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HYDROGEN OXIDATION ACTIVITY IN MEMBRANES FROM *RHIZOBIUM JAPONICUM* *

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Membranes capable of oxidizing H_2 with O_2 as terminal acceptor were obtained from free-living *Rhizobium japonicum*. Membranes contained highest H_2-uptake specific activities when isolated in the presence of an H_2 atmosphere, and when the oxygen radical scavenger butylated hydroxytoluene was included in the buffer used for rupturing cells. After breaking cells, all of the O_2-dependent H_2-uptake activity was associated with a particulate membrane-containing fraction, whereas approx. 75% of the methylene blue-dependent H_2-uptake activity was sedimented. The particulate and soluble fractions containing H_2-uptake activity with methylene blue were separated by sucrose gradient centrifugation. The particulate and soluble activities behaved identically with regard to artificial electron acceptor specificity and reversible inhibition by oxygen. The hydrogenase in membranes coupled H_2 uptake with the reduction of many positive potential electron acceptors, but not with negative potential acceptors. The optimal pH for H_2 uptake with O_2 as acceptor in membranes was approx. 7.2. H_2-uptake activity in membranes was associated with an inner (lighter) membrane fraction that also contained succinate oxidase activity. H_2-reduced minus O_2-oxidized difference spectroscopy of membranes indicated the involvement of *b* and *c*-type cytochromes in the H_2-oxidation pathway, with an absorption peak at 551.5 nm and a shoulder at 560 nm. The addition of sodium dithionite to H_2-reduced membranes caused additional *b*-type cytochrome reduction. The methylene blue-dependent H_2-uptake activity in membranes was reversibly inhibited by brief exposure to oxygen. Recovery of full activity after oxygen exposure was achieved only after several minutes of incubation under strict anaerobic conditions.

Introduction

The root nodules of most N_2-fixing plants evolve H_2 [1,2]. Hydrogen is generated from the reduction of protons via the N_2-fixing enzyme nitrogenase [2,3]. Several *Rhizobium* species produce nodules that do not evolve H_2; these Rhizobia possess an H_2-uptake system. Dixon [4,5] suggested that one function of the H_2-uptake hydrogenase in pea nodules was to recoup some of the energy lost in H_2 evolution. Quantitative studies of this loss in soybeans have been carried out in Evans' laboratory [6–8]. The possession of H_2-uptake ability by *Rhizobium japonicum* results in greater N_2-fixation rates by the soybean nodules, and subsequently greater plant weight is obtained [7,9–11].

The first enzymatic step in the oxyhydrogen reaction among all H_2-oxidizing bacteria is termed

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; DCIP, 2,6-dichlorophenolindophenol.

H_2 activation, and is carried out by hydrogenase. Dixon [5] found that the hydrogenase from *Rhizobium* bacteroids was particulate, and it could reduce methylene blue and ferricyanide rapidly. Arp and Burris [12] solubilized the particulate hydrogenase and subsequently purified the enzyme from *R. japonicum* strain 110 bacteriods. They reported many of its physical properties, including the molecular weight (approx. 64000) and reaction with various electron acceptors. The same authors recently reported other biochemical properties of the purified enzyme [13]. Other studies on hydrogenase and the complete H_2-uptake system have been confined to whole intact bacteroids [14–17]. In one such study, the use of electron-transport inhibitors suggested that cytochromes and non-heme iron proteins were involved in the H_2-oxidation pathway [16]. Recently, indirect evidence for the involvement of *b*- and *c*-type cytochromes in H_2 oxidation in whole bacteroids was provided [37]. The specific electron carriers operating between H_2 and the terminal electron acceptor O_2 are not known.

R. japonicum strains possessing H_2-oxidation activity as bacteroids are able to grow chemoautotrophically in free-living culture with H_2 as the energy source [18]. The studies on H_2 oxidation in free-living *R. japonicum* have been concerned only with the regulation of the system [19,20]. In order to identify factors involved in H_2 oxidation in *R. japonicum*, we isolated H_2-oxidizing membranes from free-living bacteria that were previously derepressed for H_2-uptake activity. We report here some of the properties of H_2-uptake activity in these membranes.

Materials and Methods

Chemicals

All of the electron acceptors reported in Table III were purchased from Sigma Chemical Co. Butylated hydroxytoluene and dithiothreitol were also from Sigma. Sodium dithionite was obtained from Fisher Scientific. Gases were obtained from Arundel Sales and Services Co., Baltimore.

Derepression for H_2-uptake activity

The wild type Hup^+ strain of *R. japonicum* used (strain SR) is a kanamycin- and streptomycin-resistant derivative of strain USDA 122. It has been described previously [19,21]. *R. japonicum* strain SR was derepressed for H_2-uptake activity similar to that described previously [19]. The strain was grown to a cell concentration of approx. $1 \cdot 10^9$ cells/ml in modified Bergersens medium [22]. The cells were harvested by centrifugation and the pellet resuspended in 0.05 M potassium phosphate, pH 7.0, containing 2.5 mM $MgCl_2$ (hereafter referred to as phosphate/$MgCl_2$ buffer) to a cell concentration of $5 \cdot 10^8$ cells/ml. 1 l of the cell suspension was placed in a 6 l flask. The flask was then sparged for 20–30 min with an anaerobic gas mixture containing 85% N_2, 10% H_2, 5% CO_2. The flask was tightly sealed with a stopper and O_2 injected to a final partial pressure of 1%. H_2 and O_2 concentrations were monitored amperometrically [23,24] and maintained near 10 and 1%, respectively. The flask was then shaken at 120–180 cycles/min for 40–48 h at 30°C to derepress H_2-uptake activity. Whole-cell H_2-uptake rates ranged from 58 to 110 nmol H_2 oxidized/min per 10^8 cells. Control cultures not subjected to derepression were grown in the modified Bergersens medium [22] as described above, and then washed once with the phosphate/$MgCl_2$ buffer.

Isolation of membranes

Cells were harvested by centrifugation, and each 1 g of wet cell paste suspended in 5 ml of the phosphate/$MgCl_2$ buffer. The cells were then broken by passage through a French pressure cell at 1100 kg/cm^2 at 4°C. The pressure cell was previously flushed with H_2, and the cell loaded with the culture under a continuous stream of H_2. The cell macerate (continuously under H_2) was transferred to an H_2-sparged centrifuge tube, the tube sealed with a stopper and centrifuged for 30 min at $34000 \times g$. All centrifugations were performed at 4°C. The supernatant solution was removed with an H_2-sparged syringe and the pellet discarded. This cell-free extract contains membranes and soluble components and is referred to as the crude extract. For harvesting of the membranes, the crude extract was added to a centrifuge bottle (under an H_2 atmosphere) and centrifuged at $110000 \times g$ for 90 min to sediment membranes. Membranes were suspended in the phosphate buffer while sparging the suspension with Ar, and

then assayed. Membranes were isolated daily for each experiment. All experiments with the exception of those of Fig. 3 were performed on membranes prepared in the presence of butylated hydroxytoluene as described in the legend to Table I. All of these experiments were also repeated with membranes prepared without butylated hydroxytoluene; similar results were obtained with both preparations. Protein concentrations for samples are given in the individual table legends and were determined by the dye-binding method of Bradford [25]. Crude extracts or membranes were hydrolyzed with NaOH (final concentration 3.0%, w/v) at 90°C for 10 min prior to analysis for protein content.

H_2-uptake assays

H_2 and O_2 concentrations were determined amperometrically [23,24]. Known amounts (50 or 100 μl) of H_2- or O_2-saturated phosphate/$MgCl_2$ buffer were injected into the chamber as standards. All assays were performed with saturating levels of H_2 and electron acceptors. Methylene blue was used at a final concentration of 200 μM.

Sucrose gradient centrifugation

The procedures for the preparation of the discontinuous sucrose density gradient and assay of the fractions are described in the legend to Fig. 1. Continuous sucrose density gradient centrifugation as described by Osborne et al. [26] was used to separate the inner (lighter) and outer (heavier) membrane fractions. H_2-sparged sucrose solutions (in the potassium phosphate buffer) of 20–70% sucrose were layered in a centrifuge tube, and allowed to equilibrate overnight (under an H_2 atmosphere) at 4°C. A 5 mg membrane protein sample was added to the top of the centrifuge tube and centrifugation was performed at 30 000 rpm in an SW50.1 rotor for 15 h at 4°C. This was sufficient time for the membrane fractions to reach equilibrium. The two visible membrane fractions were then carefully removed by use of a Pasteur pipette, and each fraction assayed for H_2 uptake or subjected to gel electrophoresis. The lighter membrane fraction rapidly took up O_2 (assayed polarographically) upon the addition of 1 mM succinate or 1 mM NADH, whereas the heavier membrane fraction did not. Other procedures are described in individual table or figure legends.

Optimal pH determination

Membranes (0.1 ml of approx. 2.0 mg protein/ml) suspended in phosphate/$MgCl_2$ buffer were added to the amperometric chamber that had been filled with Ar-sparged buffer composed of 25 mM Mes, 25 mM Hepes, 25 mM Tris, 25 mM glycine [27] at the appropriate pH. Optimal pH with O_2 (40–80 μM) or with methylene blue (200 μM) was determined.

Results and Discussion

Recovery of activity in crude extracts and membranes

We monitored the activity of both the H_2-activating hydrogenase (H_2 uptake with methylene blue as acceptor) and the complete oxyhydrogen reaction (H_2 uptake with O_2 as the acceptor) with various isolation procedures. The results are shown in Table I. We obtained greater methylene blue- and O_2-dependent H_2-uptake activity when crude extracts or membranes were isolated under an H_2 atmosphere rather than in air. Hydrogenases are commonly O_2 labile and are often stabilized when incubated under H_2 rather than air [27,28,36]. We attempted to recover more activity by adding antioxidants to the membrane isolation buffer. Butylated hydroxytoluene is known to inhibit lipid oxidation by acting as an oxygen radical scavenger. The addition of butylated hydroxytoluene to the isolation buffer (see Table I) resulted in a 106 and 93% increase in the O_2-dependent specific H_2-uptake activity recovered in crude extracts and membranes, respectively, compared to samples prepared in an H_2 atmosphere without butylated hydroxytoluene. The butylated hydroxytoluene-treated membranes recovered from 2 to 6% of the total whole cell H_2-uptake activity with O_2 as terminal acceptor. The addition of butylated hydroxytoluene did not benefit recovery of methylene blue-dependent activity (see Table I). In many experiments, membranes isolated in the presence of butylated hydroxytoluene recovered approximately twice the amount of activity (with O_2 as acceptor) as membranes prepared without butylated hydroxytoluene. This result suggests that lipid oxidation my be one factor limiting high recovery of intact H_2-oxidation electron-transport chain activity in these membranes.

TABLE I

EFFECT OF DIFFERENT ISOLATION CONDITIONS ON RECOVERY OF H_2-UPTAKE ACTIVITY IN CRUDE EXTRACTS AND MEMBRANES

H_2-uptake activity is expressed as nmol/min per mg protein, mean ± S.D. for three replicates. The general procedures for isolation of crude extracts and membranes are described in Materials and Methods. For preparation of the crude extracts approx. 4 g wet weight of the H_2-uptake derepressed cells were suspended in 20 ml of 0.05 M potassium phosphate, pH 7.0, containing 2.5 mM $MgCl_2$. The suspension was split into four 5-ml fractions for cell breakage under each of the four conditions described. Condition 1 (aerobic) means that no precautions were taken to exclude oxygen. Condition 2 means that the cell suspension was sparged with H_2 just prior to breaking cells, and the cell macerate was collected under a continuous stream of H_2. Extracts were transferred by use of syringes, and extracts were stored in serum-stoppered Ar-flushed vials. However, no O_2-scavenging reagents were added. Condition 3 was like condition 2 except the 5 ml cell suspension contained 1 mM sodium dithionite. Condition 4 was like condition 2 except that 20 mg butylated hydroxytoluene were added to the 5 ml cell suspension immediately prior to breaking cells. The butylated hydroxytoluene was prepared as a 50% (w/v) solution in ethanol. The preparation of the four different membrane isolation condition samples also began with the suspension of another 4 g wet weight sample of cells in 20 ml of the phosphate/$MgCl_2$ buffer. After isolation of the four crude extracts by the methods described above and in the text, the crude extracts were centrifuged at 110000 × *g* for 90 min at 4°C. The pellets (membranes) were each suspended in 2 ml of the potassium phosphate/$MgCl_2$ buffer. The 2 ml of buffer were then either exposed to air (condition 1), sparged with H_2 (conditions 2 and 4) or sparged with H_2 and contained 1 mM sodium dithionite (condition 3). Membranes in H_2-sparged buffer of conditions 2, 3 and 4 were then transferred by use of a syringe to serum stoppered Ar-flushed vials. Protein concentrations for the various crude extracts ranged from 2.3 to 2.8 mg/ml, and 0.25 ml fractions were assayed. Protein concentrations for the membranes ranged from 2.8 to 3.1 mg/ml and 0.2 ml fractions were assayed. All values are the mean ± S.D. of three independent amperometric assays. For assay, the electrode chamber was filled with Ar-sparged 0.05 M potassium phosphate/$MgCl_2$ buffer (pH 7.0). For methylene blue (200 μM)-dependent H_2 uptake, assays were conducted anaerobically, in the presence of 100 μM sodium dithionite. O_2-dependent H_2-uptake activity was determined in the presence of 50–120 μM O_2.

Isolation condition	Description	H_2-uptake activity			
		Crude extracts		Membranes	
		Methylene blue	O_2	Methylene blue	O_2
1	Aerobic	19 ± 3	0.8 ± 0.2	41 ± 7	3.0 ± 0.9
2	H_2 atmosphere	39 ± 7	1.7 ± 0.6	66 ± 8	6.7 ± 1.2
3	H_2 atmosphere + sodium dithionite	47 ± 6	not tested	77 ± 11	1.9 ± 0.5
4	H_2 atmosphere + butylated hydroxytoluene	37 ± 5	3.5 ± 0.6	65 ± 10	12.9 ± 1.2

After breaking cells, a substantial amount of activity (77–89% of the total O_2-dependent activity) was recovered in the pellet of the initial centrifugation. This fraction contained intact and partially broken cells and is like the result obtained by Ruiz-Argueso et al. [17] upon fractionation of H_2-uptake activity from *R. japonicum* bacteroids. The addition of other O_2-scavenging reducing agents (dithionite, 1 mM; dithiothreitol, 1 mM; ascorbate, 5 mM) did not result in greater recovery of membrane H_2-uptake specific activity with O_2 as acceptor (data for dithionite included in Table I). After cells were ruptured, the cell debris was removed by centrifugation. The supernatant solution was then centrifuged at high speed (see Materials and Methods) to sediment the membranes. All of the O_2-dependent H_2-uptake activity was sedimented, whereas approx. 25% of the methylene blue-dependent activity remained in the supernatant. Arp and Burris [12] found that 30% of the hydrogenase activity from *R. japonicum* bacteroids was not sedimented after breaking cells.

Storage, stability and pH optimum

Membranes were stored at 0–4°C in serum-stoppered vials under a 100% H_2 or Ar atmosphere. Methylene blue-dependent activity was stable for approx. 2 days under these conditions. However, the O_2-dependent H_2-uptake activity decayed rapidly. We found that membranes isolated with butylated hydroxytoluene were more stable with respect to retaining O_2-dependent H_2-

uptake activity than membranes isolated without butylated hydroxytoluene. In 24 h under an 100% Ar atmosphere, membranes isolated with butylated hydroxytoluene lost 44% of their original activity, whereas membranes prepared in the absence of butylated hydroxytoluene lost 76% in the same time period.

We consistently observed an optimal pH for H_2-uptake activity in membranes with O_2 as acceptor of approx. 7.2. Separate attempts with six different membrane preparations to obtain consistent pH optima for methylene blue-dependent activity failed. The optimal pH for methylene blue-dependent activity in these preparations ranged from 6.2 to 7.5. Arp and Burris [12] reported an optimal pH for hydrogenase in membranes of *R. japonicum* bacteroids (assayed with methylene blue) of 9.0, and Schink and Schlegel [33] reported high H_2 uptake (with methylene blue) in intact membranes of *Alcaligenes eutrophus* H16 at pH 7–8.5.

Distribution of activities among soluble and particulate fractions

We wished to investigate further the distribution of methylene blue- and O_2-dependent activity among particulate and soluble fractions of crude cell-free extracts. A crude extract was fractionated by equilibrium density gradient centrifugation in a 10–60% discontinuous sucrose gradient. Fractions were removed for assay of methylene blue- and O_2-dependent H_2-uptake activities (see Fig. 1). Oxygen-dependent H_2-uptake activity was found primarily in the 40–50% sucrose concentration only. Electron microscopy showed that this fraction contained closed membrane vesicles. The methylene blue-dependent activity was associated with this particulate fraction and also with a soluble fraction that reached equilibrium near the top of the gradient. Some H_2-oxidizing bacteria yield a soluble hydrogenase capable of reducing NAD, in addition to a membrane-bound enzyme which cannot reduce NAD [27,33,34,36]. The two hydrogenases are thought to perform different functions in cellular metabolism. We have no evidence that the two methylene blue-dependent activities (particulate and soluble) from *R. japonicum* are due to different hydrogenases (see below). Both activities behaved similarly with regard to electron acceptor specificity and oxygen inhibition. It is possible that the soluble hydrogenase is observed only as an artifact of cell rupture.

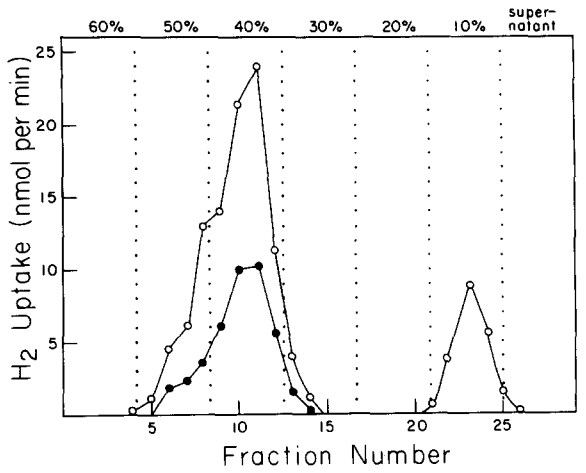

Fig. 1. Fractionation of a crude extract into O_2- and methylene blue-dependent H_2-uptake activities by discontinuous sucrose density gradient centrifugation. Sucrose density gradient centrifugation was performed similar to that described by Schneider and Schlegel [31]. H_2-sparged sucrose solutions (3.0 ml each of 60, 50, 40, 30, 20, 10% prepared in 0.05 M potassium phosphate buffer containing 2.5 mM $MgCl_2$, pH 7.0) were carefully layered into a 34 ml cellulose nitrate centrifuge tube. A 1.5 ml (10.5 mg protein/ml) crude extract (see Materials and Methods for crude extract preparations) was loaded onto the top of the gradient. The gas space above the gradient was flushed with H_2 before sealing the cap on the bucket over the tube. The tube was centrifuged at 22000 rpm for 24 h in an SW25.1 rotor. The bottom of the tube was perforated and 6-drop fractions (approx. 0.70 ml) were collected and assayed amperometrically for H_2-uptake activity. Each fraction was stored under a 100% Ar atmosphere at 4°C until assayed. The 4.8 ml amperometric chamber was filled with Ar-saturated phosphate/$MgCl_2$ buffer, and oxygen injected to approx. 50 μM. Fractions were assayed first with O_2 as acceptor (●———●), and then methylene blue was injected (200 μM) and the uptake rate determined with both methylene blue and O_2 (20–50 μM) present (○———○).

Acceptor specificity

We characterized the electron acceptor specificity of the H_2-oxidizing membranes (see Table II). Methylene blue is commonly used to assay hydrogenases and the H_2-uptake rate with methylene blue is often given the relative value of 100% [12,35,38]. The membranes from *R. japonicum* could couple H_2 uptake with the reduction of acceptors of positive potential, but poorly or not

TABLE II

MEMBRANE-BOUND HYDROGENASE ACTIVITY WITH VARIOUS ELECTRON ACCEPTORS

The electrode chamber was flushed with Ar and filled with Ar-sparged 0.05 M potassium phosphate/$MgCl_2$ buffer (pH 7.0). The electron acceptor dissolved in the phosphate/$MgCl_2$ buffer was added at the concentration indicated. All acceptor concentrations (in parentheses) were above saturating levels for H_2-uptake activity. The phenazine methosulfate-dependent reaction was performed in the dark. After addition of the acceptor, 100 μl of membranes (0.18 mg protein) were added. The chamber contained low levels (5–20 μM) of oxygen. The membranes were isolated in the presence of butylated hydroxytoluene as described in the legend to Table I. H_2 (75 nmol) as a gas-saturated solution was added to initiate the assay. Five independent replicates with each acceptor were performed, the values averaged, and relative activities with respect to methylene blue calculated. The activity $\pm$S.D. for the five methylene blue-dependent samples was 23.7 ± 2.9 nmol/min per mg protein.

Electron acceptor	E_0' (mV)	Relative activity (%)
Methylene blue (200 μM)	11	100
Phenazine methosulfate (400 μM)	80	182
Ferricyanide (1.5 mM)	360	141
DCIP (200 μM)	217	53
Oxygen (50 μM)	816	40
Cytochrome *c* (100 μM)	250	18
FMN (1 mM)	−122	<1
Benzyl viologen (1 mM)	−360	<1
Methyl viologen (1 mM)	−440	<1

at all with the negative potential dyes such as benzyl and methyl viologen. The results shown in Table II are similar to those reported by Arp and Burris [12] for the purified *R. japonicum* bacteroid hydrogenase. We also obtained no H_2 uptake with NAD, FAD, or FMN (all at 1 mM) provided as acceptors. Membranes from *R. japonicum* SR that were not derepressed for H_2 uptake did not oxidize H_2 with methylene blue or phenazine methosulfate. The nonsedimentable H_2-uptake activity was compared with the particulate activity for coupling of H_2 uptake with these electron acceptors. The nonsedimentable fraction also took up H_2 with methylene blue and with phenazine methosulfate, but not with benzyl viologen, methyl viologen, NAD, FAD, or FMN provided as acceptors. Therefore, the nonsedimentable hydrogenase was like the membrane-bound enzyme with regard to artificial electron acceptor specificity.

Separation of two membrane types and SDS gel electrophoresis

We further fractionated the membranes into inner and outer membrane by equilibrium sucrose density gradient centrifugation. This is a routine procedure for separation of the two membrane types from gram-negative bacteria. A 30–70% linear gradient distinctly separated membrane particles into two types: a lighter (presumably inner membrane) fraction in the 40–50% sucrose range, and a heavier fraction in the 55–65% sucrose. Both fractions were composed of closed vesicles as determined by electron microscopy. The lighter fraction contained H_2-uptake specific activities (nmol/min per mg protein) ranging from 8.8 to 19.5 and 29.6 to 44.7 with O_2 and methylene blue as acceptors, respectively, in three separate experiments. This fraction also rapidly took up O_2 upon the addition of 1 mM succinate or 1 mM NADH. No H_2-uptake activity (with oxygen or with methylene blue) was observed either in the heavier membrane fraction, or in the lighter membrane fraction obtained from cells not subjected to the derepression conditions.

Proteins from these membrane fractions were compared by SDS-polyacrylamide gel electrophoresis. We observed consistent differences in protein patterns between the inner membrane fractions of derepressed vs. repressed cultures. A more detailed analysis of these differences is being conducted by use of two-dimensional gel electrophoresis. Unfortunately, we could not observe a difference in inner membrane protein patterns of repressed vs. derepressed cultures in the 60 000–65 000 molecular weight range in polyacrylamide gradient gels, the area where we would expect to visualize hydrogenase. We did not observe any differences in the protein patterns from the outer membrane fractions from derepressed vs. repressed cultures.

Difference spectroscopy

We performed difference spectra on H_2-oxidizing membranes to identify cytochromes that are involved in the oxyhydrogen system. Fig. 3 shows the results of difference spectra analysis obtained

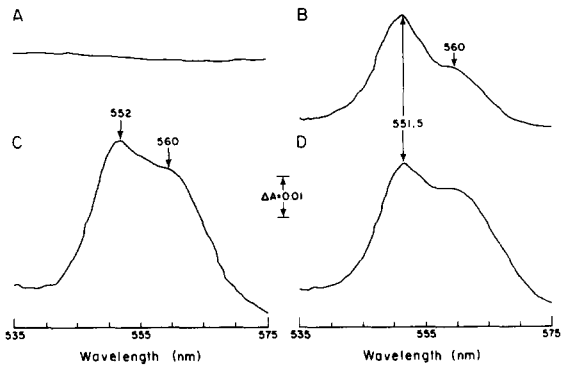

Fig. 2. Difference spectra performed on membranes from repressed (left A and C) and derepressed (right, B and D) Hup^+ strain SR. Scans A and B: H_2 reduced vs. O_2 oxidized. Scans C and D: dithionite reduced vs. O_2 oxidized. Protein concentrations were: A and C, 1.96 mg/ml; B and D, 1.22 mg/ml. Membranes were prepared in the absence of butylated hydroxytoluene but with H_2-sparged buffers as described in Materials and Methods and the legend to Table I (condition No. 2). The membranes (obtained from 1 g wet weight of cells) were then suspended in 5 ml of phosphate/$MgCl_2$ buffer, and recentrifuged at $110000 \times g$ for 90 min at 4°C to pellet the membranes. This washing step was employed to remove possible endogenous electron donors remaining in the original suspension. The membranes were resuspended in 6 ml of phosphate/$MgCl_2$ buffer, and the suspension passed through a small glass wool column to remove large nondispersed particles. The suspension was added to a quartz cuvette (10 mm path length), the cuvette sealed with a serum stopper, and the suspension sparged with O_2. The O_2-oxidized spectra vs. a blank containing buffer were recorded. The cuvette was then sparged with 100% H_2 for 10 min and the H_2-reduced vs. O_2-oxidized spectra recorded. Dithionite-reduced spectra were obtained by adding sodium dithionite (1 mM) from a 100 mM solution in the phosphate/$MgCl_2$ buffer to the H_2-flushed samples. Spectra were recorded by use of Perkin-Elmer Model 557 double-wavelength double-beam spectrophotometer. O_2-oxidized vs. Ar-sparged difference spectra revealed no peaks.

by scanning membranes for α cytochrome peaks in the 535–575 nm region. In Fig. 2, the membranes used for scans A and C were from cells not derepressed for H_2 uptake, whereas the cells used for the membrane isolation and scans B and D were previously derepressed for H_2-uptake activity. The top scans A and B are O_2-oxidized vs. H_2-reduced spectra. These scans represent the cytochromes reduced by H_2 and therefore probably involved in the H_2-uptake pathway. Scans C and D show the total cytochrome content in the 535–575 nm region as determined by O_2-oxidized vs. dithionite-reduced difference spectra. The results show that cells not subjected to derepressing conditions contain a total *b*- and *c*-type cytochrome pattern (scan C) similar to that of derepressed cells (scan D). The results directly show that the H_2-oxidation system in the membranes from derepressed cells can involve both *b*- and *c*-type cytochromes, with a difference absorption peak at approx. 551.5 nm and a shoulder at approx. 560 nm.

We consistently observed less reduction of *b*-type cytochromes by H_2 than by dithionite in H_2-oxidizing membranes of strain SR. For example, using the methods of Appleby [29,30] for calculation of cytochrome concentrations in free-living *R. japonicum*, we determined the total amount of cytochrome *b* in the membranes from derepressed cells in Fig. 1 to be 1.19 μmol heme/g protein. In contrast, the amount of *b*-type cytochrome reduced by H_2 was 0.64 μmol heme/g protein. We observed little difference in the amount of *c*-type cytochrome reduced by H_2 as compared with dithionite. Both *b*- and *c*-type cytochrome concentrations were comparable to those reported by Appleby [29,30] in membranes from *R. japonicum*. The conventional cytochrome electron-transport inhibitors antimycin A (0.02 mM), azide (1.0 mM), and potassium cyanide (0.05 mM) all significantly inhibited both H_2 uptake (greater than 50% inhibition) and H_2-dependent O_2 uptake (greater than 44% inhibition). These inhibitors did not inhibit methylene blue-dependent H_2-uptake activity.

Reversible inhibition of methylene blue-dependent H_2-uptake activity by O_2

Methylene blue is commonly used to assay H_2-uptake hydrogenases, and is an excellent electron acceptor for the purified *R. japonicum* bacteroid hydrogenase [8,24]. We consistently observed methylene blue-dependent H_2-uptake activity in the presence of O_2; however, we noticed that this activity increased when the dissolved O_2 level approached zero (as monitored amperometrically simultaneously with H_2). Fig. 3 shows a dual-pen chart recording of an experiment demonstrating this reversible O_2 inhibition effect. Initially, H_2 uptake proceeded at a low rate (6.5 nmol/min) with O_2 present. As the dissolved O_2 approached

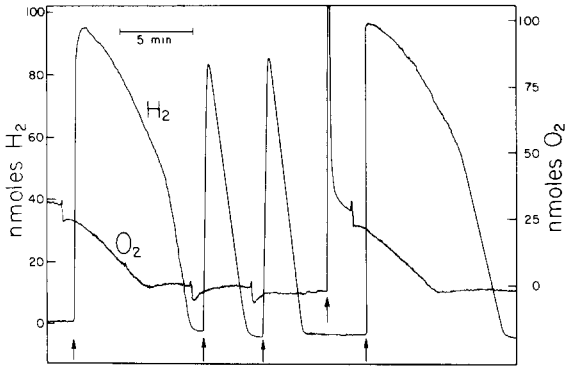

Fig. 3. Amperometric dual-pen chart recording showing the effect of oxygen on methylene blue-dependent H_2-uptake activity of membranes. The 4.8 ml amperometric chamber was filled with Ar-sparged phosphate/$MgCl_2$ buffer, 200 μM methylene blue, and 300 μl (0.53 mg protein) of membranes. Approx. 25 nmol residual O_2 remained in the chamber. To initiate the experiment 100 nmol of H_2-saturated solution (first arrow) were injected. The second, third and fifth arrows from the left are times at which additional 100-nmol aliquots of H_2 were injected. The fourth arrow shows the time of addition of 120 nmol O_2 to the chamber.

zero the H_2-uptake rate increased. When the O_2 was depleted (about 5 min after the first injection of H_2, see arrow) the H_2-uptake rate increased to 34 nmol/min. Additional 100-nmol injections of H_2 (the second and third arrows) were rapidly utilized. The addition of 120 nmol O_2 to the chamber (the fourth arrow) inhibited the H_2-uptake rate. When this additional O_2 was exhausted, the H_2-uptake rate reached 30 nmol/min. We investigated this reversible O_2 inhibition phenomena another way; we compared the H_2-uptake activities of membranes assayed anaerobically immediately after a 1 min pretreatment anaerobically vs. aerobically. The results are shown in Table III. When membranes were incubated anaerobically (and with 1 mM dithionite) for 1 min and then assayed, the H_2-uptake rate observed initially was the same as the rate obtained 5 min after the start of the assay. However, membranes that were pretreated with 1% partial pressure O_2 did not reach full activity until several minutes after all the O_2 was exhausted. This result shows the importance of assaying hydrogenase activity anaerobically and several minutes after O_2 is depleted for quantitative measurements. Reversible inhibition by low

TABLE III

EFFECT OF ANAEROBIC VS. AEROBIC INCUBATION ON H_2-UPTAKE ACTIVITY OF MEMBRANES

H_2-uptake activity is expressed as nmol/min per mg protein, mean ± S.D. of H_2-uptake activities of five replicate membrane samples in the presence of 200 μM methylene blue. A 1.0 ml sample of membranes (2.15 mg protein) was injected by use of a syringe into each of two Ar-flushed serum-stoppered vials, and the vials were then reflushed with Ar. One sample then received 100 μl of a 10 mM sodium dithionite solution to scavenge trace levels of oxygen. The other vial was injected with sufficient 100% O_2 to compose an atmosphere in the vial of 99% Ar and 1% O_2. After 1 min, 100-μl samples were removed for assay. The 4.8 ml electrode chamber was sparged with Ar, and then filled with Ar-saturated phosphate/$MgCl_2$ buffer. Methylene blue (200 μM) and H_2 (75 nmol) were added to the chamber, and then 100 μl of the membrane sample were added to initiate the reaction. Trace amounts of oxygen (less than 30 nmol) remained in the chamber after filling the chamber with Ar-sparged buffer. The initial rate in the table was measured during the 1 min period when this trace O_2 level approached and reached zero O_2 level (as determined amperometrically). Correspondingly, the other rates were measured 1 and 5 min after the time when no O_2 could be detected.

Pretreatment	H_2-uptake activity in the absence of O_2		
	Initial	1 min	5 min
Anaerobic	84±9	87±7	88±7
Aerobic	25±3	52±7	86±7

levels of oxygen was recently reported for the purified *R. japonicum* bacteroid hydrogenase [13]. Hydrogenases from several sources have been reported to be reversibly inhibited by O_2 and it is thought that this inhibition is a transitory phenomenon that eventually leads to irreversible inactivation [28]. This is apparently also true for the reversible inhibition we observed; exposure of a 0.2 ml membrane sample in a 10 ml vial overnight in air (at 0–4°C) irreversibly lost approx. 20% activity as compared to a sample incubated under 100% Ar. In addition to membranes we also studied reversible O_2 inhibition of the nonsedimentable H_2-uptake fraction. The effects of O_2 were identical to those we observed for H_2-uptake activity in membranes. Further experiments on H_2-oxidizing membranes from *R. japonicum* should reveal the biochemical components involved in H_2 uptake

and perhaps clarify the role of H_2 oxidation in symbiotic nitrogen fixation.

Note in added in proof (Received January 12th, 1983)

We have recently described the H_2-oxidation electron-transport chain in these membranes in more detail [38]. The H_2-oxidation pathway involves quinone, two *b*-type cytochromes, cytochrome *o*, cytochrome *c* and cytochromes aa_3.

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