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Some Properties of the Nickel-Containing Hydrogenase of Chemolithotrophically Grown *Rhizobium japonicum*†

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The uptake hydrogenase of chemolithotrophically grown *Rhizobium japonicum* was purified to apparent homogeneity with a final specific activity of 69 μmol of H_2 oxidized per min per mg of protein. The procedure included Triton extraction of broken membranes and DEAE-cellulose and Sephacryl S-200 chromatographies. The purified protein contained two polypeptides separable only by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. They comigrated on native polyacrylamide gels and sucrose density gradients. The molecular weights were ca. 60,000 and 30,000. Densitometric scans of the sodium dodecyl sulfate gels indicated a molar ratio of 1.03 ± 0.03. Antiserum was developed against the 60-kilodalton polypeptide for use in hydrogenase detection by an enzyme-linked immunosorbent assay. The antiserum did not cross-react with the 30-kilodalton polypeptide. Native gel electrophoresis of Triton-extracted cells grown in the presence of ^{63}Ni showed comigration of the hydrogenase and radioactive Ni.

Hydrogenase catalyzes the reversible oxidation of H_2, which may be used as a source of energy when coupled via hydrogenase to a variety of physiological electron acceptors. Certain anaerobic bacteria also evolve H_2 via hydrogenase to regenerate oxidation-reduction carriers in metabolism (3). Hydrogenases have been isolated from a variety of microorganisms, including aerobes, anaerobes, facultative anaerobes, cyanobacteria, and photosynthetic bacteria (3). The only common feature of all of these enzymes is that they contain iron-sulfur centers (3).

Recent work with hydrogenases from several sources indicates that these enzymes are more complex than was previously supposed. In 1965, Bartha and Ordal (5) showed that nickel is necessary for the chemolithotrophic growth of *Alcaligenes eutrophus*. In 1981, Friedrich et al. linked the nickel requirement to active hydrogenase formation. Since then, hydrogenases from *Vibrio succinogenes* (39), *Methanobacterium thermoautotrophicum* (19), *A. eutrophus* (17), *Desulfovibrio gigas* (25), and *Rhodopseudomonas capsulata* (10) have been shown to contain nickel.

Although the physiological electron acceptors of the uptake hydrogenases are unknown, these enzymes reduce a variety of electron acceptors, including cytochromes, in the presence of H_2 (3). The membrane-bound hydrogenase of *Xanthobacter autotrophicus* GZ29 copurifies with a cytochrome *b* component (32).

Our understanding of hydrogenases is complicated by reports that the number of polypeptides in purified preparations from different sources vary. Several hydrogenases contain a single subunit of 60,000 to 65,000 molecular weight (2, 8, 9); others contain an additional polypeptide of 26,000 to 32,000 molecular weight (33, 35, 40). The polypeptide compositions of hydrogenases from *Chromatium* spp. (18) and *Escherichia coli* (1) do not fit into either of the groups listed above.

The uptake hydrogenases of nitrogen-fixing organisms were first studied by Phelps and Wilson (28). These enzymes may play a unique role in recycling energy lost by H_2 evolution during nitrogen fixation (12). Hydrogen recycling via the hydrogenase system may increase the efficiency of energy utilization during nitrogen fixation and increase the yield of legumes (14).

Arp and Burris (4) first isolated the membrane-bound hydrogenase of the USDA 110 strain of *Rhizobium japonicum* bacteroids. The enzyme was reported to be an iron-sulfur protein composed of a single subunit of 63,300 molecular weight. Since this initial work, cytochromes have been implicated in the H_2 oxidation process under physiological conditions (13, 26). Eisbrenner et al. (15) used cyanide inhibition and difference spectrophotometry to detect a *b*-type cytochrome that appears to be specifically involved in H_2 oxidation in chemolithotrophically cultured *R. japonicum*. Klucas et al. (23) have shown that nickel is an essential micronutrient for expression of hydrogenase activity and growth of *R. japonicum* in chemolithotrophic cultures.

Cantrell et al. (7) have constructed a gene library from hydrogen uptake-positive (Hup^+) *R. japonicum* 122DES. Analysis by Tn*5* insertion (20) of several recombinant cosmids indicates that Hup-specific DNA occurs over a region of at least 16 kilobases of insert DNA. Further evidence indicates that at least three complete transcriptional units are contained in this region and are necessary for the expression of hydrogen uptake activity.

The involvement of nickel and cytochrome *b*, as well as the large expanse of DNA which codes for hydrogen uptake activity, suggests a complex biochemical system associated with hydrogenase. The purpose of this work was to isolate and purify the hydrogenase from chemolithotrophically grown *R. japonicum*, to identify the polypeptides of the purified enzyme, and to determine whether nickel and cytochrome *b* are associated with the enzyme.

MATERIALS AND METHODS

Chemicals. Genapol X-080 is a neutral detergent (polyethylene glycol isotridecanol ether) which does not absorb light at 280 nm. Initially obtained as a gift from Hoechst AG, it is now available through Calbiochem-Behring. $^{63}NiCl_2$ was purchased from New England Nuclear Corp. Molecular weight standards for sodium dodecyl sulfate-polyacyrlamide gel electrophoresis (SDS-PAGE) were purchased from Bio-

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Rad Laboratories. Other standard proteins were purchased from Schwarz/Mann.

Chemolithotrophic bacterial culture. *R. japonicum* 122DES (Hup^+ strain) was used for hydrogenase isolation. Cultures were grown on hydrogen uptake medium under derepressing conditions (21); 45 plates were incubated at 29°C for 1 week, and bacteria were washed aseptically from the plates and used to inoculate a 36-liter liquid culture containing the medium described by Repaske and co-workers (30, 31). The procedure for cell culture is described by Purohit et al. (29), except that we used an initial oxygen concentration of 0.1% rather than 1.0%. The cells were harvested by centrifugation after 28 days and suspended in 50 mM KH_2PO_4-K_2HPO_4, pH 7.0. They were collected by a second centrifugation and stored at −80°C.

Bacteria cultured in the presence of ^{63}Ni were grown in an 8-liter culture by the procedures described above, except that 2 mCi of $^{63}NiCl_2$ was used instead of $NiCl_2$ normally added to the culture medium. This is equivalent to 1.49 μmol of $NiCl_2$ with a specific activity of 10.05 mCi/mg of $NiCl_2$. The cells were harvested after 29 days.

Purification. Because of the lability of the hydrogenase in oxygen, certain precautions were taken throughout the procedure. All steps after cell breakage were performed under 4% H_2 in N_2 which had passed over a De-Oxo catalytic purifier and a BASF R3-11 catalyst heated to 100°C. All buffers and column materials were degassed and contained 1 mM dithionite and 1 mM dithiothreitol. The enzyme, except where specifically noted, was kept on ice, and the columns were equilibrated at 4°C. Protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride and 5 μM antipain) were added to all buffers. The protein was stored under H_2 at −80°C.

Portions (10 g) of cell paste were suspended in 50 ml of 50 mM Tris-hydrochloride, pH 7.5, to which 4 mg of DNase was added. The cell slurry was passed twice through a French pressure cell at 5,700 lb/in^2 (1 lb/in^2 ≅ 7 kPa). The pressure cell and collection vessel were both flushed with N_2. The crude extract containing soluble protein, membranes, and unbroken cells was centrifuged immediately in sealed tubes at 100,000 × *g* for 1 h. The pellet containing cell membranes and unbroken cells was suspended in 30 ml of 20 mM Tris-hydrochloride, pH 7.5, and centrifuged again. The pellet was resuspended in the same buffer, and Triton X-100 was added to a final concentration of 1%. This mixture was stirred at room temperature for 1 h and again centrifuged at 100,000 × *g* for 1 h. The pellet was discarded, and the Triton-solubilized supernatant was collected and concentrated to 5 ml in an ultrafiltration cell (Amicon Corp.; XM 50 membrane).

DEAE-cellulose chromatography. A column (1.5 by 28 cm) of DEAE-cellulose (Whatman DE52) was prepared and equilibrated with 20 mM Tris-hydrochloride, pH 7.5, containing 0.2% Genapol, 1 mM dithionite, and 1 mM dithiothreitol. The column equilibration was continued until the effluent would rapidly reduce a 0.1% solution of tetrazolium red (2,3,5-triphenyltetrazolium chloride). The concentrated enzyme was added to the column and eluted with a linear KCl gradient (400 ml of 0.03 to 0.15 M KCl) at a flow rate of 4 ml/h. Collection tubes were sealed with rubber serum stoppers and filled with H_2. Fractions (2 ml) were collected through a hypodermic needle connecting the column and collection tube. Excess pressure was released through a second hypodermic needle, the end of which was under water.

Sephacryl S-200 chromatography. Fractions from the DEAE-cellulose column containing hydrogenase activity were combined and concentrated to 1 ml. The concentrate was applied to a Sephacryl S-200 column (1 by 60 cm) equilibrated and monitored for reducing conditions as described above. Proteins were eluted with the equilibration buffer at a flow rate of 3 ml/h and collected in 2-ml fractions under H_2.

The column was calibrated under identical conditions with apo-ferritin, gamma globulin, bovine serum albumin, and cytochrome *c* reductase as standards.

Assays. Hydrogenase activity was measured by hydrogen-dependent methylene blue reduction as described previously (33). One unit of activity is the quantity of enzyme which catalyzes the reduction of 1 μmol of methylene blue per min.

Activity stains in native gels were performed by the method of Schneider and Schlegel (34), with the omission of NAD. In this procedure, nitro blue tetrazolium is used as the terminal acceptor.

Total protein was measured by the method of Bradford (6) with bovine serum albumin as a standard. The cytochrome content was measured by recording the difference spectrum of oxidized and reduced samples in the range of 400 to 600 nm (13). Enzyme isolated in the presence of dithiothreitol and dithionite was placed in sealed reference and sample cuvettes (0.3 ml) and flushed with H_2. The background spectrum was recorded. The reference cuvette was then sparged with water-saturated O_2 for 15 min, and the difference spectrum was recorded. The oxidation process was repeated once to ensure complete oxidation of the cytochromes in the reference cuvette.

Electrophoresis. SDS-PAGE was performed by the method of Laemmli (24). The samples were diluted 3:1 with sample buffer containing 20% glycerol, 10% β-mercaptoethanol, 5% SDS, and 0.125 M Tris-hydrochloride, pH 6.8. The gels were 1.5-mm-thick slabs of 10% acrylamide. Electrophoresis was at 20 mA per gel for 4 h.

Native PAGE was carried out by the procedure of Davis (11). The gels were the same dimensions as the SDS gels but had 5 to 20% polyacrylamide gradients. Electrophoretic conditions were the same as for the SDS gels. Samples were made to 10% glycerol before loading. All gel and tank buffers contained 0.2% Genapol to maintain enzyme solubility. All buffers were degassed. Reducing agents could not be added because they directly reduce the color reagent of the activity stain.

Two-dimensional PAGE was done by excising a lane from a native gel and treating it for 1 h at room temperature in full-strength SDS sample buffer. The treated slice was placed in a single-line well of an SDS gel and sealed in place with 1% agarose in 1/4 strength SDS stacking-gel buffer (27). The gels were electrophoresed as previously described.

The general protein stain used was 0.1% Coomassie blue in 30% methanol and 7.5% acetic acid. Protein standards for SDS gels were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

The density of protein stains on the SDS gels was determined by optical density scanning at 600 nm at 0.05 cm/min on a spectrophotometer (Gilford Instrument Laboratories, Inc.) fitted with a single-lane gel scanner. Scans were made of individual gel lanes excised from slab gels or of high-quality photographic negatives (10 by 12.5 cm) of the gels. Absorbance peaks were integrated, and the molar ratio was calculated on the basis of polypeptide molecular weights of 60,000 and 30,000.

Antibody production. Antiserum was made against the 60-kilodalton (kD) polypeptide of purified hydrogenase by the

procedures of Suissa and Reid (37). Pure protein (500 μg) was applied in a single 13-cm-wide well of an SDS slab gel and electrophoresed as described above. On completion, a 1-cm vertical slice was cut from the gel and stained for protein. The position of the 60-kD band was determined, and the corresponding horizontal slice of the remainder of the gel was removed. The gel slice was homogenized, and a portion was electrophoresed again to determine the purity and the extent of recovery. The homogenate was mixed with an equal volume of Freund complete adjuvant, and the mixture was injected into two rabbits at 20 intradermal dorsal sites per rabbit. Before injection, 5 ml of blood was drawn from each rabbit for preimmune control serum.

The immunization process was repeated after 2 and 4 weeks. The homogenate was mixed with incomplete adjuvant and injected at a single intramuscular site. After the injection series was complete, 20 ml of blood was collected by bleeding the marginal ear vein. The blood was allowed to coagulate overnight at room temperature, and the clot was removed by centrifugation at 4,000 × *g* for 15 min. Clear serum was decanted and stored in 0.5-ml aliquots at −20°C. No further processing was required for this study.

ELISA. The enzyme-linked immunosorbent assay (ELISA) procedure was adapted from the method of Towbin et al. (38). Incubation of nitrocellulose blots with the antiserum was for 3 h, usually at a dilution of 1:2,000. Incubation with horseradish peroxidase-conjugated anti-rabbit goat serum (1:3,000 dilution) was also for 3 h. The nitrocellulose blots were washed with 1 liter of Tris-saline buffer (38) in five 12-min washes between all steps. General protein staining of the nitrocellulose blots before the ELISA was in 0.25% amido black (naphthol blue black) in 50% methanol and 10% acetic acid.

Antiserum characterization. The antiserum to the 60-kD polypeptide was characterized by titration of serial dilutions of antiserum (1:1,000 to 1:20,000) against 2 μg of purified hydrogenase. Serial dilutions of protein (2 μg to 0.075 ng) were also titrated against constant antiserum concentration (1:2,000 dilution). Cross-reaction was detected by ELISA on nitrocellulose electroblots of SDS gels (38). In all cases, the controls contained the highest serum and protein concentrations with pre-immune serum substituted for the 60-kD antiserum.

Measurement of radioactive Ni. Liquid scintillation counting was done on sectioned lanes from SDS and native gels. Sections (4 mm) were dissolved in 30% hydrogen peroxide for 5 h at 60°C, and 10 ml of ScintiVerse E (Fisher Scientific Co.) was added. The samples were placed in the dark for 4 to 5 h or until the count rates were stable and counted four times on the ^{14}C channel of an LS 6800 liquid scintillation counter (Beckman Instruments, Inc.).

Autoradiography was done on ELISA blots of native and SDS gels. Kodak XAR-5 X-ray film was exposed by contact for 1 week at room temperature and processed by standard procedures.

RESULTS

Purification. Although a variety of methods and conditions have been used to purify hydrogenase from various organisms, our experience with the membrane-bound hydrogenase from *R. japonicum* indicates that there are two essential requirements: (i) strict anaerobic conditions in the presence of reducing agents, and (ii) the presence of a detergent to maintain protein solubility. Attempts to purify the protein without taking these precautions have been unsuccessful.

A summary of the hydrogenase purification is shown in Table 1. The specific activity in the most highly purified fraction was 68.8 U/mg of protein, and the yield was only 3% of the initial activity; 30% of the protein loss could be accounted for in the unbroken cells remaining after solubilization of the broken membranes. Subsequent losses are undoubtedly due to protein inactivation.

TABLE 1. Purification of hydrogenase from autotrophically grown *R. japonicum*

Purification step	Total amt of protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)
Crude extract[a]	474.3	2,272	4.8	100
Triton extract	65.6	1,238	18.9	54
DEAE 1 (fractions 30 to 34)	10.5	211	20.1	9
DEAE 2 (fractions 36 to 47)	5.9	312	52.9	14
Sephacryl 1 (fractions 13 to 17)[b]	5.2	152	29.2	7
Sephacryl 2 (fractions 13 to 17)[c]	2.8	152	54.4	7
Sephacryl 2 (fraction 15)	0.9	62	68.8	3

[a] The crude extract is a mixture of soluble protein, membranes, and unbroken cells.
[b] Sephacryl S-200 chromatography of DEAE 1.
[c] Sephacryl S-200 chromatography of DEAE 2.

The elution profile of the DEAE-cellulose column (Fig. 1) shows two activity peaks. The reasons for this separation are as yet unclear. Fractions corresponding to these two peaks were collected (fractions 30 to 34, referred to as DEAE 1, and fractions 36 to 47, referred to as DEAE 2) and chromatographed separately on Sephacryl S-200 columns (DEAE 1 on Sephacryl 1, DEAE 2 on Sephacryl 2).

The elution profiles of the Sephacryl 1 and Sephacryl 2 fractions (Fig. 2) reflect a slight increase in specific activity (Table 1). The two Sephacryl elution profiles of hydrogenase activity are essentially the same, and all detectable hydrogenase protein is in the column void volume.

The progress of purification is also illustrated by the SDS gel protein patterns of fractions from the different purification steps (Fig. 3). Lanes 7 to 9 contain two major proteins of 60,000 ± 500 and 30,000 ± 900 molecular weights. These two polypeptides are also discernible in earlier purification fractions (lanes 2, 4, and 5) and represent a significant portion of the detectable protein in these fractions. The greatest purification was obtained by DEAE-cellulose column chromatog-

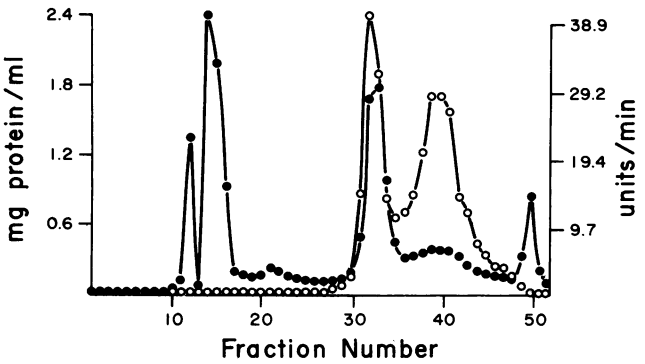

FIG. 1. Elution profile of the DEAE-cellulose column. Symbols: ○, hydrogenase activity; ●, total protein. This profile represents only the 0.03 to 0.06 M portion of the linear KCl gradient.

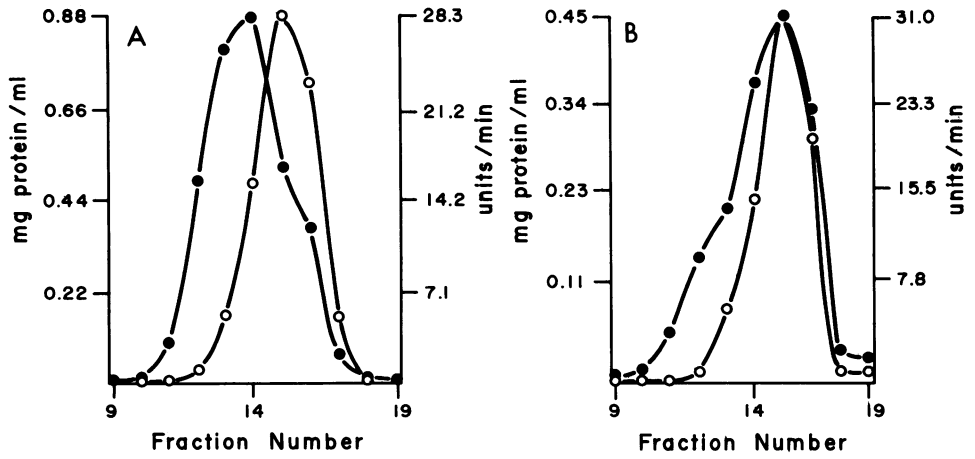

FIG. 2. Elution profiles of Sephacryl S-200 exclusion gel chromatography. (A) Sephacryl 1 (from DEAE 1: pooled fractions 30 to 34), and (B) Sephacryl 2 (from DEAE 2: pooled fractions 36 to 47). Symbols: ○, hydrogenase activity; ●, total protein. There were no detectable proteins in fractions other than fractions 9 to 19. Protein and activity peaks coincide in B.

raphy (lane 5 versus lanes 7 and 8). Fraction 15 of Sephacryl 1 (lane 9) is clearly different with respect to the 30-kD polypeptide. The prominent doublet in lane 9 is also present to a lesser extent in lanes 7, 8, and 10. All fractions of Sephacryl 1 which contained active hydrogenase showed the prominent doublet. In previous purifications, in the absence of protease inhibitors, purified hydrogenase often showed as many as four bands in the 30-kD region. We conclude that the diminished quantity of the 30-kD protein in Sephacryl 1 (lane 9) is probably due to the action of a protease present in DEAE 1 (lane 7) but not in DEAE 2 (lane 8).

There was no detectable cytochrome spectrum associated with the active hydrogenase fractions from either Sephacryl column.

Antiserum characterization. The hydrogenase antiserum was made against the 60-kD polypeptide isolated from the SDS gels. After the 6-week immunization schedule, the antiserum cross-reaction was verified by ELISA as described above. Nitrocellulose electroblots of SDS gels treated by ELISA with the 60-kD antiserum (1:1,000 dilution) (Fig. 4, lane 1) and the corresponding control serum (lane 2) are shown. The cross-reaction is specific for the 60-kD polypeptide, although both polypeptides were detected by amido black protein staining (lane 3). We conclude that the 30-kD protein is a unique polypeptide, and not a breakdown product of the larger protein.

Titrations performed with decreasing amounts of enzyme indicated detection limits of 150 ng with a 1:2,000 dilution of antiserum and 0.075 ng with a 1:200 dilution of antiserum (data not shown).

Polypeptide characterization. Native gels of the purified hydrogenase show the comigration of protein detected by the protein stain, the activity stain, and the ELISA (Fig. 5). Lanes a and b were loaded with 125 μg of purified hydrogenase and treated identically except that lane a was incubated under N_2, showing the H_2 dependence (lane b) of the

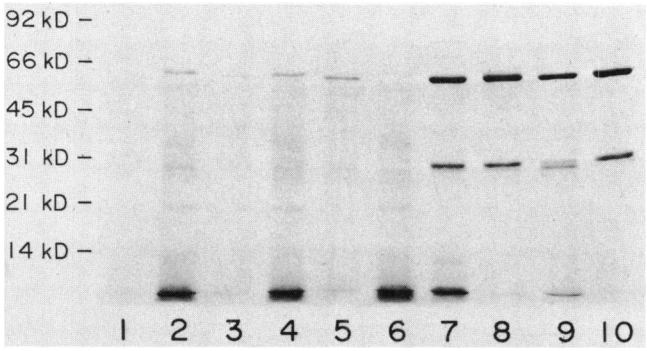

FIG. 3. SDS-PAGE of protein samples from all purification steps. Lanes: 1, Supernatant, and 2, pellet resulting from the 100,000 × *g* centrifugation of the crude extract; 3, supernatant, and 4, pellet from the 100,000 × *g* centrifugation of the pellet from 2; 5, supernatant, and 6, pellet from the 100,000 × *g* centrifugation of the Triton-treated pellet from 4 (all material collected from lane 5 was chromatographed on DEAE-cellulose); 7, fraction 32 (DEAE 1); 8, fraction 39 (DEAE 2) (the two DEAE-cellulose peak fractions were chromatographed separately on Sephacryl S-200); 9, fraction 15 of Sephacryl 1 (from DEAE 1); 10, fraction 15 of Sephacryl 2 (from DEAE 2).

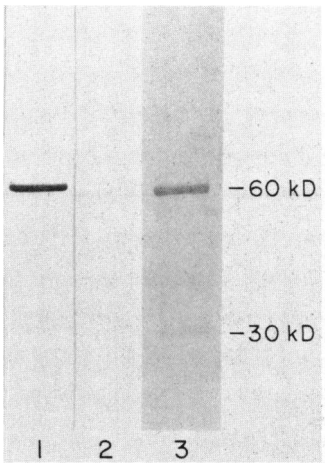

FIG. 4. ELISA and protein stain detection of purified hydrogenase from Sephacryl 2 (fraction 15). Hydrogenase (5 μg) was electrophoresed by SDS-PAGE and electroblotted to nitrocellulose. Lanes: 1, ELISA with a 1:1,000 antiserum dilution; 2, ELISA with a 1:1,000 control serum dilution, 3, amido black protein stain. The 30-kD band in lane 3 is less prominent than would be expected because of poor staining of the 30-kD polypeptide by amido black.

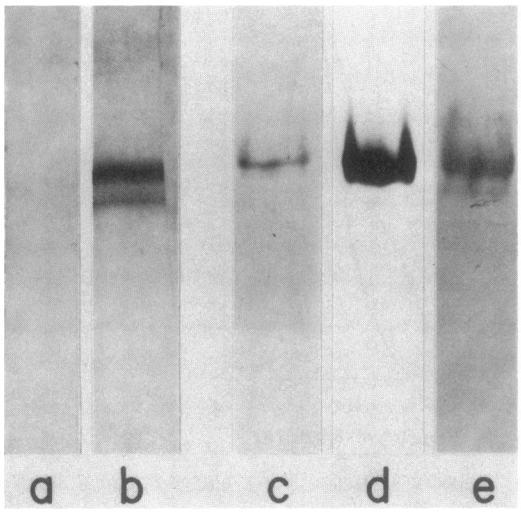

FIG. 5. Native PAGE of purified hydrogenase (Sephacryl 2, fraction 15) (lanes a and b were loaded with 125 μg of protein; lanes c to e were loaded with 23 μg of protein). Lanes: a, activity stain under nitrogen; b, c, activity stain under hydrogen; d, Coomassie blue protein stain; e, ELISA with a 1:2,000 antiserum dilution.

hydrogenase activity stain. Owing to the greatly different sensitivities of the three procedures, it is difficult to balance the protein load to achieve a well-defined ELISA and still detect activity. The ELISA (lane e) shows multiple banding, which can be shown in the activity stain (lanes b and c) only with a very heavy protein load (lane b). This is probably due to progressive inactivation of the labile protein during electrophoresis. Protein electrophoresed in the presence of higher and lower Genapol or Triton concentrations yielded similar multiple banding patterns.

Attempts at native molecular weight characterization were inconclusive. The hydrogenase could not be successfully electrophoresed without the presence of 0.2% Genapol. In gels containing Genapol, standard proteins migrated in an erratic and uninterpretable manner.

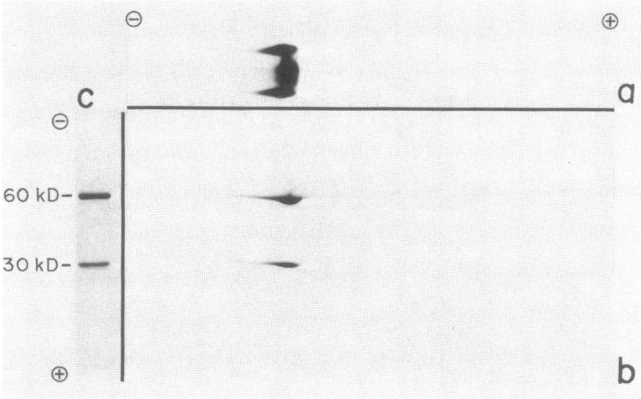

FIG. 6. Two-dimensional electrophoresis of purified hydrogenase (23 μg of Sephacryl 2, fraction 15). Lanes: a, native gradient gel from lane adjacent to that used for the second-dimension SDS gel; b, second-dimension SDS gel; c, standard SDS gel of 10 μg of Sephacryl 2, fraction 15, showing the position of the 60- and 30-kD polypeptides.

TABLE 2. Molar ratio of the 60- and 30-kD polypeptides of *R. japonicum* hydrogenase as measured by SDS-PAGE and optical densitometry

Sample description[a]	No. of samples	Molar ratio (mean ± SEM)
Crude extract	1	0.86
Triton extract	2	1.16 ± 0.21
DEAE 1 and 2 fractions	8	0.99 ± 0.05
Sephacryl fractions[b]	7	1.04 ± 0.08
Sephacryl 1 fractions	6	1.02 ± 0.09
Sephacryl 2 fractions	11	1.05 ± 0.06

[a] See Table 1 for a description of the purification steps.
[b] From purification experiments not shown in Table 1.

Subunit composition. We have shown above that the purified hydrogenase fractions contain two polypeptides (Fig. 3). Two-dimensional gels (Fig. 6) of lanes excised from the native gels described in the legend to Fig. 5 also show that the enzyme contains two components. The second-dimension SDS gel (Fig. 6b) of the native hydrogenase shows that two polypeptides were derived from the protein band corresponding to hydrogenase on the native gel (Fig. 6a). Furthermore, the migration of these two polypeptides corresponds to that of the 60- and 30-kD polypeptides derived from the purified enzyme by standard SDS-PAGE (Fig. 6c).

These two polypeptides copurify, comigrate on native gels, and comigrate in sucrose density gradients (data not shown). They are only separable by denaturation in SDS. Densitometric scans of SDS gels of protein samples from various stages of purification yield a molar ratio of 1.03 ± 0.03 (Table 2). Data from scans of gels of crude and Triton extracts often were not definitive because of heavy background staining.

Nickel content. *R. japonicum* hydrogenase from cells grown in ^{63}Ni was extracted through the Triton step de-

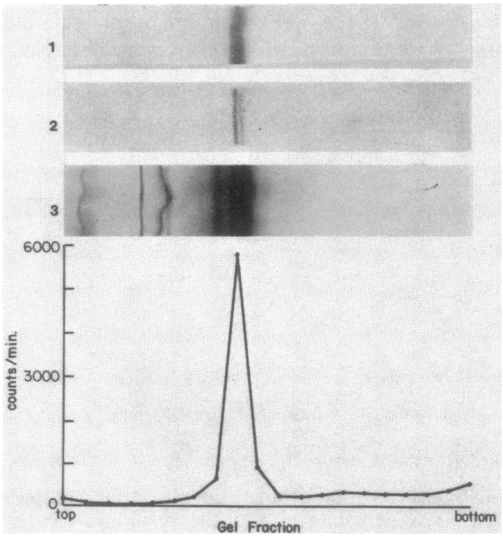

FIG. 7. Native gradient PAGE of the Triton-extracted hydrogenase from cells grown in $^{63}NiCl_2$. Protein was loaded in a single 13-cm-wide well, and the gel was cut into 2-cm lanes. One lane was cut into 20 fractions, digested, and counted by liquid scintillation; the graph shows the position of the radioactivity. Lanes: 1, nitrocellulose blot indicating the position of the 60-kD polypeptide as detected by ELISA; 2, autoradiogram of the ELISA-treated nitrocellulose blot; 3, native gel stained for total protein.

scribed above. The Triton extract had a specific activity of 14.2 U/mg of protein and contained one-third of the radioactive counts measured in the crude cell preparation. A sample of Triton extract containing 375 μg of protein was electrophoresed on a nondenaturing gel. Protein was applied in a single well 13 cm wide. After electrophoresis, lengthwise portions of the gel were excised for liquid scintillation counting, protein staining, and ELISA. After the ELISA was developed, it was placed in contact with X-ray film for autoradiography. All radioactivity comigrated with the 60-kD polypeptide as detected by the ELISA (Fig. 7).

The portion of the native gel containing ^{63}Ni was excised and electrophoresed in a second-dimension SDS gel. The 30- and 60-kD polypeptides were the only proteins contained in this region in significant amounts. All the radioactivity migrated with the buffer salt front (data not shown).

DISCUSSION

Hydrogenase isolated from *R. japonicum* shows multiple activity peaks when purified by DEAE-cellulose chromatography and multiple activity bands when subjected to nondenaturing PAGE. This type of behavior by membrane-bound hydrogenases is not without precedent. Hydrogenase from *A. eutrophus* elutes in two peaks from carboxymethyl cellulose, and both peaks have identical SDS-PAGE banding patterns (33). The hydrogenase from *M. thermoautotrophicum* is separated into two peaks on hydroxyapatite (19). The solubilized membrane hydrogenase from *R. capsulata* in the absence of Triton elutes in the void volume of a Sepharose 6B column (9). Addition of 0.05% Triton to the elution buffer results in the separation of two activity peaks, but their relative concentrations were not constant in different experiments. In the absence of 0.2% Triton in native gels, hydrogenase from *Rhodopseudomonas capsulata* precipitates at the interface of the stacking and resolving gels (9). Profiles of hydrogenase from *Chromatium vinosum* eluted from the Sephadex G-75 column are altered significantly by the concentration of detergent in the elution buffer (22). Aggregation of hydrogenase from *Paracoccus denitrificans* (36) and *X. autotrophicus* (32) has been reported. Hydrogenase from *V. succinogenes* shows multiple banding after sucrose density gradient centrifugation (39).

Hydrogenase from *R. japonicum* elutes in the void volume of the Sephacryl S-200 column in the presence of 0.2% Genapol. The same Genapol concentration causes protein standards to elute in the void volume. In previous work with *R. japonicum* hydrogenase (4), purification after Triton extraction was done in the absence of detergent. Glycerol (20%) stabilizes the enzyme but causes it to elute in the void volume of a Sephadex G-200 column.

It our experience, detergent is necessary to maintain the solubility of the hydrogenase from chemolithotrophically grown *R. japonicum*. It appears, however, to alter the mobility and elution patterns of both the hydrogenase and the standard proteins under nondenaturing conditions. Further work is necessary to understand the complex effect of the detergent on protein behavior. We conclude on the basis of consistent polypeptide constituents on SDS-PAGE and cross-reaction with the 60-kD antiserum that only one hydrogenase is present in *R. japonicum* and that the apparent complexities in native gels and column chromatography are artifacts related to the presence of the detergents.

Hydrogenase from chemolithotrophically cultured *R. japonicum* was a nickel enzyme. This result is consistent with those for other organisms (10, 17, 19, 25, 39) and the result of Klucas et al. (23), which showed the requirement for nickel in hydrogenase expression. The molar ratio of nickel to hydrogenase protein has not been determined.

Although cytochromes *b* and *c* are abundant in the Triton-solubilized membrane extract, they are rapidly removed by DEAE-cellulose and Sephacryl S-200 chromatography. There is no detectable cytochrome in the active hydrogenase fractions of the Sephacryl columns. These results support earlier work done in this laboratory by Liang-Shu Xu and coworkers (unpublished data), but differ from those obtained with the hydrogenase of *X. autotrophicus*, where a cytochrome *b* component was associated with the homogenous hydrogenase preparation (32). Our observations do not preclude the importance of cytochrome in electron transport from H_2 to O_2 as suggested by Eisbrenner et al. (15), but indicate that none of the cytochromes involved in electron transport remains attached to the hydrogenase during the purification procedure.

Evidence from SDS-PAGE indicates that *R. japonicum* hydrogenase is composed of two subunits of approximate molecular weights of 60,000 and 30,000, respectively. This subunit composition is similar to that reported for several other hydrogenases (32, 33, 35, 39, 40). The ELISA characterization of the 60-kD antiserum (Fig. 4) provides no evidence that the 30- and 60-kD polypeptides are related. If the smaller polypeptide were a domain of the larger, one would expect a degree of cross-reaction and detection by ELISA. The two polypeptides copurify and comigrate on native gels and sucrose density gradients. The consistent molar ratio of ca. 1:1 of the subunits throughout purification seems to preclude copurification of the two components by random aggregation of lipophilic polypeptides.

From experiments in which protease inhibitors were omitted during the purification, we conclude that the multiple bands in the 30-kD range of some SDS gel experiments are products of proteolysis. Previous work with *R. japonicum* (4) reported a single subunit of 63 kD with major contaminants near 30 kD. The lack of significant quantities of 30-kD protein may have been due to proteolysis or to the differences between hydrogenases from *R. japonicum* bacteroids and those from *R. japonicum* grown chemolithotrophically.

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