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REDUCTANT AND ENERGY LEVEL OF BACTEROIDS OF *RHIZOBIUM LEGUMINOSARUM*

NISHA GARG and H. S. NAINAWATEE

Department of Chemistry & Biochemistry Haryana Agricultural University, Hisar–125004, India

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Key Word Index—*Rhizobium leguminosarum*; Cyanophyceae; adenylate charge; bacteroids; hydrogen uptake.

Abstract—Low redox potential electron carriers such as benzyl viologen and NAD^+ were reduced by bacteroids prepared from nodules of pea inoculated with *hup*$^+$ *R. leguminosarum* strain Vp1, whereas, the bacteroids of plasmid cured *hup*$^-$ derivative Vm1 did not exhibit the reducing activity. The bacteroids prepared from nodules of *hup*$^+$ strain Vp1 at 46d stage, where nitrogenase activity was maximum, exhibited 1.5-fold higher levels of NADH/NADPH, two-fold higher levels of ATP while the level of ADP and AMP were lower as compared to the bacteroids of *hup*$^-$ strain Vm1. Also the level of adenylate energy charge in *hup*$^+$ bacteroids was 29% higher than the *hup*$^-$ bacteroids.

INTRODUCTION

Uptake hydrogenase catalyses the oxidation of hydrogen evolved during nitrogen fixation process. The oxidation of exogenously supplied hydrogen by *hup*$^+$ bacteroids is coupled to energy release, conservation of host photosynthates and protection of nitrogenase from oxygen damage [1–3]. Additional energy produced by hydrogen oxidation has been reported to support nitrogenase activity in bacteroids [4], *Azotobacter chroococcum* [5] and blue green algae [6]. However, the mode of energy release during the hydrogen oxidation process is not clearly understood. A few reports indicate the production of ATP by uptake hydrogenase activity [7, 8], yet generation of NAD(P)H and the low redox potential electron carrier has not been established. The lack of inhibition of acetylene reduction by iodoacetate in the presence of hydrogen in *R. japonicum* raises the possibility that the reductant could have been supplied by a reaction such as hydrogen uptake which is insensitive to iodoacetate [9]. An ATP dependent hydrogen oxidation by the reversed respiratory electron flow has been reported in *Azotobacter* and *Bradyrhizobium* [10, 11]. The possible role of uptake hydrogenase in providing low redox potential reductant for nitrogenase in bacteroids of wild type *hup*$^+$ groundnut strain has also been shown [12]. From studies on *hup*$^+$ strain of *R. leguminosarum* and its plasmid cured *hup*$^-$ mutant, we report the hydrogen dependent increased level of reductant and ATP in the bacteroids.

RESULTS AND DISCUSSION

The role of uptake hydrogenase in providing reductant was studied by *in planta* comparison of a *hup*$^+$ wild type field isolate Vp1 of *R. leguminosarum* and *hup*$^-$ strain Vm1 constructed by curing the plasmids of Vp1, by using *Pisum sativum* as the host. The isolate Vp1 exhibited hydrogenase activity under both *ex planta* and *in planta* conditions, whereas, Vm1 showed no activity under both conditions [13].

It has been suggested that the oxidized hydrogen could be used for the generation of reductant [14]. The bacteroids prepared from 46-day-old nodules of pea inoculated with *hup*$^+$ Vp1 and *hup*$^-$ Vm1, when N_2 fixing efficiency was maximum, were capable of using both NADH and NADPH as reductant for the reduction of redox dye benzyl viologen (BV), thus exhibiting the presence of BV reductase activity (Table 1 row a). However, NADH served as a better electron donor than NADPH. This activity was used as a check for the production of reductant due to hydrogenase activity. In *hup*$^-$ Vm1 bacteroids, the reduction of BV with NAD^+ as electron carrier in an H_2 atmosphere was not appreciable. The *hup*$^+$ bacteroids significantly reduced BV with NAD^+ as electron carrier in the H_2 atmosphere. When H_2 was replaced with Ar, there was a six-fold decrease in the reducing activity (Table 1 row b) indicating a positive role of *hup* in providing a reductant. The generation of reductant due to *hup* activity was further confirmed by conducting bacteroid dependent BV-NAD reduction in the presence of iodoacetate, which inhibited the endogenous respiration. The rate of NAD^+ dependent BV reduction was nearly equal to that in the H_2 atmosphere in the presence of iodoacetate (Table 1 row b). The *hup*$^+$ bacteroids of Vp1 were also capable of reducing NAD^+ directly in the H_2 atmosphere (Table 1 row c). The rate of reduction of NAD^+ directly in H_2 was *ca* 12-fold greater as compared to that in argon. These results are supported by the observations of *hup* associated reductant production in groundnut bacteroids [12]. The level of NADH/-NADPH in Vp1 bacteroids was also 1.5 fold higher as compared with Vm1 bacteroids, thus favouring the contention that hydrogen might serve as a reductant for nitrogenase activity in pea bacteroids as well (Table 2). These results are in agreement with earlier reports showing the production of reductant due to *hup* activity in other nitrogen fixing organisms [5, 6]. Hydrogen was also reported to supply reducing equivalents to nitrogenase in photosystem I dependent reaction in *Anabaena cylindrica* [6]. However, in Cyanobacteria, the uptake

Table 1. Reducing activity of bacteroids of *Rhizobium leguminosarum*

Substrate	Reductant	Atmosphere	*A*/hr/mg protein Vp1	Vm1
a) BV	NAD + Bacteroid	Air	0.0704 ± 0.004	0.0693 ± 0.003
BV	NADPH + Bacteroid	Air	0.003 ± 0.0005	0.0042 ± 0.0003
b) BV-NAD	Bacteroid	Ar	0.0019 ± 0.0002	0.0023 ± 0.0002
BV-NAD	Bacteroid	H_2	0.0119 ± 0.001	0.0017 ± 0.0002
BV-NAD	Bacteroid	H_2 + Iodoacetate	0.0111 ± 0.001	0.0021 ± 0.0001
c) NAD	Bacteroid	Ar	0.0015 ± 0.0002	0.0018 ± 0.000
NAD	Bacteroid	H_2	0.0178 ± 0.002	0.0018 ± 0.000

Values are mean of 4 replicates ± s.d.

Table 2. Level of metabolites in bacteroids prepared from 46-day-old nodules of pea inoculated with *R. leguminosarum hup*$^+$ strain Vp1 or plasmid cured *hup*$^-$ strain Vm1

Metabolite	Concentration (nmol/mg protein) Vp1	Vm1
NADH	18.8 ± 2.3	10.4 ± 1.6
NADPH	40.3 ± 1.0	30.5 ± 2.7
ATP	13.5 ± 1.4	8.66 ± 0.59
ADP	6.25 ± 0.34	21.2 ± 1.0
AMP	11.2 ± 1.0	16.5 ± 0.8
Pi	271 ± 7.8	247 ± 4
Adenylate energy charge	0.537	0.418

Values are mean of 4 replicates ± s.d.

hydrogenase dependent reduction of $NADP^+$ was not observed [15].

The amount of ATP and adenylate energy charge was respectively 50 and 29% greater, whereas the level of ADP and AMP were respectively 3.4- and 1.5-fold smaller in *hup*$^+$ bacteroids of Vp1 as compared to *hup*$^-$ Vm1 bacteroids (Table 2). These results are supported by reports indicating that uptake hydrogenase is associated with greater ATP concentration in nodules of several other crops [1, 3, 7, 9]. Higher nitrogenase activity in *hup*$^+$ strain of *R. leguminosarum* was also attributed to the increased production of ATP due to uptake hudrogenase activity [14]. However, in *Azotobacter* and *Bradyrhizobium* the hydrogen oxidation has been reported to be ATP dependent which takes place by reversed respiratory electron flow [10]. In Vp1 *hup*$^+$ bacteroids, ATP did not influence the hydrogen oxidation. In soybean and lupin nodules also, an ATP independent hydrogenase activity is reported to be present [16]. Although the evidence reported here by using *hup*$^+$ and plasmid cured *hup*$^-$ strains of *R. leguminosarum*, prove the beneficial role of uptake hydrogenase in providing reductant and energy for nitrogenase, there exists a possibility that other characters prescribed by the plasmid might have also affected the metabolite status of the nodules.

EXPERIMENTAL

Strain. The wild type *hup*$^+$ *R. leguminosarum* strain Vp1 was isolated in this laboratory and the *hup*$^-$ strain Vm1 was produced by plasmid curing of Vp1 [13].

Bacteroid preparation. Bacteroids were prepared from nodules of 46-day-old pea plants inoculated with either *hup*$^+$ Vp1 or *hup*$^-$ Vm1. Nodules (10 g) were macerated in a pestle and mortar in 25 ml of 50 mM K-Pi buffer, pH 6.8, containing 0.2 M Na ascorbate and 1.7 g polyvinyl-polypyrrolidone (PVP). The homogenate was passed through 4 layers of cheese cloth, centrifuged at 9000 *g* for 15 min, the pellet of bacteroid was carefully separated from PVP, washed × 3 with 50 mM K-Pi buffer, pH 7, containing 2.5 mM $MgCl_2$ and finally suspended in 50 mM K-Pi buffer, pH 7.5. The protein content of the bacteroids was determined by using Folin reagent [17].

Redox dye reduction. The reduction of redox dye benzyl viologen under Ar or H_2 atmosphere was observed in the Thunberg tubes. The reaction mixture (3.5 ml) contained 60 μmol K-Pi buffer, pH 7.5, 2.5 μmol BV, 1.5 μmol NAD^+ in the main arm and bacteroids (0.5 ml, ~6 mg protein) in the side arm [18]. The Thunberg tubes were flushed with Ar or H_2. Reaction was started by tipping the bacteroids in the main arm, incubated for 4 hr at 30° and *A* was read at 610 nm.

To check the activity of BV-reductase, an experiment was conducted using 5 μmol NAD(P)H in the main arm in place of NAD^+, and the amount of bacteroids was reduced to 0.2 ml.

NAD^+ reduction. The reaction mixture (2.5 ml) for direct hydrogen dependent reduction of NAD^+ by bacteroids, contained 50 μmol K-Pi buffer pH 7.5, 1.5 μmol NAD^+ in the main arm of Thunberg tube and bacteroids (0.5 ml, ~6 mg protein) in the side arm. The reaction was started by tipping the bacteroids in the main arm and after incubation for 3 hr at 30°, terminated by addition of 5 ml saturated $(NH_4)_2SO_4$ soln and centrifuged at 10000 *g* for 15 min. The amount of NAD^+ reduced was measured in the supernatant at 340 nm.

Metabolites extraction/estimation. Bacteroids for the estimation of metabolites were prepared from fresh nodules frozen in liquid N_2. The bacteroid pellet was transferred to chilled 0.8 M $HClO_4$ and neutralized with 5 mM K_2CO_3 at the time of metabolite estimation. The bacteroid suspension was mixed with 20 mM Tris–Cl buffer, pH 7.7, containing 2 mM EDTA, the mixture was kept at 100° for 90 sec, centrifuged at 12000 *g* for 15 min. The supernatant was used for the estimation of metabolites. The assay mixture (1.5 ml) for estimation of reductants NAD^+/$NADP^+$ contained 0.5 ml bacteroid extract (0.8 mg protein) 20 μl EtOH and 100 μmol Tris–Cl buffer, pH 8 (ref. [19]).

Increase in A at 340 nm on addition of 2 IU of alcohol dehydrogenase was taken as a measure of NAD^+ concn. The reaction mixture (1.5 ml) for $NADP^+$ estimation contained 0.5 ml bacteroid extract (0.8 mg protein), 10 μmol $MgSO_4$, 60 μmol Tris–Cl buffer pH 7.6 and 1 μmol glucose-6-phosphate. The A increase at 340 nm on addition of 2 IU of glucose-6-phosphate dehydrogenase was used for the estimation of $NADP^+$ concentration.

ATP was estimated using ATP dependent $NADP^+$ reduction in a coupled assay for hexokinase-glucose-6-phosphate dehydrogenase [20]. The assay mixture (1.5 ml) contained 0.5 ml bacteroid extract (0.8 mg protein), 60 μmol Tris–Cl buffer pH 7.8, 10 μmol $MnSO_4$ and 1 μmol $NADP^+$. After addition of 2 IU of glucose-6-phosphate dehydrogenase, the A (E_1) was recorded at 340 nm. The reaction was initiated by 2 IU of hexokinase and 5 μmol glucose and after 30 sec A (E_2) was recorded. The concn of ATP in the sample was obtained by the difference between E_2 and E_1.

For the estimation of ADP and AMP, the reaction mixture (1.5 ml) contained 0.5 ml bacteroid extract (0.8 mg protein), 60 μmol Tris–Cl buffer pH 7.6, 0.25 μmol NADH, 3 μmol phosphoenol pyruvate, 10 μmol $MgSO_4$ and 30 μmol KCl (ref. [21]). The A (E_1) at 340 nm was recorded after addition of 15 IU of lactate dehydrogenase. The pyruvate kinase (10 IU) was then added and A (E_2) was recorded. To this 10 IU of myokinase was added and A (E_3) was recorded. Amounts of ADP and AMP were computed respectively from the A differences ($E_2 - E_1$) and ($E_3 - E_2$).

Pi was estimated according to ref. [22]. Equal vols of bacteroid extract and molybdate reagent (0.5 M H_2SO_4 containing 40% $FeSO_4$ and 1% ammonium molybdate) were mixed thoroughly, the A was read at 700 nm against a suitable blank.

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