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ROLE OF UPTAKE HYDROGENASE IN PROVIDING REDUCTANT FOR NITROGENASE IN *RHIZOBIUM LEGUMINOSARUM* BACTEROIDS ¶

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The role of uptake hydrogenase in providing reducing power to nitrogenase was investigated in *Rhizobium leguminosarum* bacteroids from nodules of *Pisum sativum* L. (cv. Homesteader). H_2 increased the rate of C_2H_2 reduction in the absence of added substrates. Malate also increased nitrogenase (C_2H_2) activity while decreasing the effect of H_2. At exogenous malate concentrations above 0.05 mM no effect of H_2 was seen. Malate appeared to be more important as a source of reductant than of ATP. When iodoacetate was used to minimize the contribution of endogenous substrates to nitrogenase activity in an isolate in which H_2 uptake was not coupled to ATP formation, H_2 increased the rate of C_2H_2 reduction by 77%. In the presence of iodoacetate, an ATP-generating system did not enhance C_2H_2 reduction, but when H_2 was also included, the rate of C_2H_2 reduction was increased by 280% over that with the ATP-generating system alone. The data suggest that, under conditions of substrate starvation, the uptake hydrogenase in *R. leguminosarum* could provide reductant as well as ATP in an isolate in which the H_2 uptake is coupled to ATP formation, to the nitrogenase complex.

Introduction

It has been established that in a *Rhizobium*-legume symbiosis the presence of an uptake hydrogenase capable of oxidizing H_2 can lead to recycling of the H_2 produced by the nitrogenase complex [1,2]. Furthermore, this uptake of H_2 may be coupled to ATP formation [1,3].

Bothe et al. [4] reported that H_2 oxidation may provide both ATP and reductant for nitrogenase activity in blue-green algae. Data indicating that the uptake of H_2 will contribute to transfer of electrons to nitrogenase have also been presented for *Azotobacter* [5]. The lack of inhibition of C_2H_2 reduction by iodoacetate when H_2 was present in *Rhizobium japonicum* led Emerich et al. [3] to raise the question of whether the reductant could have been supplied by reactions, such as hydrogen uptake, which are insensitive to iodoacetate.

Our previous studies with the *R. leguminosarum* pea symbiosis led us to conclude that uptake hydrogenase was coupled to ATP formation in only a few of the rhizobial isolates tested [6]. The main role of uptake hydrogenase in our experiments appeared to be in providing protection against O_2 damage. In agreement with the data of Emerich et al. [3], we also observed a greater effect of H_2 on C_2H_2 reduction rate in samples where C_2H_2 reduction was inhibited by iodoacetate than in samples without the inhibitor.

This led us to investigate further the role of uptake hydrogenase in the *R. leguminosarum*–pea

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Abbreviations: Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino}ethanesulphonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

symbiosis. In this paper we present data consistent with the notion that the uptake of H_2 can provide reducing power to the nitrogenase complex regardless of whether or not H_2 uptake is coupled to ATP formation.

Materials and Methods

Cultures. R. leguminosarum isolates 128C30, in which the uptake of H_2 was shown to be coupled to ATP formation, and 128C52, in which it was uncoupled [6], were obtained from J. Burton, Nitragin, Milwaukee, WI. In the coupled isolate the addition of H_2 resulted in an increase in the ATP level in aerobic samples, in which nitrogenase was inactive, whereas in the uncoupled isolate no difference was found between samples with or without H_2, even though the uptake hydrogenase was found to be active.

Plant culture. Pisum sativum L. (cv. Homesteader) seeds were surface sterilized, planted in sterile Leonard jar assemblies containing Turface, a granular montmorillonite clay support (Wyandotte Chemicals, Wyandotte, MI) and N-free medium, inoculated with one of the rhizobial cultures and grown in a controlled environment cabinet (Enconaire System, Winnipeg, Canada) as described by Nelson and Child [7].

Preparation of bacteroids. Nodules (200–300 mg nodule tissue per ml grinding medium) from 4-week-old plants were placed in a plastic bag, Atmos Bag (Terochem Laboratories, Edmonton, Canada), under argon flow and ground with a mortar and pestle using the grinding medium of Salminen [8]. The homogenate was filtered through two Miracloth (Chicopee Hills, Milltown, NJ) discs in a 10 ml syringe and collected into a centrifuge tube. The tube was capped while under argon flow and centrifuged at $2000 \times g$ for 10 min. The bacteroid pellet was resuspended in fresh grinding medium under argon flow.

Acetylene reduction assay. Acetylene reduction was used as an indirect measure of nitrogenase activity. The reactions were carried out in 1.5×10 cm test tubes, containing 1.5 ml liquid assay mixture. The gas phase contained 0.05 atm C_2H_2, 0.03 or 0.05 atm O_2 and, when required, 0.01 atm H_2.

Uptake hydrogenase activity. The $^3H_2$ uptake method was used as described by Nelson and Salminen [6]. A mixture of $^3H_2$ and H_2 (Matheson Canada, Whitby, Canada, specific activity 210 μCi $\cdot$ ml^{-1}) was stored in a lecture bottle equipped with a single-stage regulator and a septum permitting withdrawal of $^3H_2$. The reactions were carried out in 22 ml scintillation vials capped with Suba seals (William R. Freeman, Barnsley, U.K.). Each vial contained 0.5 ml of bacteroid preparation and 1 ml 80 mM Tes buffer (pH 7.1) with 0.01 atm $^3H_2$ in the gas phase. After shaking the vials at 240 rpm at 30°C for 30 min, the reaction was stopped by addition of 1 ml methanol. H_2 was bubbled through the reaction mixture for 30 s to remove any dissolved $^3H_2$ and 10 ml of Phasar scintillation cocktail (Amersham Corp., Oakville, Canada) was added. Radioactivity in the samples was then determined in a Philips liquid scintillation counter (model PW4700).

ATP determination. The levels of ATP were determined by the luciferin-luciferase method as described by Nelson and Salminen [6] using reagents from Beohringer-Mannheim Canada (Dorval, Canada). The ATP was extracted by the rapid addition of 0.5 ml of the reaction mixture used for C_2H_2 reduction assays to 4.5 ml boiling Tris/EDTA buffer (20 mM Tris/2 mM EDTA, pH 7.7), and the mixture was heated at 100°C for 90 s. The mixture was centrifuged at $12000 \times g$ for 15 min and 0.1 ml of the supernatant fluid was assayed for ATP. The extract was mixed with 0.5 ml Tris/EDTA buffer in a 10 ml mini-scintillation vial and 0.4 ml of freshly prepared luciferin-luciferase reagent (20 μg luciferase, 1 mg luciferin in 10 ml Tris/EDTA buffer) was added. The sample was immediately shaken and luminescence determined in the Philips liquid scintillation counter (model PW4700). ATP standards were always run concurrently.

Results

The effects of H_2 and malate on C_2H_2 reduction were determined in bacteroid preparations of the coupled (128C30) and uncoupled (128C52) isolate. At supraoptimal O_2 level (0.05 atm), in the absence of any added substrate, both isolates showed increased rates of C_2H_2 reduction when H_2 was added (Table I). A similar effect of H_2 was shown by Nelson and Salminen [6]. This increase

TABLE I

EFFECT OF H_2 AND MALATE ON THE RATE OF ACETYLENE REDUCTION

The reaction mixture contained 0.5 ml bacteroid preparation in 1.5 ml 80 mM Tes buffer (pH 7.1). Malate was added as indictaed at the concentrations given below. The gas phase contained 0.05 atm C_2H_2, 0.05 atm O_2 and 0.01 atm H_2, when indicated below. Means ± S.E. of duplicate samples.

Isolate	Malate (mM)	C_2H_2 reduced (nmol/g nodule fresh wt. per h)			
		Buffer	Buffer + H_2	Malate	Malate + H_2
128C30	5	286 ± 9	565 ± 2	1090 ± 12	982 ± 42
128C52	1	56 ± 9	212 ± 21	470 ± 31	528 ± 0

was not as great, however, as in the presence of an exogenous substrate, malate, added to provide the bacteroids with a potential source of both reducing power and ATP. One mM malate inhibited the rate of H_2 uptake by 10–20% after 30 min (data not shown). In shorter time intervals or with malate concentrations less than 1 mM no inhibitory effect was seen.

Malate concentrations were varied to determine whether H_2 could enhance C_2H_2 reduction in the presence of low levels of malate in 128C30 (Table II). At 0.03 atm O_2, H_2 increased the C_2H_2 reduction rate by 48% over the buffer-control rate. The addition of even low levels of malate led to a decrease in this effect, and no enhancement of C_2H_2 reduction by H_2 was seen at malate levels above 0.050 mM. The magnitude of the increase in C_2H_2 reduction rate by in the presence of H_2 varied between bacteroid preparations, presumably as a result of varying levels of endogenous substrates present in the bacteroids. A consistent effect of H_2 on C_2H_2 reduction rate was observed only when malate concentrations were below 0.05 mM.

TABLE II

EFFECT OF MALATE CONCENTRATION ON H_2 STIMULATION OF C_2H_2 REDUCTION IN 128C30

The reaction mixtures were as in Table I and malate was added as indicated below. Results are the ratio of rates of C_2H_2 reduction with 0.01 atm H_2 in the gas phase to that without H_2 in the gas phase, expressed as a percentage. Means ± S.E. of duplicate samples. The actual rates in different experiments for the buffer controls varied from 204 to 286 nmol/g nodule fresh wt. per h.

Malate (mM)	C_2H_2 reduced (% of buffer control)
0	148 ± 16
0.002	118 ± 3
0.005	118 ± 4
0.010	121 ± 13
0.025	115 ± 6
0.050	109 ± 5
0.50	98 ± 5
1.0	87 ± 16
5.0	93 ± 3

In order to determine whether providing the bacteroids with a source of ATP would abolish the enhancing effect of H_2 in buffer, we added an ATP-generating system, phosphocreatine and creatine phosphokinase, without exogenous ATP (Table III). The results are expressed as a percentage of the buffer control; this enabled us to combine several experiments with different treatments in a single table. When an O_2 level below the optimum for C_2H_2 reduction was used (0.03 atm) to minimize the protective effect of H_2 against O_2 damage [6], H_2 did not enhance the rate of C_2H_2 reduction over that in buffer alone in either the coupled or uncoupled isolate. We have previously observed an effect of H_2 on C_2H_2 reduction under these conditions in a coupled isolate (Table II) [6]. Variations in the levels of endogenous substrates in different bacteroid preparations may account for this differential effect of H_2. The ATP-generating system inhibited the rate of C_2H_2 reduction by 61% in the coupled isolate without significantly affecting the uncoupled isolate. Addition of H_2 increased the rate in the coupled isolate by 34% and in the uncoupled isolate by 59%. When malate, a potential source of reductant, was

TABLE III

EFFECT OF H_2, MALATE AND ATP-GENERATING SYSTEM (GS) ON C_2H_2 REDUCTION RATE AND ATP CONTENT OF BACTEROIDS

The reaction mixture was as in Table I. H_2 at 0.01 atm, malate at 1 mM, and ATP-generating system (45 μmol creatine phosphate and 600 μg creatine phosphokinase) were added as indicated below. The bacteroids were incubated under 0.03 atm O_2. Means ± S.E. of two or three experiments with duplicate samples in each. The table is a composite of several experiments; values are percentages of buffer control, normalized by assigning the rate in buffer the value 100%. The actual rates for both isolates varied from 192 to 321 nmol/g nodule fresh wt. per h, except for one experiment with 128C52 where a rate of 56 ± 9 was measured (see Table I).

Reaction mixture addition	Isolate 128C30		Isolate 128C52	
	C_2H_2 reduced	ATP content	C_2H_2 reduced	ATP content
Buffer + H_2	92 ± 8	144 ± 8	95 ± 5	106 ± 7
Malate	381 ± 4	176 ± 13	839 ± 55	100 ± 0
GS	39 ± 7	466 ± 72	111 ± 6	140 ± 10
GS + H_2	73 ± 4	569 ± 23	159 ± 12	150 ± 7
GS + malate	656 ± 47	573 ± 28	223 ± 1	127 ± 6
GS + malate + H_2	619 ± 15	587 ± 23	201 ± 5	125 ± 13

included with the ATP generating system, C_2H_2 reduction rates increased 12-fold in the coupled and 2-fold in the uncoupled isolate over that with ATP-generating system alone. No further increase in activity was seen on addition of H_2. Curiously, in the case of 128C52, the relative increase in C_2H_2 reduction activity was greater with malate alone than with the ATP generating system and malate. The table is a composite of several experiments and in the experiments with malate alone, the C_2H_2 reduction rate in buffer was rather low, lower than in the experiments with ATP generating system and malate. This variation between bacteroid preparations may thus account for these differences.

Addition of H_2 led to a 44% increase in ATP level over that in buffer alone in 128C30 (Table III). The addition of ATP-generating system appeared to saturate the ATP pool, as no significant increases were seen in the remaining treatments. Addition of H_2 to the buffer reaction mixture had no significant effect on ATP in isolate 128C52, in which H_2 uptake is not coupled to ATP formation [6]. The ATP-generating system increased ATP levels by 40% and no further increases were seen with any of the other treatments.

As the ATP levels measured could be affected by the turnover of ATP used in C_2H_2 reduction, bacteroids were prepared aerobically to inactivate nitrogenase before addition of substrates (data not shown). ATP levels in aerobically prepared bacteroids were very similar to those obtained in anaerobic preparations (Table III).

As endogenous substrates could contribute to the ATP and reductant pools, their contribution was minimized by using iodoacetate, a general inhibitor of dehydrogenases. The uncoupled isolate, 128C52 was used to clarify further the role of H_2 uptake, since H_2 would not increase the level of ATP. Iodoacetate reduced the rate of C_2H_2 reduction by 50% and the ATP level by 40% (data not shown) [6]. In the presence of iodoacetate, H_2

TABLE IV

EFFECT OF H_2 AND ATP-GENERATING SYSTEM (GS) ON C_2H_2 REDUCTION IN THE PRESENCE OF IODOACETATE (IA) IN ISOLATE 128C52

Reaction mixture was as in Table I with the addition of 16 mM iodoacetate, ATP-generating system (45 μmol creatine phosphate and 600 μg creatine phosphokinase) and 0.01 atm H_2 as indicated below. The bacteroids were incubated under 0.03 atm O_2. Means ± S.E. of duplicate samples.

Reaction mixture addition	C_2H_2 reduced (nmol/g nodule fresh wt. per h)	ATP content (nmol/mg dry wt.)
IA	61 ± 20	0.53 ± 0.03
IA + H_2	108 ± 17	0.54 ± 0.04
IA + GS	85 ± 18	0.69 ± 0.04
IA + GS + H_2	238 ± 6	0.78 ± 0.02

TABLE V

EFFECT OF H_2 AND NADH+H^+ ON C_2H_2 REDUCTION RATE IN THE PRESENCE OF ATP-GENERATING SYSTEM (GS) IN ISOLATES 128C30 AND 128C52

Reaction mixture was as in Table I with the addition of ATP-generating system (45 μmol creatine phosphate and 600 μg creatine phosphokinase), NADH+H^+ (0.46 mM) and H_2 (0.01 atm), as indicated below. Bacteroids were incubated at 0.03 atm O_2. Means ± S.E. of duplicate samples. The actual rates for the ATP-generating system controls varied from 390 to 570 nmol/g nodule fresh wt. per h in both isolates.

Reaction mixture addition	C_2H_2 Reduced (% of GS alone)	
	128C30	128C52
GS+H_2	214± 7	188±10
GS+NADH+H^+	125± 1	118± 9
GS+NADH+H^++H_2	210±17	238±23

almost doubled the rate of C_2H_2 reduction without any observable effect on the level of ATP (Table IV). When ATP-generating system was included there was a 30% increase in ATP content, but no significant alteration in the rate of C_2H_2 reduction. Only when H_2 was added along with ATP-generating system did the C_2H_2 reduction rate increase to a level more than twice that with iodoacetate and H_2, suggesting ATP limitation in the absence of the ATP generating system.

The nature of the reductant for bacteroid nitrogenase is not clear. When NADH + H^+ was used as reductant, the rate of C_2H_2 reduction was increased by 25% over that with ATP-generating system alone in the coupled isolate, 128C30, and by 18% in the uncoupled isolate, 128C52 (Table V). When both H_2 and NADH + H^+ were provided, the rate was the same as with H_2 alone, which was approximately double that with ATP-generating system alone.

Discussion

Earlier studies led us to suggest that the main function of uptake hydrogenase at supraoptimal O_2 levels appears to be in protecting nitrogenase against O_2 damage [6]. The enhancing effect of H_2 on C_2H_2 reduction was masked by malate which also gave a several-fold increase in C_2H_2 reduction (Table I). This effect of exogenous substrate on C_2H_2 reduction has been described before [8,9]. In *R. japonicum*, H_2 did not stimulate C_2H_2 reduction when succinate was present [3].

As the results with malate were similar at suboptimal O_2 levels (Table III), it is unlikely that the effect of malate could be accounted for by O_2 protection alone. The uptake hydrogenase system was still functional, although the inhibitory effect of substrate was a factor and has been reported before in *R. japonicum* [10]. However, the amount of malate required to abolish the enhancement of C_2H_2 reduction by H_2 (Table II) was much less than 1 mM, the level at which significant inhibition of uptake hydrogenase has been observed.

Addition of nothing but ATP-generating system will not necessarily lead to increased C_2H_2 reduction (Tables III, IV). Indeed, in the coupled isolate, 128C30, a strong inhibition was observed. No inhibition of C_2H_2 reduction was seen with the uncoupled isolate, 128C52. The ATP-generating system had a relatively small effect on the ATP level in this isolate (Table III) and this may account for the differences in response of the two isolates. The inhibitory effect of ATP on nitrogenase activity has been described by others [8,11].

H_2 failed to stimulate the rate of C_2H_2 reduction by isolate 128C30 in buffer reaction mixture in some experiments (Table III). This may have been due to the presence of endogenous substrates which masked H_2 enhancement. We have observed that the greater the rate of C_2H_2 reduction in buffer, the smaller the effect of H_2. In 128C30, adding H_2 in the presence of the ATP-generating system doubled the rate of C_2H_2 reduction (Table III). Unless there is a small separate pool of ATP which we were unable to resolve, these data suggest that here H_2 was providing reductant rather than ATP. Addition of malate with the ATP-generating system resulted in a 6-fold increase in C_2H_2 reduction and here H_2 had no further effect. This suggests that malate acts more as a source of reducing power than as a source of ATP, although the presence of an ATP pool which supports C_2H_2 reduction more directly than ATP provided by the generating system cannot be ruled out. If the enhancement of C_2H_2 reduction by H_2 or malate is due to the provision of reductant, then clearly malate is more efficient in doing so.

In the uncoupled isolate 128C52, where we were

unable to detect any effect of H_2 on ATP level (Table III) [6], the explanation of a separate pool of ATP is less tenable and if true would require a very small pool. The simpler hypothesis is that H_2 uptake and ATP formation are uncoupled. It is interesting to note that malate did not significantly increase the level of ATP in this isolate (Table III); this supports the idea that malate acts more as a source of reducing power than of ATP. Results with ATP-generating system suggest that the uncoupled isolate has a smaller adenosine nucleotide pool than the coupled isolate and this agrees with measured values (0.95 ± 0.07 nmol ATP/mg dry wt. for 128C52 and 1.28 ± 0.04 for 128C30). The addition of H_2 or malate with ATP-generating system increased the rate of C_2H_2 reduction without affecting the level of ATP (Table III), again suggesting these substrates were providing reducing power. The results obtained with iodoacetate (Table IV) were similar and strongly support the view that H_2 oxidation is able to provide reductant for nitrogenase.

$NADH + H^+$ has been implicated in providing reducing power to support nitrogenase activity [12–14]. We observed a stimulation of C_2H_2 reduction by $NADH + H^+$ in both isolates (Table V) but it appeared to be less effective than H_2 when ATP-generating system was a source of ATP.

The contention that H_2 might serve as a reductant for nitrogenase in pea bacteroids agrees with earlier reports by Bothe et al. [4] and Walker and Yates [5] in other N_2-fixing organisms. Our data also indicate that the effect of H_2 can be masked by the presence of endogenous or exogenous substrates, which could provide both reducing power and ATP. Therefore, the importance of uptake hydrogenase in *R. leguminosarum*, as suggested earlier [6], would be limited, except under starvation conditions where it could supply both ATP and reducing power in coupled isolates or reductant alone in uncoupled isolates, as well as protecting the nitrogenase complex from O_2 damage.

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