (Fig. 1a) or the single band from the higher pH gel (Fig. 1b) both possessed hydrogenase activity when assayed directly, and each gave identity only with the hydrogenase of arc 1. In the same manner, elution of hydrogenase activity from gel slices encompassing both bands 2 and 3 together indicated the presence of only the hydrogenase of arc 2, suggesting that these bands represent the arc 2 hydrogenase in different physical forms. However, although the activity eluted from band 2 alone was shown to have identity with the arc 2 hydrogenase, no activity or antigen could be detected in this manner after elution of band 3 alone. Possibly, there was too little activity and antigen present in band 3, or the elution was too inefficient for detection. Alternatively, antibodies to this hydrogenase species are absent in the crude antisera. A different approach demonstrated the latter explanation was not the case. Figure 3 shows an activity-stained crossed immunoelectrophoresis plate in which the Triton X-100–dispersed membrane has been separated in the first dimension by electrophoresis in a non-dissociating polyacrylamide gel. The plate clearly shows that the activity arcs corresponding to bands 2 and 3 have fused, indicating identity. Therefore the hydrogenase activities of both bands

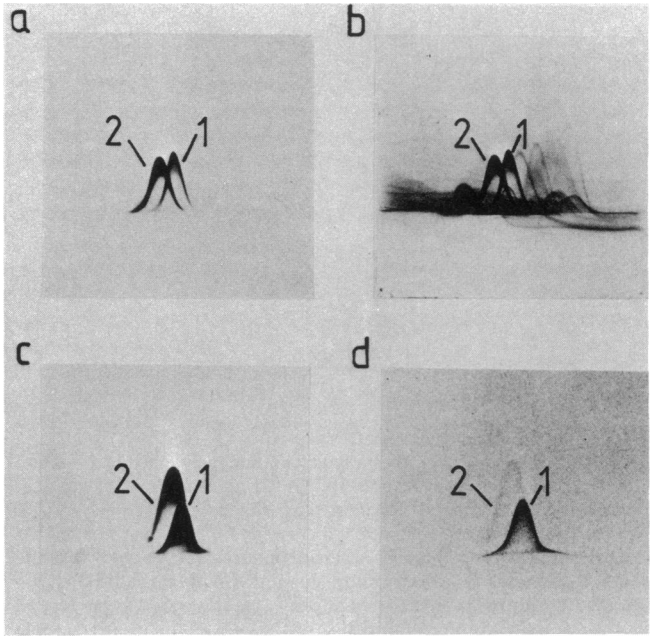

FIG. 2. Crossed immunoelectrophoretic analysis of Triton X-100–dispersed membranes. Between 20 and 40 μg of Triton X-100-dispersed membrane protein was electrophoresed in the first, horizontal dimension. Electrophoresis in the second, vertical, dimension was into agarose containing antiserum raised to membrane vesicles. Panels: (a) immunoplate stained for hydrogenase activity; (b) plate in a after subsequent staining for protein (the heavy staining of arcs 1 and 2 is attributable almost exclusively to the prior activity stain); (c) immunoplate stained for hydrogenase activity (the membrane sample electrophoresed in this case was prepared from cells grown in the presence of $^{63}NiCl_2$); (d) autoradiograph of the plate in c.

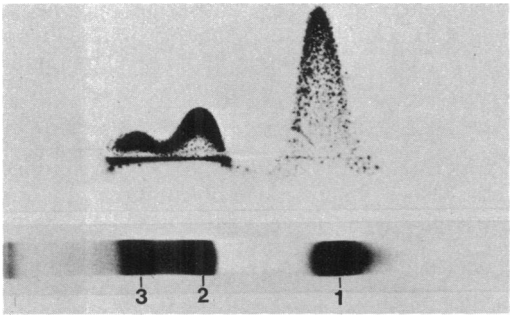

FIG. 3. Immunoplate showing immunological identity between the hydrogenases of bands 2 and 3. Triton X-100–dispersed membranes (25 μg of protein) were fractionated by electrophoresis in a pH 8.0, non-dissociating, cylindrical, 5.5% (wt/vol) polyacrylamide gel in the first, horizontal dimension and then electrophoresed into agarose containing antiserum to membrane vesicles in the second, vertical, dimension. The immunoplate was stained for hydrogenase activity. A control gel not subjected to second-dimension electrophoresis and stained for hydrogenase activity is shown below the immunoplate.

2 and 3 are due to the presence of the arc 2 hydrogenase. Note that the majority of the arc 2 antigen is present in band 2. The isoenzymes giving rise to arcs 1 and 2 are hereafter referred to simply as hydrogenases 1 and 2, respectively.

Exposure to high pH irreversibly inactivates hydrogenase 2. The number of activity bands seen after gel electrophoretic analysis of the Triton X-100–dispersed membranes depends upon the buffering system used (Fig. 1a and b). Only hydrogenase 1 appears on the high-pH gel system. A possible reason for the loss of hydrogenase 2 activity is that it is alkali labile, since the discontinuous buffering systems of the gels used resolve at pH 8.0 and 9.5 (13). The effect of pH variation on hydrogenase activity was investigated; 75% of the hydrogenase activity present after incubation at pH 7.0 was progressively lost between pH 8.0 and 10.0 (Fig. 4). Concomitant gel electrophoretic analyses with the neutrally buffered system revealed that the activities of bands 2 and 3 were correspondingly progressively lost over this pH range. Figure 1c and d depicts the activity-stained gels obtained after the pH 7.0 and 10.0 incubations, respectively. No apparent loss of hydrogenase 1 activity was noted from the gels. We conclude that hydrogenase 2 is inactivated after incubation at high pH and that this inactivation is irreversible, since the activities of bands 2 and 3 are not recovered on subsequent neutralization. Furthermore, up to 75% of the total hydrogenase activity as assayed (hydrogen:benzyl viologen oxidoreductase) is attributable to hydrogenase 2 despite it being the isoenzyme of lesser abundance.

Both hydrogenase isoenzymes contain nickel. The transition metal nickel has been found in hydrogenase from several sources, including methanogenic bacteria (9), *Alcaligenes eutrophus* (8), and *Desulfovibrio gigas* (17). We explored whether nickel is associated with *E. coli* hydrogenase isoenzymes. Cells were grown in the presence of $^{63}Ni^{2+}$, and membranes were prepared. Approximately 2% of the radioactivity added to the growth medium was incorporated into the cells; of this, about 20% was associated with the membrane fraction. Dispersion of the membranes with Triton X-100 released greater than 90% of this radioactivity.

Shown in Fig. 2c is an immunoplate stained for hydrogenase activity in which a membrane sample prepared from the $^{63}Ni^{2+}$ radioactive growth has been electrophoresed. The corresponding autoradiograph (Fig. 2d) revealed only two radioactive arcs, which exactly corresponded to the activity staining arcs (Fig. 2c). Analysis of the $^{63}Ni^{2+}$–Triton X-100–dispersed membranes by gel electrophoresis was also performed (data not shown). Autoradiographs of the neutrally buffered gels revealed a major radioactive band exactly corresponding to band 1, a minor band corresponding to band 2, and a very small amount of radioactivity corresponding to band 3. With the higher-pH gel system, only a single radioactive band appeared that was coincident with the activity band. It is clear from these results that both hydrogenases 1 and 2 contain nickel. They are likely to be the only significant nickel-containing proteins in the membranes. The majority of membrane-bound nickel is attributable to hydrogenase 1. The smaller proportion found associated with hydrogenase 2 would be in keeping with its low abundance. Since the isoenzymes are distinct, there is no necessary relationship between their respective precipitin arcs for apparent activity and abundance as assessed by relative protein and nickel content. However, we anticipate that isolated hydrogenase 2 would possess a greater hydrogen:benzylviologen oxidoreductase specific activity than isolated hydrogenase 1.

Derivative of hydrogenase 2 released from membranes by limited proteolysis. Exposure of membranes to trypsin (1, 25) and pancreatin (2) has been used in earlier studies on the nature of membrane-bound hydrogenase from *E. coli* with conflicting results. Crossed immunoelectrophoretic analysis of a Triton X-100–dispersed membrane sample that had been incubated with trypsin showed that the hydrogenase 2 arc was depleted, and a new, faster-migrating arc, which fused with that of hydrogenase 2, was produced (Fig. 5a). The hydrogenase 1 arc appeared unaltered. When a centrifugation step to recover the membranous material was performed immediately after the trypsin treatment of the membranes, the faster-migrating hydrogenase 2 derivative was found exclusively in the supernatant (Fig. 5b). After prolonged exposure to trypsin, the activity in the supernatant fraction accounted for between 50 and 60% of the remaining hydrogenase activity. Note that the formation of the hydrogenase 2 isoenzyme fragment was incomplete (Fig. 5a), but further incubation with trypsin did not lead to further release of activity. Heterogeneity in the sidedness of the membrane vesicles could account for this.

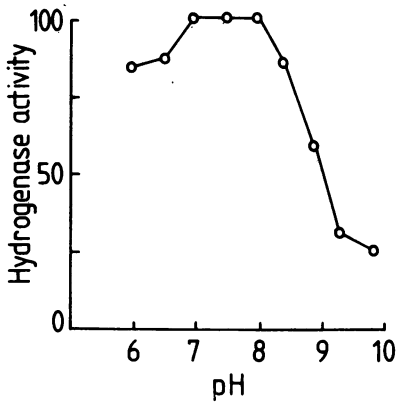

FIG. 4. Influence of pH on hydrogenase activity. Triton X-100–dispersed membranes were incubated in media of variable pH and assayed for hydrogenase activity as described in the text. The activities are plotted as percentages of that of a control preparation that was incubated in 100 mM potassium phosphate (pH 7.0).

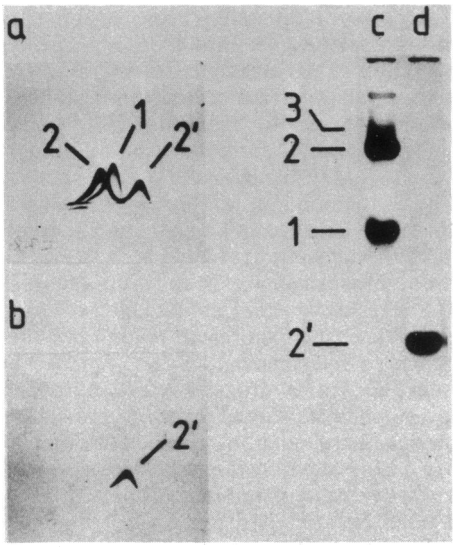

FIG. 5. Susceptibility of hydrogenase 2 to proteolysis by trypsin. Membranes, trypsin treated as described in the text, were either dispersed with Triton X-100 or recovered by centrifugation. Shown above are two crossed immunoelectrophoresis plates (a and b) obtained with antiserum raised to unfractionated membrane vesicles and two tracks (c and d) from a neutrally buffered, non-dissociating, 5.5% (wt/vol) polyacrylamide gel, each stained for hydrogenase activity. The samples analyzed were (a) Triton X-100–dispersed membranes after trypsin-treatment, (b) supernatant from trypsin-treated membranes, (c) untreated Triton X-100–dispersed membranes, and (d) supernatant from trypsin-treated membranes.

Analysis of the supernatant fraction by gel electrophoresis revealed that the new species had a much enhanced electrophoretic mobility, R_f 0.54 (Fig. 5c and d). Hydrogenase 1 remained exclusively membrane bound after treatment, but it also was trypsin susceptible, since its electrophoretic mobility revealed in polyacrylamide gels of the membrane residue was slightly enhanced (data not shown). No differential susceptibility to trypsin was found for bands 2 and 3. Trypsin treatment of the membrane fraction led to cleavage of hydrogenase 2 to produce an active, soluble derivative. No such soluble fragment was found for hydrogenase 1. A similar series of experiments, which gave similar results leading to the same conclusions, was performed with pancreatin in place of trypsin.

Identity of hydrogenase 1. Researchers for this laboratory have previously described a hydrogenase species from *E. coli* with a polypeptide subunit of M_r 64,000 (10). The relationship of that enzyme to those described in this paper was examined. We attempted to isolate the two hydrogenases from ^{35}S-labeled cultures. This was effected by elution from gels of the hydrogenase activity associated with bands 1 and 2, followed by rocket immunoelectrophoresis of the eluted material. Activity-stained arcs cut from the immunoplates were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. For hydrogenase 1 a single radioactive band of M_r 64,000 was found. This approach was not fruitful for hydrogenase 2, since too little radioactivity was recovered in the hydrogenase 2-specific arc. This is consistent with the low abundance of hydrogenase 2. The subunit molecular weight of hydrogenase 1 and its relatively high abundance establish its identity with the enzyme we have previously described (10). Further confirmation was obtained with the previously used procedure (10, 22) for raising specific antibodies to antigens in precipitin arcs to obtain antiserum specific for hydrogenase 1. This antiserum immunoprecipitated a polypeptide of M_r 64,000; in agreement with our above findings, it did not cross-react with hydrogenase 2.

DISCUSSION

This work establishes that there are two membrane-bound hydrogenase isoenzymes in anaerobically grown *E. coli*. The isoenzymes are most clearly distinguished by staining for hydrogenase activity after crossed immunoelectrophoretic analysis. The distinct nature of these isoenzymes is revealed by both their immunological and electrophoretic properties and is confirmed by their differential stability at high pH and their behavior after exposure to proteases. Since much of our evidence involves the interpretation of the results of somewhat lengthy staining of gels and immunoplates for hydrogenase activity, it is important that this method of detection can be related to direct enzymatic assay—hydrogen:benzyl viologen oxidoreductase activity in this work. We have demonstrated this qualitatively by direct assay of eluted material from gels and more quantitatively with respect to hydrogenase 2, from the correlation of the instability of hydrogen:benzyl viologen oxidoreductase activity at high pH with the activity-stained profile of the material analyzed by gel electrophoresis. It appears unlikely from our work that a further enzyme possessing significant hydrogen:benzyl viologen oxidoreductase activity is present in the organism when grown anaerobically. However, we cannot discount this possibility since our data are not fully quantitative, and since inactivation during electrophoretic and immunological analysis cannot be eliminated.

This is the first report that nickel is a component of hydrogenases from *E. coli*. Nickel has been shown to be associated with several hydrogenases from a variety of organisms (5, 8, 9, 17), and it appears to be centrally important for hydrogenase activity (8, 19). The hydrogenase isoenzymes described here appear to be the major nickel-containing components in the cytoplasmic membrane. Our data suggest that the lability of hydrogenase 2 at high pH may be due to the irreversible loss of nickel.

Adams and Hall (2) purified a membrane-bound hydrogenase from aerobically grown *E. coli* that was labile at high pH and whose solubilization from the membrane was assisted by incubation with pancreatin. This enzyme, therefore, probably corresponds to hydrogenase 2. This interpretation is supported by our findings that, although hydrogenase activity is hardly measurable in aerobically grown *E. coli*, hydrogenase 2 is by far the more predominant isoenzyme present (G. Sawers, S. P. Ballantine, and D. H. Boxer, unpublished observation). The similar subunit molecular weights for the Adams and Hall enzyme and that for the enzyme identified previously by ourselves (10), hydrogenase 1, of 56,000 and 64,000 respectively, which raised the possibility that they were the same enzyme, is probably fortuitous.

In *E. coli* hydrogenase activity is involved both in hydrogen evolution during fermentative growth and in energy-conserving hydrogen uptake when the organism grows anaerobically with a suitable nonfermentable carbon source under a hydrogen-containing atmosphere. The possible relationship of the two hydrogenase isoenzymes described here to the above physiological roles will only be evident after further investigation. Since it is now unclear whether hydrogenase 1 functions as the uptake, energy-conserving enzyme, the significance of its transmembraneous location (10) within the membrane remains to be established.

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