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PURIFICATION AND PROPERTIES OF THE PARTICULATE HYDROGENASE FROM THE BACTEROIDS OF SOYBEAN ROOT NODULES

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Summary

The uptake hydrogenase (hydrogen:ferricytochrome c_3 oxidoreductase, EC 1.12.2.1) from the bacteroids of soybean root nodules infected with *Rhizobium japonicum* 110 has been purified and characterized. Bacteroids were prepared, then broken by sonication. The particulate enzyme was solubilized by treatment with Triton X-100 and further purified by polyethylene glycol fractionation, DEAE-cellulose and Sephadex G-100 chromatography. The specific activity has been increased 196-fold to 19.6 units/mg protein. The molecular weight is 63 300 as determined by gel filtration and 65 300 as determined by SDS-polyacrylamide gel electrophoresis, indicating that the enzyme is a monomer. The enzyme is O_2 sensitive, with a half-life of 70 min when exposed to air.

The pH optimum of the solubilized enzyme is near 5.5; the K_m for H_2 is 1.4 μM. Suitable electron acceptors are methylene blue, ferricyanide, 2,6-dichlorophenolindophenol, and cytochrome *c*. Benzyl viologen is reduced slowly; methyl viologen, $NAD(P)^+$, FAD, FMN, and O_2 are not reduced. The optimum temperature for activity is 65—70°C with an activation energy of 9.2 kcal. H_2 evolution by the enzyme has been demonstrated. The hydrogenase is well-suited to function in an environment where all the available H_2 is generated in situ.

Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

The nodules of most N_2-fixing plants evolve H_2 via the nitrogenase system [1,2]. This process wastes a considerable amount of the energy flux through nitrogenase. A few species of *Rhizobium* and all of the actinorhizal non-legume systems studied form nodules that do not evolve H_2 and are capable of taking up H_2 from the atmosphere [2,3]. A hydrogenase (hydrogen:ferricytochrome c_3 oxidoreductase, EC 1.12.2.1) of the H_2-oxidizing type is responsible for this activity. The presence of this enzyme in pea root nodules was first described in 1941 by Phelps and Wilson [4] and confirmed by Dixon [5] in 1967. Dixon suggested three possible functions for the enzyme: (1) O_2 scavenging through the oxyhydrogen reaction; (2) elimination of inhibition of nitrogenase by H_2, and (3) as part of a recycling system to recoup some of the energy lost to H_2 evolution [6]. Dixon found that the hydrogenase of *Rhizobium* bacteroids was particulate, could reduce methylene blue and ferricyanide rapidly and benzyl viologen slowly and could not reduce methyl viologen, NAD^+, $NADP^+$, nitrate or fumarate. He also showed that H_2 oxidation was coupled to phosphate esterification. Purification and characterization was limited by the instability of the hydrogenase and the small quantity present in the bacteriods.

Hydrogenases have been extensively purified and characterized from *Clostridium pasteurianum* [7,8], *Desulfovibrio vulgaris* and *Desulfovibrio gigas* [9—11], *Chromatium* [12], *Rhodospirillum rubrum* [13], *Alcaligenes eutrophus* [14], *Pseudomonas ruhlandii* [15], and *Thiocapsa roseopersicina* [16] and are found to be a heterogenous group. They may be particulate or soluble, H_2 oxidizing and/or H^+ reducing, from aerobic, anaerobic, or facultative organisms. Most of the reported purifications are from anaerobes. The hydrogenases purified from the aerobes *A. eutrophus* and *P. ruhlandii* are soluble, but no particulate hydrogenases from aerobic organisms have previously been purified. We report here on the extensive purification and characterization of the catalytic properties of the hydrogenase from bacteroids of soybean root nodules infected with *Rhizobium japonicum* 110, a strain that produces an uptake hydrogenase.

Materials and Methods

Chemicals. The chemicals used were obtained from: Whatman, DEAE-cellulose (DE 52); Pharmacia, Sephadex G-100 and G-200; Bio-Rad, sodium dodecyl sulfate (SDS), Bio-Beads SM-2, acrylamide, *N,N'*-methylene bisacrylamide; Mann Research Lab., benzyl viologen; Sigma Chemical Co., methyl viologen, methylene blue, polyvinylpolypyrrolidone, cytochrome *c* (from horse heart), all coenzymes, all buffers, and all molecular weight standards (except *Azotobacter vinelandii* dinitrogenase and dinitrogenase reductase which were a gift from Bob Hageman); Research Products Int. Co., Triton X-100; J.T. Baker Co., sodium dithionite; Calbiochem, dithiothreitol. All other chemicals were of the highest purity available.

Plant growth. Soybeans (Hodgson) were treated with a peat-based inoculum of *R. japonicum* 110 (culture supplied by W.J. Brill), planted in Perlite and grown in a glasshouse. Nitrogen-free nutrient solution [17] was applied once a

week and the plants were watered twice weekly. Auxiliary light was supplied by sodium vapor lamps for 16 h/day. Within 6—7 weeks the plants flowered and the nodules were harvested within the next seven days. Nodules were stored in liquid N_2. About 300 mg of nodules were obtained/plant.

Assays for hydrogenase activity. Routine assays of H_2 uptake were performed in double-serum-stoppered cuvettes similar to those described by Averill et al. [18] except that no side arms were present and all evacuations and additions were done through the serum stoppers with syringe needles. Assay reaction mixtures contained 38 μmol of MES buffer at pH 6.0 and 200 nmol of methylene blue in 2 ml. Reactions were run at 30°C under 1 atm H_2 and absorbance was measured at 600 nm with an experimentally determined extinction coefficient. The reaction also could be followed amperometrically [19], and under the same conditions the two methods gave the same results. H_2 evolution was determined amperometrically. A unit of activity is the amount of enzyme required to catalyze the oxidation of 1 μmol of H_2 in 1 min at 30°C.

Reduction of various electron acceptors was followed spectrophotometrically at appropriate wavelengths. Extinction coefficients for methyl viologen, benzyl viologen, NAD(P)H, FAD, FMN, and ferricyanide were accepted from the literature. Values for cytochrome *c* and methylene blue were determined experimentally. To determine whether or not O_2 was an acceptor, H_2 uptake was followed amperometrically.

Estimation of molecular weight and purity. The molecular weight was determined by Sephadex G-100 and G-200 chromatography with myoglobin, ovalbumin, bovine serum albumin, and alcohol dehydrogenase as molecular weight standards. Subunit composition and molecular weight were determined with SDS-polyacrylamide gel electrophoresis by the method of Laemmli [20] with bovine serum albumin, ovalbumin, myoglobin, and *A. vinelandii* dinitrogenase and dinitrogenase reductase as standards.

Gels for native proteins were run as described [21] under anaerobic conditions. Hydrogenase activity was localized by slicing the gel into 2.5-mm sections; each section was crushed in anaerobic buffer (20 mM MES, pH 6.0) and and aliquot was assayed. Recovery of activity was from 30% to 100%.

Protein assay. Protein was estimated by the dye-binding method of Bradford [22] or by the microbiuret method [23] with bovine serum albumin as a standard.

Enzyme purification. Because of the oxygen sensitivity of the enzyme, all steps in the purification were performed anaerobically. All buffers contained 1 mM dithionite and 1 mM dithiothreitol. All operations were at room temperature except where indicated otherwise; centrifugations were at 4°C.

Bacteroids were prepared by a modification of the procedure described by Klucas et al. [24]. 200 g of nodules were thawed in 400 ml of 100 mM potassium phosphate buffer (pH 7.0) that was 0.2 M in ascorbate and contained 70 g of polyvinylpolypyrrolidone. The mixture was homogenized for 1 min in a Waring blendor and then was poured into a basket centrifuge. The perforated walls of the 200 ml basket were lined with two layers of 68 mesh/cm silk cloth. This retained the bulk of the nodule debris and polyvinylpolypyrrolidone while allowing the bacteroids and cytosol to pass. The supernatant was collected in

250-ml centrifuge bottles and centrifuged at 40 × *g* for 5 min to remove the remaining debris and polyvinylpolypyrrolidone. The supernatant then was centrifuged at 15 000 × *g* for 10 min. The pellet, containing the bacteriods, was washed and centrifuged in 100 ml of 20 mM (pH 7.4) Tris buffer. The pellet was resuspended in sufficient 50 mM Hepes (pH 7.5) to make a total of 70 ml. The suspension was sonicated in two portions at full power (350 W) for a total of 5 min with an Ultrasonics model 350 sonicator operating in the pulsed mode at 30% duty time. The sonicate was transferred to 25-ml centrifuge tubes and centrifuged for 1 h at 130 000 × *g* to sediment the particles.

70% of the hydrogenase activity was sedimented. This hydrogenase was solubilized by resuspending the pellet in 20 ml of 20 mM Mes (pH 6.0) that contained 1% Triton X-100. The suspension was shaken gently at 30° C for 1 h, then heated to 50° C for 15 min. The tubes were cooled and again centrifuged at 130 000 × *g* for 1 h. 70—80% of the remaining hydrogenase was now in the supernatant; it was made to 25% (w/v) with polyethylene glycol 4000 (Union Carbide), gently shaken for 15 min, and centrifuged for 30 min at 27 000 × *g* to sediment the hydrogenase. The pellet was resuspended in a minimum volume of 20 mM Tris (pH 7.4) and passed through a 1 cm × 7 cm column of Sephadex G-25 with a 3 cm layer of Bio-Beads (to remove Triton X-100) [25] on top. The column was equilibrated with 20 mM Tris (pH 7.4).

The eluant was then loaded onto a 2.5 cm × 15 cm column of DEAE-cellulose equilibrated with 20 mM Tris (pH 7.4), 50 mM NaCl. Hydrogenase was eluted with the same buffer. The column fractions with the highest specific activities were concentrated with an Amicon ultrafiltration cell and UM-20 membrane.

The concentrated solution was loaded onto a 1.5 × 100 cm Sephadex G-100 column equilibrated with 20 mM Tris (pH 7.4). The column was run with upward flow with the 20 mM Tris, and protein elution was followed at 280 nm with an ISCO model UA5 column monitor. The material from the symmetrical hydrogenase peak was collected and concentrated as before. The enzyme was stored in a double-serum-stoppered vial at 4° C.

Results and Discussion

Stability and storage

In whole nodules frozen in liquid N_2 hydrogenase is stable for at least several months. When purification is started there is a rapid loss of activity (half-life of about 5 h) to about 30% of the initial activity. The remaining activity decays slowly upon storage at 4° C (half-life of several days). The cause of this biphasic loss of activity is unknown. The enzyme can be stored indefinitely in liquid N_2, but it loses activity upon repeated freezing and thawing. When 0.1 ml of the enzyme is opened to the atmosphere in a 9 ml vial and shaken occasionally, O_2 inactivates the enzyme with a half-life of 70 min; hydrogenases commonly are O_2 sensitive [26].

Molecular weight and purity

As shown in Table I, purification increased the specific activity nearly 200-fold to 19.6 units/mg protein, but multiple bands still appeared on SDS and

TABLE I

PURIFICATION OF HYDROGENASE FROM SOYBEAN NODULE BACTEROIDS

PEG: polyethylene glycol.

	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)	Yield (%)
Broken bacteroids	3190	320	0.10	1	100
Triton treatment	384	160	0.40	4	50
PEG fractionation	55	60	1.09	11	19
DEAE-cellulose	8.8	34	3.9	39	11
Sephadex G-100	1.1	22	19.6	196	7

native gels. Fig. 1 illustrates the progess of the purification, and indicates that the purest fraction yielded a single major band and several minor bands on an SDS gel. Hydrogenase activity was recovered from native gels in a single peak corresponding to the position of the major stained protein (Fig. 2). The molecular weight of the enzyme is 63 300 ± 5600 as determined by gel filtration and

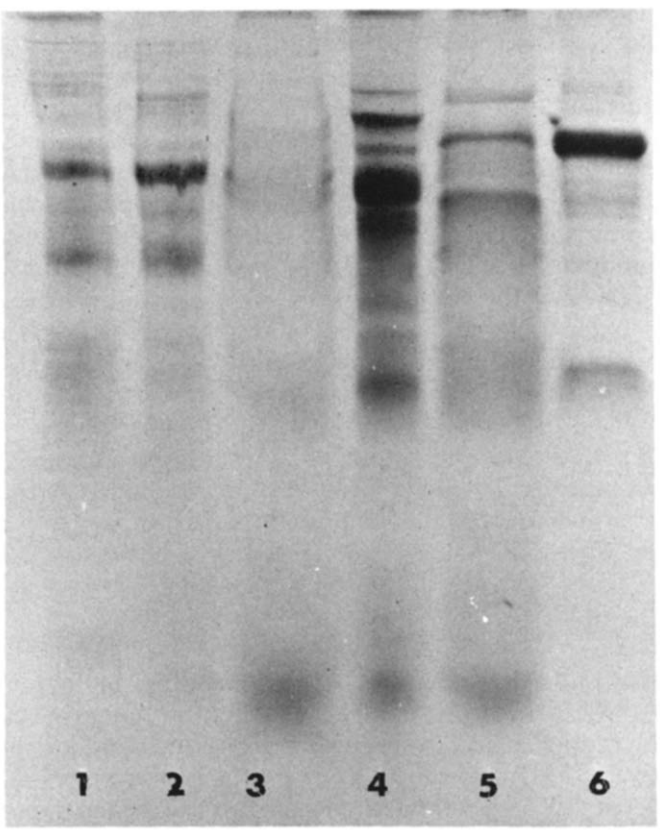


Fig. 1. SDS slab gel showing progress of purification. (1) Broken bacteroids; (2) 1st 130 000 × *g* supernatant; (3) 2nd 130 000 × *g* supernatant; (4) 25% polyethyleneglycol fractionation; (5) concentrate from DEAE column, and (6) Sephadex G-100 concentrate.

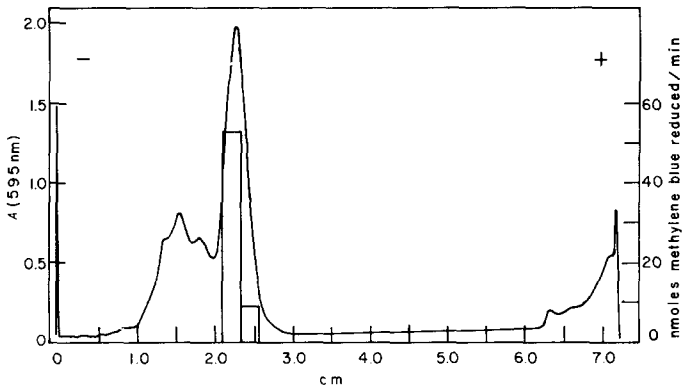


Fig. 2. Native gels stained for protein with Coomassie brilliant blue G-250 (———) and analysed for hydrogenase activity (bar graph) as described in Materials and Methods. The bar graph indicates the total hydrogenase activity/gel section. The cathode (—) was at the top of the gel.

65 300 ± 2000 as determined by SDS gel electrophoresis; this indicates that the enzyme is a monomer.

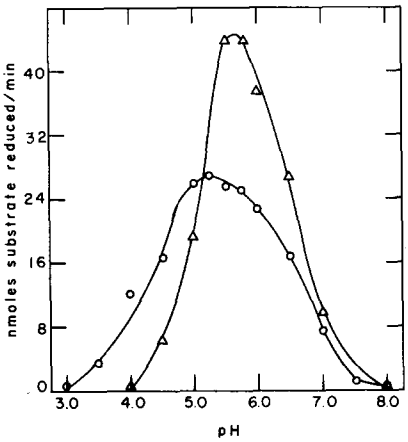
Soluble and solubilized activity

When bacteroids are broken by sonication and then are centrifuged, 30% of the hydrogenase activity stays in the supernatant and the rest is sedimented. This response is not uncommon for hydrogenases [26]. Hydrogen bacteria often yield a soluble hydrogenase capable of reducing NAD^+ and a membrane-bound enzyme which cannot reduce NAD^+ [14]. Comparisons of the soluble and particulate enzymes from *T. roseopersicina* indicated that they were the same enzyme [16]. It generally is assumed that the particulate and soluble enzymes performed different functions in the cell [27], but in some cases the soluble enzyme may be an artifact of the cell-breakage procedure. We have only characterized the soluble enzyme to show that it reduces methylene blue, does not reduce NAD^+, behaves like the solubilized enzyme on DEAE-cellulose, is heat stable, O_2 sensitive, and is reversible; thus, the two enzymes are similar in all respects examined.

Triton X-100 is a non-ionic detergent commonly used in enzyme solubilization [28]. Lauryl dimethylamine oxide (Onyx Chemical Co.) solubilized the enzyme but gave lower yields than Triton X-100. Cholate (tried up to 1%) or 1 M NaCl failed to release any enzyme. 1% deoxycholate inactivated the enzyme.

pH profile

The optimum pH is 5.3 for reduction of methylene blue and 5.7 for reduction of ferricyanide (Fig. 3). The enzyme is rapidly inactivated in the presence of ferricyanide, both while reducing ferricyanide and while incubated with ferricyanide in the absence of H_2. Addition of a second aliquot of enzyme restores the initial rate of ferricyanide reduction. The particulate hydrogenase from *A. vinelandii* has a pH optimum at 8.0 [29], as does the soluble hydrogenase from *A. eutrophus* [14]. Particulate hydrogenase from soybean bacteroids taken up H_2 optimally near pH 9.0 (data not shown), but the



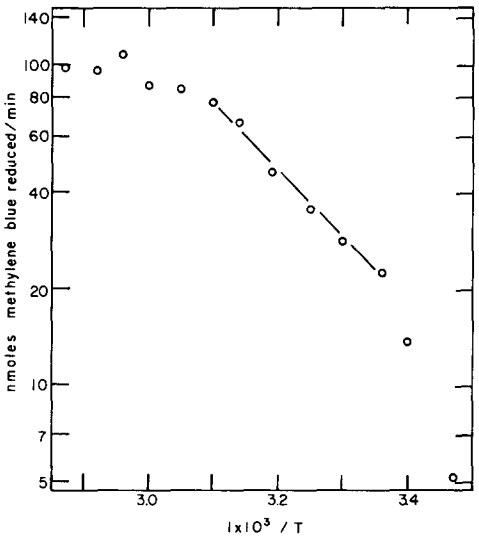


Fig. 3. pH profile. Reactions were performed at 30°C under 1 atm H_2. Substrate concentration was 100 μM. ○———○, methylene blue; △———△, ferricyanide. The results are typical of three experments.

Fig. 4. Arrhenius plot to determine energy of activation. Reactions were run as described in Materials and Methods and were initiated by the addition of 5 μl of enzyme solution. Each point is the average of trplicate assays. The results are typical of two experiments. The temperature is used as K.

optimum shifts to near 5.3 upon solubilization. Perhaps the pH optimum for the hydrogenase bound to the membranes of the bacteroids merely reflects the optimum for an endogenous electron acceptor that still is coupled to hydrogenase, although the act of solubilization may cause the optimum for hydrogenase to shift.

Temperature profile

The optimal temperature range for hydrogenase activity is 65—70°C, and above this the enzyme is unstable and the activity falls off rapidly. The high thermal stability and temperature optimum are characteristic of most hydrogenases. The linear Arrhenius plot from 25°C to 55°C (Fig. 4) indicates an

TABLE II

UPTAKE OF H_2 COUPLED TO VARIOUS ACCEPTORS

Reactions were performed as described in Materials and Methods at pH 6.0, 30°C, 1 atm H_2 and 100 μM acceptor. Each value is an average of triplicate samples; results are typical of two experiments. DCPIP, 2,6-dichlorophenolindophenol (Sigma Chemical Co.).

Acceptor	Relative activity (%)	E'_0 (mV)
Methylene blue	100	11
Ferricyanide	134	360
Cytochrome *c*	80	250
DCPIP	78	217
Benzyl viologen	0.3	−360
Methyl viologen	<0.1	−440

activation energy of 9.2 kcal. Values of 7—15.5 kcal have been reported for hydrogenases [26]. Hyndman et al. [29] found 15.5 kcal for *A. vinelandii* hydrogenase.

Acceptor specificity

Table II records the relative activity of a series of electron acceptors for hydrogenase. In general, positive potential electron acceptors couple best. FAD, FMN, NAD^+, and $NADP^+$ also were tested and were not reduced under the conditions specified in the table. The purified enzyme did not take up H_2 with O_2 as the electron acceptor. These results are in agreement with those reported by Dixon [6] for *Rhizobium phaseoli* bacteroids and by Hyndman et al. [29], for *A. vinelandii*. The inability to reduce methyl viologen is in contrast to other hydrogenases and indicates a more positive effective potential for this type of hydrogenase.

K_m for H_2

The K_m for H_2 is low; we have observed values from 0.9 μM to 2.5 μM with an average value of 1.4 μM. This is in reasonable agreement with the value of 2.8 μM in intact bacteriods reported by McCrae et al. [30]. We have obtained similar results by measuring the K_m either spectrophotometrically or amperometrically with methylene blue as the electron acceptor. Few K_m values for H_2 from purified hydrogenases have been reported. The K_m for H_2 for hydrogenase from *C. pasteurianum* varies with pH from 218 to 562 Torr at 25°C (222—574 μM) [8]; that from *A. eutrophus* is 37 μM [14].

Reversibility

Dixon reported that the hydrogenase action of *Rhizobium* bacteroids and *A. vinelandii* could not be reversed [6]; this agreed with previous findings for *A. vinelandii* [31]. Both attempts were with cell-free extracts but the pH was not specified. We found H_2 evolution at pH 6.0 at all stages of the purification with reduced methyl viologen as the electron donor; values were from 0.5% to 2% of the uptake rate at the same temperature and pH. The reaction is pH dependent (Table III), much slower at pH 7.0 than at pH 6.0, and the rate increases with descent to pH 4.0.

TABLE III

H_2 EVOLUTION FROM REDUCED METHYL VIOLOGEN

H_2 evolution was detected amperometrically and reactions were run at 30°C. Reaction mixtures contained 10 μg of protein and 1 mM methyl viologen. Methyl viologen was reduced with dithionite. Each value is an average of duplicate assays, and results are typical of three experiments.

pH	nmol H_2 evolved/min
7.0	0.27
6.0	0.70
5.0	1.44
4.0	2.53
3.0	0

Aggregation

We found that addition of 20% glycerol to the buffer reduced the rate of hydrogenase inactivation, but the behavior of the enzyme during purification was altered. Two peaks of activity were observed during chromatography on Sephadex G-200, one at the usual elution volume and one in the void volume.

In a separate experiment, a treatment of the solubilized hydrogenase at 60°C for 10 min caused the enzyme to precipitate while still in the active state. Both of these phenomena can be explained in terms of aggregation of the protein and reflect the normal particular nature of the enzyme.

Conclusions

The O_2 lability and high thermal stability of the hydrogenase from soybean nodules are characteristic of hydrogenases [26]. The coupling of the hydrogenase to a variety of positive potential acceptors, but only slowly or not at all to negative potential acceptors, but only slower or not at all to negative potential acceptors, indicates that the enzyme functions at an unusually positive potential. Such a potential could explain slow evolution of H_2 by the enzyme; as the pH decreases H_2 evolution rates increase.

The low K_m of this hydrogenase for H_2 is advantageous, because all of the H_2 available in the nodule is generated in situ by nitrogenase and any H_2 that escapes represents a dissipation of potentially usable energy. The specific activity of the purified hydrogenase, 19.6 units/mg protein is low compared to many hydrogenases [7—16]. Despite its relatively low specific activity, the hydrogenase still is sufficiently active to oxidize all of the H_2 produced by the nodules.

The hydrogenase is present in nodules in very low amounts, so the quantities isolated have not permitted detailed analysis of the physical and chemical properties of the enzyme. Iron analysis has shown 12.2 Fe/molecule of 62 000 molecular weight. Although this is very similar to the value of 12 found by Mortenson and Chen [26] for the hydrogenase from *C. pasteurianum*, the agreement should not be stressed, because our preparation was not pure. The enzyme forms a brown band on polyacrylamide gels. Because the nodule hydrogenase is similar, the hydrogenase system from *A. vinelandii* may provide a useful mode for comparison.

Note added in proof (Received July 23rd, 1979)

The purification and properties of the particulate hydrogenase from the aerobic bacterium, *Alcaligenes eutrophus*, has been described recently [32].

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