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PROPERTIES OF THE HYDROGENASE SYSTEM IN RHIZOBIUM JAPONICUM BACTEROIDS

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SUMMARY

The hydrogenase system which catalyzes the oxyhydrogen reaction in soybean nodules produced by strains of Rhizobium japonicum is located in the bacteroids. The hydrogenase complex in intact bacteroids has an apparent K_m for H_2 of 2.8 μM and an apparent K_m for O_2 of 1.3 μM. The addition of hydrogen to bacteroids increases oxygen uptake but decreases respiratory CO_2 production, indicating a conservation of endogenous substrates. After correction for the effect of hydrogen on endogenous respiration a ratio of 1.9 ± 0.1 for H_2 to O_2 uptake was determined. Bacteroids from greenhouse or field-grown soybeans that evolved hydrogen showed no measurable oxyhydrogen reaction activity whereas consistent activity was demonstrated by bacteroids from soybean nodules that evolved little or no H_2.

INTRODUCTION

ATP-dependent H_2 evolution by nitrogenase occurs in vivo in many N_2-fixing nodulated symbionts (1,2). Until recently the possible importance of hydrogen evolution and uptake in the efficiency of N_2 fixation by nodulated N_2-fixing plants was not fully appreciated (1,3). Hydrogen evolution via the nitrogenase system not only consumes ATP but also reducing power that is needed for the operation of an efficient N_2-fixing process. Symbionts with an ability to oxidize H_2 via the hydrogenase complex may recapture at least part of the energy that is lost through H_2 evolution. In 1941 Phelps and Wilson (4) reported the presence of hydrogenase in pea root nodules. These findings were not confirmed by Shug et al. (5), but in 1967, Dixon (6) demonstrated H_2 uptake by excised pea root nodules. The following year Dixon (7) showed that the hydrogenase system in pea nodules was located in the bacteroids. He suggested the following three roles for the hydrogenase complex in the N_2 fixation process (8): (a) protection of the O_2 sensitive nitrogenase by utilization of O_2 within the bac-

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teroids; (b) prevention of H_2 inhibition of nitrogenase through the H_2 uptake mechanism; and (c) recovery of part of the energy lost by H_2 evolution through ATP formation associated with H_2 oxidation. No hydrogenase complex from nodules of other legumes has yet been characterized.

Research has been conducted with a series of soybean cultivars and Rhizobium japonicum strains to determine the most efficient H_2-recycling combinations (2,9). From these experiments, Carter et al. (9) concluded that the capacity to recycle H_2 evolved by the nitrogenase system in soybean nodules appears to be determined by the R. japonicum strain. Nodules on soybeans formed by strains USDA 110, USDA 122, USDA 136, 3I1b 6, 3I1b 142, and 3I1b 143 lost little or no H_2 in air and showed a capacity to take up this gas when it was placed over them. In the same experiments (9) soybeans inoculated with strains USDA 16, USDA 31, USDA 117, USDA 120, USDA 135 and 23 other strains produced nodules that lost H_2 in air at rates that averaged approximately 36% of the electron flow through the nitrogenase system. It is the purpose of this paper to define some of the major characteristics of the hydrogenase complex in bacteroids from the nodules of soybeans.

MATERIALS AND METHODS

R. japonicum strains and soybean seeds were obtained from Drs. George Ham and Deane Weber. In greenhouse experiments where different strains were used, seeds of Glycine max (cv. Anoka) were surface disinfected (10) and germinated on water agar plates. Young seedlings were inoculated with 7-10 day-old yeast extract mannitol broth cultures of R. japonicum and planted in a sand-vermiculite mixture in modified Leonard jars (10). A nitrogen-free nutrient solution (11) was provided to the plants, which were kept in a greenhouse near 27°C during the day and 21°C at night. Supplemental light equivalent to 5380 Lux was provided during a 16-hour light period. Uninoculated soybean controls in Leonard jar assemblies did not form nodules.

Nodules were collected from a field experiment which was conducted to determine the relationship between activity of the hydrogenase system in nodules and yield of soybeans. In this experiment surface disinfected soybean seeds (cv. Portage) were treated with a series of inocula prepared from sterile peat and pure cultures of R. japonicum strains. Of the eight strains, four were hydrogenase-positive and four were hydrogenase-negative (2,9). Sufficient inoculum was added to insure that each seed received at least 10^3 bacteria (10). Uninoculated control plots were included and showed only sparse nodulation.

Bacteroids were prepared from 27-36 day-old greenhouse-grown plants as described by Dixon (7), except that anaerobic conditions were not maintained during the preparation procedure. No difference was found in hydrogenase activity of aerobically and anaerobically prepared bacteroids, however the high respiratory rate of the concentrated bacteroids maintained O_2 at a very

low level. Bacteroids prepared in this way exhibited no nitrogenase activity. Heat-treatment of bacteroids at 80°C for 15 minutes under anaerobic conditions destroyed hydrogenase activity. Protein contents of the washed bacteroid suspensions were determined as described by Goa (12).

H_2 uptake rates by bacteroid suspensions were measured amperometrically according to the method of Wang, Healey, and Myers (13). O_2 uptake was measured by a Clark type O_2 probe purchased from Yellow Springs Instrument Company, Yellow Springs, Ohio. CO_2 evolution by bacteroid suspensions was determined with a Carle 8500 gas chromatograph. The 3.2 mm x 45.7 cm column was packed with Porapak Q (Waters Associates, Inc., Framington, Massachusetts). Helium was used as the carrier gas at a flow rate of 17 ml/min. The column temperature was 72°C.

RESULTS AND DISCUSSION

Characterization of the Hydrogenase System. Initial evidence of activity of the hydrogenase system in nodules of Anoka soybeans inoculated with USDA 110 was shown by O_2-dependent H_2 uptake by intact nodules (2). Bacteroids prepared from nodules formed by USDA 110 consistently exhibited activity of the hydrogenase system. The effect of H_2 concentration on the rate of H_2 uptake at an initial O_2 concentration of 22.1 µM is presented in Figure 1. H_2 saturation of the bacteroid hydrogenase system from nodules formed by strain USDA 110 occurred at 30 µM. The apparent K_m for H_2 as determined from a linear regression analysis of an Eadie-Hofstee plot (14) of the data in Figure 1 was approximately 2.8 µM. At saturating concentrations of H_2, 10 µM O_2 or greater concentrations resulted in maximum rates of H_2 uptake (Fig. 2). The apparent K_m for O_2 was approximately 1.3 µM. Methylene blue also functioned as an acceptor; however, under the same conditions 5.4 mM methylene blue produced a rate of H_2 uptake that was only 5.6% of the rate with 44 µM O_2. The ineffectiveness of methylene blue and some other acceptors with freshly prepared bacteroids may be associated with problems of the permeability of cells to these compounds and further work is in progress to clarify these matters. Nodules formed by R. japonicum strain USDA 31 lost H_2 at rapid rates and showed no capacity for H_2 uptake (2). Bacteroid preparations from nodules formed by USDA 31 also exhibited no activity of the hydrogenase system. Mixtures of bacteroids from nodules formed by strain USDA 31 with bacteroids formed by USDA 110 caused no inhibition of the activity of the USDA 110 hydrogenase system. This observation provided no evidence

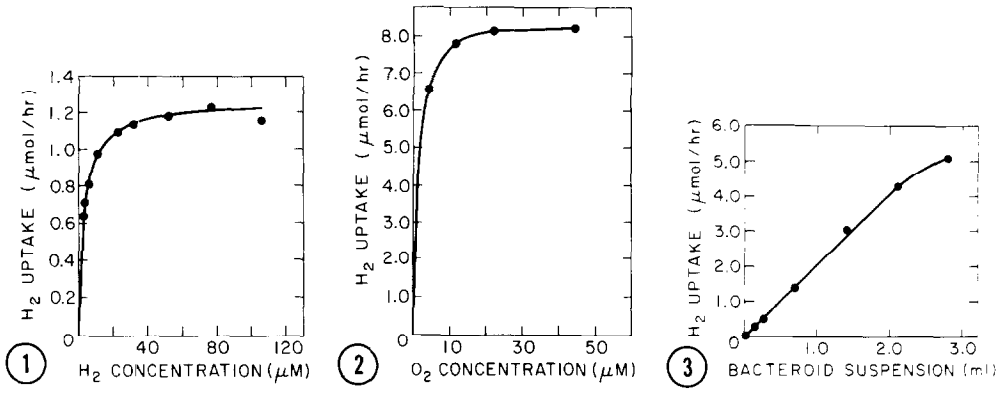

Figure 1. H_2 uptake by bacteroids of soybean nodules as a function of H_2 concentration. The bacteroid suspension, prepared from nodules formed by R. japonicum strain USDA 110, contained 111 µg protein per ml in a reaction volume of 2.8 ml at 23°C. The initial concentration of O_2 in each assay was 22.1 µM.

Figure 2. H_2 uptake by bacteroids of soybean nodules as a function of O_2 concentration. The bacteroid suspension, prepared from nodules formed by R. japonicum strain USDA 110, contained 555 µg protein per ml in a reaction volume of 2.8 ml at 23°C. The initial concentration of H_2 in each assay was 52.1 µM.

Figure 3. Proportionality between H_2 uptake and volume of bacteroid suspension (450 µg protein per ml) added to a reaction volume of 2.8 ml at 23°C. Bacteroids were obtained from nodules formed by R. japonicum strain USDA 110. The initial H_2 and O_2 concentrations in each assay were 39.1 µM and 22.1 µM, respectively.

for the presence of an inhibitor of the hydrogenase system in the bacteroids formed by strain USDA 31.

In reactions containing less than 2.2 ml of bacteroid suspensions and saturating amounts of H_2 and O_2, a linear relationship exists between the rate of H_2 uptake and the concentration of bacteroids in the reaction mixture (Fig.3). The nonlinearity at bacteroid concentrations greater than 2.2 ml may be due to incomplete mixing of the more viscous bacteroid suspensions.

By use of bacteroids formed by USDA 110, measurements were made of the concomitant H_2 and O_2 uptake rates in an attempt to define the stoichiometry of the nodule bacteroid hydrogenase system. Table 1 shows the results obtained from three replicate determinations. Subtraction of the endogenous rate of

Table 1. The Stoichiometry of the Hydrogenase Reaction in Bacteroids from Nodules of Soybeans Inoculated with Strain USDA 110 of R. japonicum[a]

Expt.	H_2 uptake	O_2 uptake with H_2	Endogenous O_2 uptake without H_2	Calculated endogenous O_2 uptake in presence of H_2[b]	H_2 dependent O_2 uptake	$\frac{H_2 \text{ uptake rate}}{H_2 \text{ dependent } O_2 \text{ uptake rate}}$[c]
	(nmoles per minute)					(ratio)
1	19.7	13.4	8.52	3.41	9.99	2.0
2	18.6	12.5	6.91	2.76	9.74	1.9
3	19.5	13.5	6.48	2.59	10.91	1.8

[a]The reaction chamber contained 2.8 ml of bacteroid suspension (0.13 mg protein per ml). Initial concentrations of H_2 and O_2 for measurement of H_2 uptake rates were 52.1 µM and 22.1 µM, respectively. For measurements of O_2 uptake rates in the absence of H_2, 44.2 µM O_2 was initially present. The figures given represent three replicate determinations. The mean ratio of H_2 to O_2 uptake is 1.9 ± 0.1.

[b]Inhibition of endogenous respiration was determined by measurement of CO_2 evolution by bacteroids in the presence and absence of H_2. The bacteroid concentration was 0.13 mg protein per ml. Initial concentrations of H_2 (when added) and O_2 in the N_2 atmosphere above the bacteroids were 0.10 and 0.05 atm, respectively. The mean inhibition of endogenous CO_2 production was 60%. It was then assumed that the endogenous O_2 uptake with H_2 present was 40% of the rate of endogenous O_2 uptake rate without H_2 present.

[c]The ratio of H_2 to O_2 taken up by the bacteroids was calculated by dividing H_2 uptake by H_2-dependent O_2 uptake.

O_2 uptake where H_2 was not present, from the O_2 uptake rate with H_2 present did not yield an expected 2 to 1 ratio in the uptake of H_2 and O_2. By use of gas chromatography CO_2 evolution rates by soybean root nodule bacteroids were measured in the presence of 70 µM H_2 and in the absence of H_2. From these experiments it was established that the rate of CO_2 evolution in the presence of 70 µM H_2 was only 40% of the rate of CO_2 evolution in the absence of H_2. This indicates that not only does H_2 inhibit the oxidation of exogenous substrates as Dixon has shown with pea root nodule bacteroids (7,15), but also that added H_2 acts to conserve endogenous substrates in intact bacteroids. As shown in

Table 2. Activity of the Hydrogenase System in Bacteroids from Nodules of Soybeans Inoculated with Different Strains of R. japonicum[a]

Strains	H_2 loss from nodules[b]	H_2 uptake (μmol/hr x mg protein)
Field-grown plants		
USDA 16	+	<0.002
USDA 117	+	<0.002
USDA 120	+	<0.002
USDA 135	+	<0.002
USDA 110	-	8.770
USDA 122 (CB 1809)	-	3.730
3I1b 6	-	9.680
3I1b 143	-	6.134
Greenhouse-grown plants		
USDA 31	+	<0.002
USDA 110	-	4.083

[a]The reaction chamber contained 2.8 ml of bacteroid suspension (0.14 to 1.95 mg protein per ml). The initial concentrations of H_2 and O_2 in all cases were greater than 39 μM and 16 μM, respectively. The values for field-grown nodules are the means of determinations on samples from two replicate plots. The values for greenhouse-grown nodules are means of three replicate determinations. Bacteroids were prepared from field-grown soybeans (cv. Portage) and greenhouse-grown soybeans (cv. Anoka) as described in Materials and Methods.

[b]Data from Carter et al. (9).

Table 1 H_2-dependent O_2 uptake rate, which was obtained by subtraction of the corrected endogenous respiration rate in the presence of H_2, was approximately 50% of the rate of H_2 uptake. The ratio of rates of H_2 uptake to H_2-dependent O_2 uptake was 1.9 ± 0.1 (standard error of the mean). It is clear therefore that the bacteroid hydrogenase system catalyzes the oxyhydrogen reaction.

Activities of the Bacteroid Hydrogenase System in Relation to Strains. Nodules were collected from field and greenhouse experiments in which strains that produce nodules showing H_2 loss were compared with strains that form nodules that lose little or no H_2 (9). No measurable rate of H_2 uptake could be detected in bacteroids from nodules that lost H_2, whereas all bacteroid preparations from nodules that evolved little or no H_2 actively took up H_2 (Table 2).

No activity was observed when O_2 was omitted from the reaction chamber. These results are consistent with those reported previously for intact nodules (2,9) and provide strong evidence that the hydrogenase complex, in bacteroids of nodules that do not evolve H_2, participates in an H_2 recycling process both in greenhouse-grown and in field-grown plants.

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REFERENCES

1. Schubert, K.R., and Evans, H.J. (1976) Proc. Nat'l. Acad. Sci., USA 73, 1207-1211.
2. Schubert, K.R., and Evans, H.J. (1976) in Proceedings of the II International Symposium on N_2 Fixation, Interdisciplinary Discussions. Salamanca, Spain, September 13-17, 1976. In press.
3. Dixon, R.O.D. (1976) Nature 262, 173.
4. Phelps, A.S., and Wilson, P.W. (1941) Proc. Soc. Exp. Biol. (NY) 47, 473-476.
5. Shug, A.L., Hamilton, P.B., and Wilson, P.W. (1956) in Inorganic Nitrogen Metabolism (McElroy, W.D., and Glass, B., eds.) pp. 344-360, The Johns Hopkins Press, Baltimore.
6. Dixon, R.O.D. (1967) Ann. Bot. N.S. 31, 179-188.
7. Dixon, R.O.D. (1968) Arch. Mikrobiol. 62, 272-283.
8. Dixon, R.O.D. (1972) Arch. Mikrobiol. 85, 193-201.
9. Carter, K.R., Jennings, N.T., and Evans, H.J. (1977) Western Section Meeting of the American Society of Plant Physiologists, San Francisco, June 12-16, 1977.
10. Vincent, J.M. (1970) A Manual for the Practical Study of Root-Nodule Bacteria. International Biological Programme. Blackwell Scientific Publications, Oxford. 164 p.
11. Jensen, H.L. (1942) Proc. Linn. Soc. N.S.W. 66, 98-108.
12. Goa, J. (1953) Scand. J. Clin. and Lab. Investigation 5, 218.
13. Wang, R., Healey, F.P., and Myers, J. (1971) Plant Physiol. 48, 108-110.
14. Hofstee, B.H.J. (1952) Science 116, 329-331.
15. Dixon, R.O.D. (1975) in Nitrogen Fixation by Free-Living Micro-Organisms (Stewart, W.D.P., ed.) pp. 421-435, Cambridge University Press, Cambridge.