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HYDROGENASE SYSTEM IN LEGUME NODULES: A MECHANISM OF PROVIDING NITROGENASE WITH ENERGY AND PROTECTION FROM OXYGEN DAMAGE

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SUMMARY

Some strains of rhizobia possess a hydrogenase system which catalyzes the oxidation of the H_2 that is evolved from nitrogenase during N_2 fixation. Oxidation of H_2 by a hydrogen uptake positive strain of Rhizobium japonicum provides energy for support of the N_2 fixation reactions and protects nitrogenase from O_2 damage

INTRODUCTION

The evolution of H_2 from nitrogenase in legume root nodules has been identified as a source of inefficiency in the Rhizobium-legume symbiosis. Energy losses through H_2 evolution from most of the nodulated legumes was estimated at 20 to 40 percent of the energy supplied to nitrogenase (1-4). Instead of losing energy as H_2, a few nodulated symbionts possess a system that recycles the H_2 produced by nitrogenase in nodules (3-7). The extent to which the utilization of H_2 by the nodule hydrogenase recovers the energy lost via nitrogenase-dependent H_2 production and provides other benefits to nodulated legumes has not been established. Dixon (8) has demonstrated ATP synthesis associated with the oxidation of H_2 by cell-free bacteroid preparations from pea nodules. Increases in yield and nitrogen content of soybeans from inoculation with strains containing hydrogenase has been obtained (6, 9). We report here that the oxidation of H_2, mediated by the bacteroid hydrogenase complex supported nitrogenase activity and provided respiratory protection for the nitrogenase in soybean nodule bacteroids. These results explain why the hydrogenase system in legume nodules would be expected to benefit the nitrogen-fixing process.

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MATERIAL AND METHODS

The bacteroids used in this study were prepared from nodules of 30 day-old soybean plants (Glycine max. cultivar Wilkin) inoculated with a colony derivative (DES) of Rhizobium japonicum strain USDA 122 which was isolated in this laboratory. Nodules produced by this strain evolved little or no H_2 in air and took up H_2 from external sources. Nodules for control experiments were produced by inoculation with the H_2-uptake negative strain USDA 117 of R. japonicum (3,7). Plants were grown, nodules removed and bacteroids prepared by the methods described by Klucas et al. (10), with the exception that the bacteroids were washed twice in Mg-phosphate buffer (50 mM K_2HPO_4, 2.5 mM $MgCl_2$, pH = 7.0) and resuspended finally in N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES)-Mg-phosphate buffer at pH 7.5 (50 mM HEPES, 1 mM K_2HPO_4, 1 mM $MgCl_2$). H_2 and O_2 uptake rates were measured simultaneously by use of the amperometric method described by Hanus et al. (11). H_2 and O_2 were added to the electrode chamber (2.8 ml) as H_2 and O_2 saturated HEPES-Mg-phosphate buffer solutions. Nitrogenase activity was determined by the rate of C_2H_2 reduction. C_2H_4 formation was measured on a Hewlett-Packard HP-5830A gas chromatograph equipped with a 1.8 m x 3.2 mm diameter column of Porapak R. Other details of the assays are given in legends.

RESULTS AND DISCUSSION

Washed R. japonicum bacteroids (strain USDA 122, DES) showed a capacity for O_2 dependent H_2 uptake (Table 1). The oxidation of H_2 by these bacteroids produced an approximate 3-fold increase in the rate of respiration above that resulting from oxidation of endogenous substrates. A similar increase in respiration was produced by adding succinate at a concentration of 10 mM, which saturates the system for O_2 uptake. In the presence of both H_2 and succinate (10 mM) the rate of O_2 uptake was higher than with either substrate alone.

The rates of C_2H_2 reduction by USDA 122 (DES) bacteroids throughout a series of increasing partial pressures of O_2, in the presence and the absence of 0.1 atm of H_2, are presented in Figure 1. The maximal rate of C_2H_2 reduction in the presence of H_2 was three-times the rate observed when only endogenous substrates were utilized. Addition of H_2 also increased the optimal O_2 partial pressure for the maximal rate of C_2H_2 reduction. Adding H_2 stimulated C_2H_2 reduction and allowed the nitrogenase in bacteroids to function at higher O_2 partial pressures than was possible in a suspension of bacteroids to which no H_2 was supplied.

The effect of H_2 on C_2H_2 reduction could be due to an increased supply of either ATP or reductant for support of nitrogenase activity. The

Table 1. Effect of substrates on H_2 and O_2 uptake by washed soybean bacteroids from R. japonicum USDA 122 DES[a]

Substrate added	H_2 uptake μmoles x h^{-1}	O_2 uptake μmoles x h^{-1}
none	-	0.36
H_2, 27 μM	1.57	0.94
Succinate, 10 mM	-	0.87
Succinate, 10 mM and H_2, 27 μM	1.22	1.39

[a] Assays were conducted in an electrode chamber containing 2.8 ml of bacteroid suspension (0.24 mg dry weight per ml). The initial concentration of O_2 was 22.1 μM.

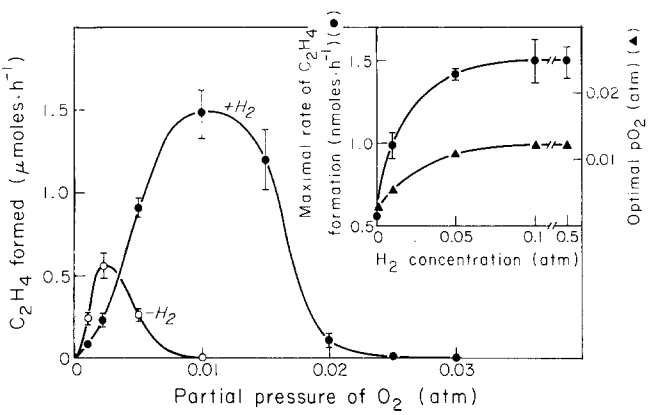

Fig. 1. Effect of O_2 and H_2 on the rates of C_2H_2 reduction by soybean nodule bacteroids (R. japonicum USDA 122 DES). Assays were conducted in 20 ml vials containing 2.4 ml of HEPES-Mg-phosphate buffer. The gas phase consisted of 0.1 atm C_2H_2, 0.1 atm H_2 as indicated and sufficient argon to obtain 1 atm. The reactions were initiated by injecting 0.1 ml of bacteroids (5.7 mg dry weight) into each vial. Vials were incubated in a shaking bath (150 strokes/min) at 23º and 0.5 ml gas samples were removed for C_2H_4 determination by gas chromatography after 15 and 30 minutes of incubation. Data presented are the averages of two replicate experiments ± standard error of the mean.

The inserted graph represents the maximal rates of C_2H_2 reduction and the optimal O_2 partial pressures for maximal C_2H_2 reduction obtained from experiments identical to that depicted in the main graph except that H_2 at the partial pressures indicated was added.

initial positive response in the rates of C_2H_2 reduction to increasing amounts of O_2 either with or without H_2 suggests that nitrogenase activity was limited by the generation of energy, through oxidative phosphorylation (12) (Fig. 1). Direct measurement of the steady-state content of ATP in aerobically prepared bacteroids have shown that the addition of H_2 increased ATP synthesis (9). These results are consistent with those obtained with R. leguminosarum bacteroids (8), blue-green algae (13-15) Rhodopseudomonas capsulata (16) and Azotobacter (17). The possibility that H_2 oxidation also provided reductant for bacteroid nitrogenase has not been excluded.

At partial pressures of O_2 above 0.002 atm, the rates of C_2H_2 reduction that were supported by endogenous substrates were suppressed and at 0.02 atm, C_2H_2 reduction was almost completely inhibited (Fig. 1). In contrast, when 0.1 atm of H_2 was added, the rate of C_2H_2 reduction was maximal at 0.012 atm O_2. Addition of H_2 caused an increase in O_2 consumption and therefore, respiratory protection for nitrogenase. The decline in nitrogenase activity at O_2 partial pressures above the optimum may be explained by assuming that the O_2 input into the bacteroid suspension exceeded the oxidative capacity of the bacteroids, and the excess of O_2 inactivated the nitrogenase system (18, 19). When the partial pressure of O_2 over the nitrogenase-inactivated bacteroids (in the absence of H_2) was lowered from 0.01 to 0.002 atm, the C_2H_2 reduction rates were increased from 0.6 to 426 nmoles per hour. The inactivation of nitrogenase by O_2 under these conditions, therefore, was reversible.

C_2H_2 reduction rates and respiratory protection of nitrogenase in bacteroid suspensions increased with increasing partial pressures of H_2 in the gas phase up to a saturating partial pressure of 0.1 atm H_2 (Fig. 1, inserted graph). H_2 produced no effect on the rate of C_2H_2 reduction by bacteroid suspensions from nodules formed by the H_2-uptake negative R. japonicum strain USDA 117.

H_2-dependent C_2H_2 reduction decreased as the succinate concentration in the bacteroid suspensions increased (Fig. 2). The effect of H_2 was maximal at succinate concentrations below 1 mM. No H_2-supported C_2H_2 reduction was observed at succinate concentrations higher than 1 mM. Protection of nitrogenase from O_2 demage, however, was provided by H_2 at a succinate concentration of 50 mM. The additional respiratory

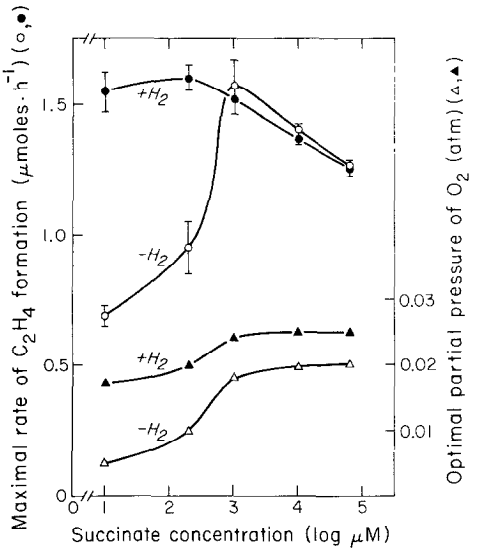

Fig. 2. Effect of succinate with and without H_2 on the maximal rates of C_2H_2 reduction and on the optimal O_2 partial pressures for maximal C_2H_2 reduction. A series of experiments analogous to that in Figure 1 (major graph) were conducted at different succinate concentrations. From the curves obtained the maximal rate of C_2H_2 reduction and the optimal O_2 partial pressures for maximal rates of C_2H_2 reduction were determined and plotted against the succinate concentration. The assays were carried out as described in the legend of Figure 1 except that succinate was included in the HEPES-Mg-phosphate buffer as indicated an the pH was adjusted to 7.5 with KOH.

protection resulting from supplying H_2 to suspensions containing 10 mM or 50 mM succinate is consistent with the observation that the addition of H_2 increased the rate of O_2 uptake by bacteroids supplied with succinate (Table 1). H_2 obviously functions effectively as a respiratory substrate for R. japonicum bacteroids even at saturated concentrations of succinate.

These results provide direct evidence for the existence of a metabolic interaction between hydrogenase and nitrogenase in bacteroids and provide a rational basis for expecting a beneficial effect of hydrogenase on N_2 fixation in nodulated legumes.

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