Reconstitution of $H_2$ Oxidation Activity from $H_2$ Uptake-negative Mutants of *Rhizobium japonicum* Bacteroids*

(Received for publication, August 10, 1981)

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An *in vitro* reconstitution of both methylene blue and oxygen-dependent $H_2$ uptake activity from extracts of $Hup^+$ (H$_2$ uptake-negative) mutant strains of *Rhizobium japonicum* bacteroids is described. Cell-free extracts prepared from bacteroids formed from two different $Hup^+$ mutants were mixed, and active $H_2$ oxidizing particles formed. Extracts from each mutant alone did not oxidize $H_2$. The source of the components required for the complementation were soluble. Mixing of membrane particles from the two mutants did not result in reconstituted activity. The development of activity required an incubation period of several hours under anaerobic conditions, and maximal activity was obtained ~10 h after the mixing of the two extracts. Along with the development of $H_2$ uptake activity with time, the soluble extract mixture became turbid. The turbidity could be correlated with an increase in the appearance of membrane structures, including closed vesicles. After reconstitution, 65% of the methylene blue-dependent $H_2$ uptake activity was recovered in a particulate fraction.

The bacterium *Rhizobium japonicum* enters into a $N_2$ fixing symbiotic association with soybean plants. The factors which limit $N_2$ fixation rates in the symbiosis may be determined by the bacterial symbiont, the plant host, or they may be environmental. The efficient use of photosynthate by the bacteria in the nodule is thought to be one important limiting factor in the $N_2$ fixation process. Some strains of *R. japonicum* that appear to be efficient in $N_2$ fixation possess an $H_2$ uptake hydrogenase system. The $H_2$ oxidation system is capable of utilizing $H_2$ produced by the nitrogenase reaction, and $H_2$ oxidation by the bacteroids (the bacteria in the root nodule) can provide ATP to support $N_2$ fixation (1–3). The possession by the *R. japonicum* strain of $H_2$ oxidizing ability results in significant increases in plant dry matter and total $N_2$ fixed (2, 4, 5).

The hydrogenase system in bacteroids is complex, catalyzing the activation and oxidation of $H_2$ presumably through a series of unidentified electron transport components. Oxygen is the terminal electron acceptor, yielding water as the final product (2, 6). Little information is available concerning the specific components involved in the electron transport sequence between $H_2$ and $O_2$. The first component of the $H_2$ oxidation pathway is the $H_2$ activating hydrogenase. This hydrogenase has been solubilized and purified from *R. japonicum* bacteroids (7). It is associated with a particulate fraction, is oxygen labile, and apparently is a monomer with a molecular weight of ~65,000 (7).

In order to study the factors involved in $H_2$ oxidation and nitrogen fixation in *R. japonicum*, mutant strains unable to oxidize $H_2$ have been obtained (8). These $H_2$ uptake-negative strains are unable to oxidize $H_2$ as bacteroids isolated from soybean nodules. We attempted to reconstitute $H_2$ uptake ability by mixing together extracts from bacteroids of $Hup^+$ mutants. Such *in vitro* complementation experiments would be useful for categorizing the many $Hup^-$ mutants available (8) as well as providing information on the biochemical factors involved in $H_2$ oxidation. Reconstitution-type experiments have been useful to study mitochondrial and liver microsomal electron transport processes (9–12), as well as bacterial membrane electron transport processes (12–14). *In vitro* complementation using extracts from mutants has been useful for the study of assembly of nitrate reductase components in *Escherichia coli* (15–21).

**MATERIALS AND METHODS**

**Chemicals**—All chemicals were of reagent grade and were obtained from Sigma or J. T. Baker Chemical, Phillipsburg, N.J. High purity gases were obtained from Arundel Sales and Service, Baltimore, MD.

**Rhizobium Strains and Growth Conditions**—The $Hup^+$ mutants analyzed here were obtained from *R. japonicum* strain SR based on their inability to grow chemosynthetotrophically with $H_2$. The isolation of mutants by this procedure has recently been described (8). *R. japonicum* strain SR is resistant to kanamycin (100 µg/ml) and streptomycin (250 µg/ml), as are all of the mutants described. All of the mutants are able to form effective $N_2$-fixing nodules on soybean plants. *R. japonicum* strains were grown in the medium described by Bishop et al. (22). Turbid cell suspensions were used to inoculate surface-sterilized soybean seedlings.

**Growth of Soybeans and Harvesting of Bacteroids**—Maryland certified soybean seed (Essex) were surface-sterilized (23) and germinated in the dark for 48 h. Germinated seedlings were planted into sterile Leonard jar assemblies (23, 24). The Leonard jars contained a mixture of sand and vermiculite and contained sterile nutrient solution (23). Each seed was then inoculated with 0.5 ml of a turbid cell suspension of the appropriate strain, and the seeds covered with paraffin-coated sand (23). The Leonard jars were incubated in the greenhouse for 4 weeks with both natural and supplemental light (supplied by 250-watt Mercury-Vapor lights, Super Plant Lite, Duro-Lite, Fair Lawn, NJ). Supplemental light ([µE m$^{-2}$ s$^{-1}$], measured at

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* This work was supported by Grant 59-2243-0-1-435-0 from the United States Department of Agriculture Science and Education Administration Competitive Grants program, and by BRSG grant 5-S07-RR07041 awarded by the Biomedical Research Support Grant Program Division of Research Resources, National Institutes of Health. This publication is 1228 from the Department of Biology and McCollum Pratt Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 R. J. Maier, unpublished observation.

2 The abbreviation used is: $Hup^+$, $H_2$ uptake-negative.
the top of the pot) was supplied only when the natural light intensity dropped below 966 μE m⁻² s⁻¹ as controlled automatically by an electronic photosensor and only for a maximum period of 18 h/day. Control Leonard-jar assemblies containing uninoculated seedlings were routinely examined for nodulation to insure that *R. japonicum* was absent from the seeds or potting mixture. Nodules were picked from the plants and used the same day. Nodules (18-26 g) were crushed in 0.05 M potassium phosphate buffer, pH 6.8, containing 0.2 M sodium ascorbate (5 ml of buffer/g of nodules) and acid-washed polyvinylpyrrolidone (1 g of polyvinylpyrrolidone/3 g nodules) as described (1, 24). The bacteroids were then harvested by filtration through cheesecloth and centrifugation (1, 24). Bacteroids were washed twice in 0.05 M potassium phosphate buffer, pH 7.0, containing 2.5 mM MgCl₂ (1). Between and after washings, the pelleted bacteroids were carefully separated from the polyvinylpyrrolidone layer, and then resuspended in the potassium phosphate buffer. All of these steps were performed under aerobic conditions.

**Preparation of Cell-free Extracts—Washed bacteroids** were suspended in H₂-sparged 0.05 M potassium phosphate buffer containing 2.5 mM MgCl₂ (each gram of wet cell plate suspended in 5 ml of buffer) and broken by passage through a French pressure cell at 1100 kg/cm² at 4 °C. The pressure cell was previously flushed with H₂ and the cell loaded with the culture under a stream of H₂. The macerate (continuously under a stream of H₂) was transferred to an H₂-sparged centrifuge tube, the tube sealed with a stopper, and centrifuged for 30 min at 34,000 × g. The supernatant solution was removed with an H₂-sparged syringe and the pellet discarded. This cell-free extract is referred to as the crude extract. Initial experiments (Table I) were performed with this extract. The crude extract was then centrifuged at 110,000 × g for 1.5 h in the H₂-sparged 0.05 M potassium phosphate buffer, pH 7.0 containing 2.5 mM MgCl₂. The pellet from this fractionation is called the particulate (membrane-containing) fraction and the supernatant solution is referred to as the membrane supernatant fraction.

After breaking the cells, tubes containing extracts were flushed with an anaerobic gas mixture (85% N₂, 10% H₂, 5% CO₂). Care was taken to exclude oxygen during the preparation of the various fractions, but no O₂ scavenging reagents were added to the extracts. These precautions were taken because we had observed that H₂-oxidizing membrane particles from bacteroids of the wild type (strain SR) were more stable when incubated in O₂-free and H₂-containing buffer. Rates of H₂ uptake were determined amperometrically as described (20–27) in argon-sparged 0.05 M potassium phosphate buffer, pH 7.0 containing 2.5 mM MgCl₂. The electron acceptor concentrations were saturating (but not inhibitory) for all assays. H₂ uptake was measured directly. Hydrogen (37.7 nmol) was added as 50 μl of gas-saturated buffer to initiate the assay. These concentrations were sufficient to saturate the H₂ uptake reaction.

**Table I**

<table>
<thead>
<tr>
<th>Hup⁺ strains used for complementation mixture</th>
<th>H₂ uptake activity (nmol min⁻¹ mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR114 + SR127</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>SR114 + SR146</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>SR114 + SR118</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>SR127 + SR146</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>SR127 + SR118</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>SR146 + SR118</td>
<td>4.4</td>
</tr>
<tr>
<td>SR (Hup⁺ parent)</td>
<td>17.5</td>
</tr>
</tbody>
</table>

**RESULTS**

*R. japonicum* mutant strains that were unable to oxidize H₂ when derepressed for H₂ uptake in free-living culture (8) were tested amperometrically for H₂ oxidizing ability when isolated as bacteroids from soybean nodules. None of the mutant strains used here were able to oxidize H₂ as bacteroids. In order to better characterize these mutant strains, we attempted to reconstitute a H₂ uptake system in *vitra* by mixing extracts from bacteroids of various Hup⁺ mutants.

Bacteroids were harvested, washed, and ruptured as described under "Materials and Methods" with the exception that each gram of bacteroid cell paste was suspended in 10 ml (rather than 5 ml) of the potassium phosphate buffer just prior to breaking the cells. After disruption of the cells, the cell macerate was immediately flushed with H₂ and then centrifuged at 34,000 × g for 30 min in tightly sealed centrifuge tubes. One-mL samples of each of the two extracts to be tested (extracts contained 2.9 to 3.9 mg/ml of protein) were transferred to a 15-mL serum vial, the vial stoppered, and then flushed with an aerobic gas mixture containing N₂ (85%), CO₂ (5%), and H₂ (10%). The vials were incubated for 6 h at 30 °C, and 0.5-ml samples were removed for direct assay of the H₂ uptake activity. The 0.5-ml samples were added to a 4.5-mL amperometric chamber containing argon-sparged 0.05 M potassium phosphate buffer, pH 7.0, and 2.5 mM MgCl₂, and H₂ uptake recorded directly in the presence of 0.6 mM methylene blue. Hydrogen (37.7 nmol) was added as a saturated solution to initiate the assay. These concentrations were sufficient to saturate the H₂ uptake reaction.

**Methylene blue-dependent hydrogenase activity obtained after mixing cell-free bacteroid extracts from four different Hup⁺ mutant strains**

Bacteroids were harvested, washed, and ruptured as described under "Materials and Methods" with the exception that each gram of bacteroid cell paste was suspended in 10 ml (rather than 5 ml) of the potassium phosphate buffer just prior to breaking the cells. After disruption of the cells, the cell macerate was immediately flushed with H₂ and then centrifuged at 34,000 × g for 30 min in tightly sealed centrifuge tubes. One-mL samples of each of the two extracts to be tested (extracts contained 2.9 to 3.9 mg/ml of protein) were transferred to a 15-ml serum vial, the vial stoppered, and then flushed with an aerobic gas mixture containing N₂ (85%), CO₂ (5%), and H₂ (10%). The vials were incubated for 6 h at 30 °C, and 0.5-ml samples were removed for direct assay of the H₂ uptake activity. The 0.5-ml samples were added to a 4.5-ml amperometric chamber containing argon-sparged 0.05 M potassium phosphate buffer, pH 7.0, and 2.5 mM MgCl₂, and H₂ uptake recorded directly in the presence of 0.6 mM methylene blue. Hydrogen (37.7 nmol) was added as a saturated solution to initiate the assay. These concentrations were sufficient to saturate the H₂ uptake reaction.

**Hydrogenase activity in the SR146 plus SR118 mixture** did not occur immediately upon mixing the two extracts, but required an incubation period of several hours. We found that an incubation time of 6 h at 30 °C was sufficient to obtain easily detectable H₂ uptake activity. Another important requirement for complementation of H₂ oxidizing activity in these two strains was incubation of the mixture in a H₂-containing and oxygen-free atmosphere during the 6-h incubation period. An atmosphere composed of 85% N₂, 10% H₂, and 5% CO₂ was routinely used. A 100% H₂ atmosphere was also satisfactory. When extracts were mixed and then incubated in air for 6 h at 30 °C, no H₂ uptake activity was observed.

The distribution of the activities in the crude extracts were...
of interest, and the extracts were therefore centrifuged at 110,000 x g for 1.5 h, yielding a pellet (membrane) and supernatant fractions from SR118 and SR146. The various fractions were mixed as shown in Table II, and the mixtures were incubated for 8 h at 30 °C (anaerobically) and assayed for H2 uptake activity. Surprisingly, no activity was observed in the complementation experiment using the membrane fractions from the two mutants, but significant activity was found in the mixture containing the supernatant fractions. It was possible that the membrane supernatant fractions contained membrane particles that were not sedimented by the 110,000 x g centrifugation period, and these residual particles were responsible for the reconstitution activity. However, recentrifugation of this membrane supernatant fraction at 110,000 g did not increase activity. The amount of H2 uptake activity in crude extract and membrane supernatant fractions from the Hup+ parent strain (after the 8 h reconstitution incubation period) is also shown in Table II. The SR146 plus SR118 membrane supernatant mixture in Table II was 44% as active in methylene blue-dependent H2 uptake as a comparable fraction from the Hup+ parent strain.

As shown in Table II, O2-dependent H2 uptake activity was also observed in the crude extract and membrane supernatant complementation fractions. The SR146 plus SR118 membrane supernatant mixture was 67% as active in O2-dependent H2 uptake as a comparable fraction from the Hup+ parent strain (see Table II). Although the O2-dependent H2 uptake activity of the reconstituted mixture was lower than methylene blue-dependent activity we have consistently observed O2-dependent H2 uptake activity in reconstituted crude extract and membrane supernatant fractions. The O2-dependent H2 uptake activity was completely abolished by the addition of 10 μM potassium cyanide. This result indicates that a complete H2-oxidizing electron transport chain has been reconstituted.

All fractions listed in Table II from strains SR118 or SR146 were also tested for H2 oxidation without mixing the two fractions.

**Table II**

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Methylene blue</th>
<th>O2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(0.5 mM)</td>
<td>(0.02 mM)</td>
</tr>
<tr>
<td>Crude extract</td>
<td>114</td>
<td>19</td>
</tr>
<tr>
<td>Particulate</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Membrane supernatant</td>
<td>5.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Hup+ SR</td>
<td>13.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Membrane supernatant</td>
<td>11.7</td>
<td>2.1</td>
</tr>
</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Description</th>
<th>Total H2 uptake activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reconstituted mixture</td>
<td>123</td>
</tr>
<tr>
<td>2</td>
<td>Non sedimentable fraction from Fraction 1</td>
<td>&lt;4</td>
</tr>
<tr>
<td>3</td>
<td>Particulate fraction from Fraction 1</td>
<td>84</td>
</tr>
<tr>
<td>4</td>
<td>Mixture of Fractions 2 and 3</td>
<td>84</td>
</tr>
</tbody>
</table>

**Fig. 1.** Kinetics of reconstituted H2 uptake activity and turbidity. The membrane supernatant fractions from strains SR118 and SR146 were prepared as described under "Materials and Methods." Two ml samples of the membrane supernatant fractions (in H2-sparged 0.05 M potassium phosphate, pH 7.0, containing 2.5 mM MgCl2) of each strain were added to a H2-flushed and serum-stoppered vial. The 4 ml mixture was then split into two 1.8-ml fractions by transferring each 1.8-ml sample (by use of a syringe) to a H2-flushed, serum-stoppered cuvette (10-mm pathlength). The two cuvettes were incubated at 30 °C. Samples (0.2 ml) were removed from one cuvette every 2 h for measurement of methylene blue-dependent H2 uptake activity (O—O). The absorbance at 600 nm (O—O) in the other cuvette was measured at 2-h intervals in a Beckman DU spectrophotometer. The membrane supernatant fractions contained 3.79 and 3.82 mg of protein/ml of extract for strains SR118 and SR146, respectively. Methylene blue was added to a final concentration of 0.5 mM. Each point represents a single H2 uptake measurement or absorbance measurement. Other experiments showed similar results.
extracts, and after the 8-h incubation period. These fractions had undetectable activity. Boiling of reconstituted extracts (5 min, 100 °C) abolished H₂ uptake activity. Mixing of filter-sterilized membrane supernatant fractions of SR118 and SR146 still gave the reconstituted activity comparable to the activities in Table II. Therefore, the activity was not caused by the growth of H₂-oxidizing bacteria in the extracts.

After mixing the membrane supernatant fractions from SR118 and SR146, H₂ oxidation activity developed only after several hours of incubation. During this incubation period, activity was accompanied by an obvious increase in turbidity of the mixture. We therefore followed the kinetics of reconstitution of hydrogenase activity and of turbidity. The results are shown in Fig. 1. Both the hydrogenase activity and turbidity increased in parallel. No hydrogenase activity was detected in the individual membrane supernatant extracts of SR118 or SR146 incubated in the H₂-containing O₂-free atmosphere (tested at 6 and 10 h). However, individual SR146 or SR118 membrane supernatant fractions incubated alone still became turbid. Therefore, the formation of larger particulates (i.e. increased turbidity) was insufficient by itself to restore activity to the individual mutant supernatants. Both supernatants were required.

Since the increase in reconstituted H₂ uptake activity was accompanied by increased turbidity, it was possible that membrane fragments in the membrane supernatant fractions were aggregating to form particulate material. This was a likely possibility since we found that in addition to protein, phospholipid was present in the membrane supernatant fractions (phospholipid determination kindly performed by Dr. Mark Kuhlenschmidt of the Department of Biology, The Johns Hopkins University). Consequently, we fractionated a reconstituted mixture into particulate and soluble fractions and assayed the fractions for activity as shown in Table III. No activity was detected in the nonsedimentable fraction (Fraction 2, Table III). However, 65% of the total reconstituted activity was recovered in a particulate fraction (Fraction 3, Table III). This H₂-uptake activity was dependent upon the presence of methylene blue as an electron acceptor; oxygen-dependent H₂-uptake could not be detected in either the particulate or soluble fraction. This loss of O₂-dependent activity is probably related to the loss of oxyhydrogen activity which we normally observe upon repeated centrifugation and subsequent resuspension of Hup⁺ wild type membrane parti-
with the hydrogenase reconstitution system we have observed solubilization of a significant amount of particulate fraction back to the particulate fraction (see Table III, Fraction 4) did not restore 
O\textsubscript{2}-dependent H\textsubscript{2} uptake or significantly enhance methylene blue-dependent activity.

Electron microscopic studies of the reconstituted particulate material (8 h after mixing the extracts) showed a structurally heterogeneous population of membrane components. These included small aggregates, filaments, and closed vesicles. Typical examples of the type of membrane structures observed after thin sectioning are shown in Fig. 2, A and B. Many attempts to obtain particulate material or observe membrane structures like those shown in Fig. 2 immediately after mixing the membrane supernatant extracts failed. Since the mixing of the membrane supernatant fractions resulted in H\textsubscript{2} oxidizing activity and corresponding increases in turbidity with time, we followed the appearance of membrane structures with time of incubation. We found that the increase in turbidity could be correlated with an increase in the amount of membrane structures observed.

**DISCUSSION**

Cell-free extracts of *R. japonicum* bacteroids from two mutant strains unable to oxidize H\textsubscript{2} can be mixed, and active H\textsubscript{2}-oxidizing particles formed. Mixing of particulate fractions from the two mutants does not result in the formation of hydrogenase activity. However, mixing of the nonsedimentable fractions from the two mutants and incubation of the mixture for several hours results in hydrogenase activity.

The increase in reconstituted hydrogenase activity with time is accompanied by increased turbidity of the mixture. This turbidity can be attributed to the appearance of membrane-like structures. Therefore, we suggest that the nonsedimentable hydrogenase components from the two mutants come together to form active hydrogenase, and particulate formation may be involved in the reconstitution process. We cannot be certain that membrane vesicle formation is a requirement for the reconstituted activity.

Arp and Burris (7) solubilized and purified the membrane-bound hydrogenase from *R. japonicum* bacteroids. They found that a significant amount (30%) of the total hydrogenase activity was solubilized upon breakage of the bacteroids. We have also observed solubilization of a significant amount (~25%) of the total hydrogenase activity upon breakage of Hup\textsuperscript{+}, wild type bacteroids under our conditions. Therefore, it is likely that we have solubilized some membrane-bound hydrogenase components from the two mutants, and these components are responsible for reconstituted activity.

A nitrate reductase reconstitution system using extracts from nitrate reductase-negative mutants has some similarities with the hydrogenase reconstitution system we have described. The *E. coli* nitrate reductase reconstitution system involved the incorporation of a number of nonsedimentable components into phospholipid vesicles, including nitrate reductase subunits, ATPase, and a b-type cytochrome (20, 21, 30). Both the hydrogenase reconstitution system and the nitrate reductase system require the mixing of nonsedimentable components, an incubation period to obtain the activity, and activity is recovered in a particulate fraction. The reconstitution of 
O\textsubscript{2}-dependent H\textsubscript{2} uptake activity means that other electron transport components, in addition to hydrogenase, are present in the reconstituted system. This may include cytochromes which are probably involved in the complete H\textsubscript{2} uptake system in *R. japonicum* bacteroids (2). The nature of the hydrogenase components contributed by each strain will be determined by fractionation of the extracts, and the subsequent incubation of the various fractions with extracts from the complementary mutant strain.

**Acknowledgments**—We wish to thank Drs. Saul Roseman and Mark Kuhlenschmidt for helpful suggestions and critical review of the manuscript.

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Reconstitution of H2 oxidation activity from H2 uptake-negative mutants of Rhizobium japonicum bacteroids.
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