Properties of the Peribacteroid Membrane ATPase of Pea Root Nodules and Its Effect on the Nitrogenase Activity

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Peribacteroid membrane vesicles from pea (Pisum sativum) root nodules were isolated from membrane-enclosed bacteroids by an osmotic shock. The ATPase activity associated with this membrane preparation was characterized, and its electrogenic properties were determined. The pH gradient was measured as a change of the fluorescence intensity of oxonol VI, bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol. It was demonstrated that the ATPase generates a pH gradient as well as a membrane potential across the peribacteroid membrane. The reversibility of the ATPase was demonstrated by a light-dependent ATP synthesis by peribacteroid membrane vesicles fused with bacteriorhodopsin-phospholipid vesicles. The light-driven ATP synthesis by the peribacteroid membrane ATPase was completely inhibited by a proton-conducting ionophore. The proton-pumping activity of the peribacteroid membrane ATPase could also be demonstrated with peribacteroid membrane-enclosed bacteroids, and effects on nitrogenase activity were established. At pH values below 7.5, an active peribacteroid membrane ATPase inhibited the nitrogenase activity of peribacteroid membrane-enclosed bacteroids. At pH values above 8, at which whole cell nitrogenase activity was inhibited, the proton-pumping activity of the peribacteroid membrane ATPase could partially reverse the pH inhibition. Vanadate, an inhibitor of plasma membrane ATPases, also inhibited nodular nitrogenase activity. It will be proposed that the proton-pumping activity of the peribacteroid membrane ATPase in situ is a possible regulator of nodular nitrogenase activity.

In the root nodules of legumes, the nitrogen-fixing bacteroids are separated from the cytoplasm of the plant cell by a plant-derived membrane called the peribacteroid membrane. This membrane provides the plant with a possible means for regulating nutrient exchange with the microsymbiont and potentially a way to control nitrogen fixation. The peribacteroid membrane contains several transport systems of which the dicarboxylic acid transport system is the best studied (Ou Yang et al., 1990; Udvardi et al., 1991). It has also been reported that the isolated peribacteroid membrane contains an ATPase (Robertson et al., 1978; Verma et al., 1978; Blumwald et al., 1985; Bassarab et al., 1986; Domigan et al., 1988). The latter enzyme was also demonstrated in intact soybean symbiosomes (bacteroids enclosed by the peribacteroid membrane) (Udvardi and Day, 1989; Ou Yang et al., 1990; Udvardi et al., 1991).

In broken root nodule protoplasts, energization of the peribacteroid membrane by ATP hydrolysis was demonstrated by a protonophore-sensitive acidification of the peribacteroid space (Blumwald et al., 1985), and with isolated symbiosomes or with peribacteroid membrane vesicles, the formation of a positive ∆Ψ across the membrane was shown (Udvardi and Day, 1989; Ou Yang et al., 1990; Udvardi et al., 1991). Also, a slight stimulation of the malate uptake into soybean symbiosomes by MgATP was demonstrated (Ou Yang et al., 1990). However, attempts to measure a ΔpH across the peribacteroid membrane of soybean with fluorescent probes were not successful (Blumwald et al., 1985; Udvardi and Day, 1989), in contrast to other plant-derived membrane vesicles that are also supposed to contain a H+-pumping ATPase (Vara and Serrano, 1982). Later, Udvardi et al. (1991) reported that a ΔpH was formed only slowly and that the rate and the extent of formation were stimulated by the presence of permeant anions. The reason why the ΔpH was formed slowly was unclear, since the permeant anions used dissipated the ΔΨ immediately. Possible explanations are a low ATPase activity in combination with a highly buffered peribacteroid space of large volume. It is also possible that due to transport systems present in the peribacteroid membrane of soybean, no significant ΔpH is formed.

A striking difference between the soybean and lupin membrane preparations is the difference in specific activity. The reported values for soybean varied between 7 nmol Pi released min⁻¹ mg⁻¹ protein at 25°C (Udvardi and Day, 1989) and 300 nmol Pi released min⁻¹ mg⁻¹ protein at 37°C (Blumwald et al., 1985). The activity for lupin peribacteroid membrane vesicles is 750 nmol Pi formed min⁻¹ mg⁻¹ protein at 27°C (Domigan et al., 1988). The specific activity of the pea peribacteroid membrane ATPase is in the same order as the lupin enzyme (this paper). Based on the specificity of the enzyme, it is proposed that the peribacteroid membrane ATPase is the same as the enzyme isolated from the whole cell.

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; ∆Ψ, membrane potential; ΔpH, pH gradient; oxonol VI, bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol; TTFB, 4,5,6,7-tetrafluoro-2-trifluoromethylbenzimidazol.
cific activity, pea or lupin peribacteroid membrane vesicles are better suited than soybean vesicles to the study of the effects of the acidification of the peribacteroid space on nitrogen fixation of the bacteroids. To our knowledge, no information is available about the effects of an active peribacteroid membrane ATPase on the nitrogenase activity of the symbiosomes.

In this report it will be shown that the ATPase activity of peribacteroid membrane vesicles from pea root nodules generates a \( \Delta \phiH \) and \( \Delta V \) across its membrane. In addition it will be demonstrated that the enzyme is able to catalyze the reverse reaction: synthesis of MgATP from MgADP, Pi, and a proton motive force. Experimental data will be presented indicating that the ATPase activity, via the pH of the peribacteroid space and dependent on the pH of the medium, stimulates or inhibits nitrogenase activity of the enclosed bacteroids. Furthermore, it will be shown that vanadate, an inhibitor of the P-type ion-motive ATPases, stimulates nitrogenase activity of intact root nodules. A possible role for the peribacteroid membrane ATPase in the regulation of nodular nitrogen activity will be discussed.

**MATERIALS AND METHODS**

**Growth Conditions and Protein Preparations**

Root nodules were produced under controlled conditions on *Pisum sativum* cv Rondo by inoculation with *Rhizobium leguminosarum* strain PRE. Bacteroids were anaerobically isolated as described by Appels and Haaker (1988), with the following changes. The isolation buffer consisted of 50 mM Tes/KOH, 0.48 M Suc, 50 mM Glc, 5 mM DTT, and 1 mg/mL Glc oxidase grade III, pH 7.4. The bacteroids were obtained by 10 min of centrifugation at 2000g and were washed once with washing buffer (50 mM Tes/KOH, 0.48 M Suc, 50 mM Glc, and 1% [w/v] BSA, pH 7.4). The bacteroids (surrounded by the peribacteroid membrane, also called symbiosomes) were suspended in the washing buffer at a protein concentration of 40 to 50 mg bacteroid protein/mL.

Peribacteroid membrane was prepared from symbiosomes by an osmotic shock. A bacteroid preparation (4.5 mg of peribacteroid membrane or 30 pg of bacteriorhodopsin) was resuspended with rapid stirring in 30 mL of buffer containing 50 mM Tes/KOH and 10 mM MgCl\(_2\), pH 7.4. This suspension was centrifuged at 2,000g for 20 min. The supernatant containing peribacteroid membrane was centrifuged at 100,000g for 60 min. The pellet of peribacteroid membrane was resuspended at approximately 1.8 mg protein/mL in 25 mM Tes/Tris, pH 7.4, and used immediately or stored in liquid nitrogen. This preparation was used in the experiments described and was further characterized by Suc gradient centrifugation. The peribacteroid membrane preparation was layered onto a continuous Suc gradient (20–50%, w/v) containing 50 mM Tes/KOH, pH 7.4, and layered onto a continuous Suc gradient (20–50%, w/v) containing 50 mM Tes/KOH, pH 7.4, as described for peribacteroid membrane.

Peribacteroid membrane vesicles prepared by differential centrifugation or purified on a Suc gradient and cytoplasmic membrane vesicles were sealed before use by sonication (four times, 1 s) as described above.

**Co-Reconstitution of Peribacteroid Membrane and Bacteriorhodopsin**

Purple membrane of *Halobacterium halobium* containing bacteriorhodopsin was isolated by the method of Oesterhelt and Stoeckenius (1976). The bacteriorhodopsin concentration of the purple membrane was determined spectrophotometrically (molar extinction coefficient \( \varepsilon = 63,000 \text{ M}^{-1} \cdot \text{cm}^{-1} \) at 560 nm [Oesterhelt and Stoeckenius, 1976]). Crude soybean phospholipids (Sigma, L-\( \alpha \)-phosphatidylcholine type II) were suspended at 50 mg/mL in 0.15 M NaCl and sonicated to clarity under argon as described by Racker (1979). Co-reconstitution of the bacteriorhodopsin with peribacteroid membrane or with cytoplasmic membrane from bacteroids was achieved by a two-step procedure. In the first step the purple membrane was sonicated with phospholipids as described earlier (Kasahara and Hinkle, 1976). The peribacteroid membrane or cytoplasmic membrane was fused with the purple membrane-phospholipid vesicles in a second step by two freeze-thaw-sonication cycles (Racker, 1979). The co-reconstituted samples were illuminated with a tungsten projector lamp (L151, Fiberoptic-Heim AG, Uetikon am See, Switzerland) with a cut-off filter (520 nm). The light-dependent ATP formation from ADP and Pi was determined by trapping ATP with Glc, hexokinase, Glc-6-P dehydrogenase, and NADP\(^+\) in 50 mM Tes/KOH, 100 mM NaCl, 10 mM Glc, 2.5 mM Na\(_2\)HPO\(_4\), 5 mM MgCl\(_2\), 5 mM ADP, 0.5 mM NADP\(^+\), 30 units/mL hexokinase and 12 units/mL Glc-6-P dehydrogenase, 0.2 mg of peribacteroid membrane or 30 \( \mu \)g of cytoplasmic membrane, 0.5 mg of bacteriorhodopsin, and 5.6 mg of phospholipids per mL. The NADPH formation was measured with an Aminco (Silver Spring, MD) DW2A spectrophotometer in the dual-wavelength mode (340–380 nm) at an A of 0.1, full scale.

**Enzymatic Assays**

The ATPase activity was measured in the standard mixture (50 mM Tes/KOH, 5 mM MgCl\(_2\), 5 mM ATP, 50 mM K\(_2\)SO\(_4\), final pH 7.4) as ADP (Cordewener et al., 1988) or as phosphate formation (Ottolenghi, 1974) at 30°C.

Nitrogenase activity of bacteroids was measured at 30°C as described earlier (Haaker and Wassink, 1984) in 50 mM with a Soniprep 150 Ultrasonic disintegrator (MSE Scientific Instruments, Crawley, Sussex, UK) four times for 30 s each with an amplitude of 26 \( \mu \)m and a frequency of 23 kilohertz under argon at 0°C. The broken cells were removed by centrifugation for 10 min at 4,000g. The supernatant containing cytoplasmic membranes was centrifuged at 100,000g for 60 min, and the pellet was resuspended in 50 mM Tes/KOH, 40 mM d-Glc, and 5 mM MgCl\(_2\), pH 7.4, and layered onto a continuous Suc gradient (20–50%, w/v) containing 50 mM Tes/KOH, pH 7.4, as described for peribacteroid membrane.
Tes/KOH, 2 mM MgCl₂, 300 mM Suc, 0.2 mM myoglobin, 3% (w/v) fatty acid-free BSA, 5 mM sodium L-malate, 10 mM creatine phosphate, and 0.29 mg/mL creatine kinase described by Wittenberg et al. (1974). The optimal nitrogenase activity was determined by measuring the nitrogenase activity at different O₂ values in the gas phase (0.07–3.5%, v/v). Bacteroids were added at a protein concentration of 0.9 mg/mL to the incubation mixture with the following additions when indicated: 5 mM MgATP, 10 mM NH₄Cl, 50 mM Tes/NaOH, pH 7.4, 10 mM D-Glc supplemented with 15 mM NaCl (control), or 15 mM Na₃VO₄ in a sealed serum bottle of 7.0 mL. Both bottles were evacuated and filled with argon. Nitrogenase activity (C₅H₄ reduction) was measured with a gas phase of 90% (v/v) O₂ and 10% (v/v) C₂H₂ and was linear for at least 20 min. To minimize the effect of preparation differences, the consequence of vanadate addition was always compared with the results of a control experiment with NaCl in the medium measured with the same root nodule preparation.

**Analytical Methods**

The percentage of cytoplasmic membrane originating from the bacteroids present in peribacteroid membrane preparations was determined from redox spectra. The spectra were recorded with an Aminco DW2 spectrophotometer. Spectra were collected from 400 to 700 nm, with regular Suba-seal stoppered, 1-cm light-path cuvettes in the split-beam mode with a slit of 1 nm. Oxidized spectra were recorded in 50 mM Tes/KOH, 40 mM GIC, and 5 mM MgCl₂, pH 7.4. After evacuation, filling with argon, and the addition of 1 mM dithionite, reduced spectra were obtained. From the reduced minus oxidized difference spectrum, the A₅₅₈ difference (Cyt c) and the line connecting the troughs at 538 and 572 nm were calculated. The value obtained for peribacteroid membrane preparations was compared with that obtained for cytoplasmic membrane preparations isolated from bacteroids as described above.

The fluorescence changes of the acridine dye ACMA were registered at 22°C with an Eppendorf Fluorometer 1101 M. The excited light was filtered through a 405- to 435-nm filter, and the emitted light was measured through a 500- to 3000-nm filter. The A changes of oxonol VI were measured at the same temperature with an Aminco DW2 spectrophotometer in the dual-wavelength mode, sample wavelength at 608 nm, reference wavelength at 630 nm. The protein concentration was determined by the method of Sedmak and Grossberg (1977) (used for protein determination of peribacteroid membrane preparations) or with the biuret reaction (used for bacteroids) after a precipitation step with deoxycholic acid and TCA (Bensadoun and Weinstein, 1976). BSA was used as a standard. The Suc concentration was determined using a refractometric analysis.

**Chemicals**

PEP, pyruvate kinase, lactate dehydrogenase, Glc oxidase grade III (40 μmol Glc mg⁻¹ protein min⁻¹), NADH, NADP⁺, ATP (disodium salt), ADP (disodium salt), hexokinase, Glc-6-P dehydrogenase, DTT, and BSA were obtained from Boehringer; valinomycin was obtained from Janssen Chimica (Tilburg, The Netherlands); myoglobin and sodium orthovanadate were obtained from Sigma; oxonol VI was purchased from Molecular Probes (Eugene, OR); and ACMA and TTFB were gifts from Prof. R. Kraaijenhof (Free University, Amsterdam, The Netherlands). All other chemicals were of analytical grade.

**RESULTS AND DISCUSSION**

**ATPase Activity**

Peribacteroid membranes were isolated from membrane-enclosed bacteroids by an osmotic shock and purified by differential centrifugation as described for lupin root nodules (Robertson et al., 1978). The purity of the preparations was analyzed by density centrifugation. The main ATPase activity was found in fractions with a Suc concentration of 28 to 35% (not shown). A second ATPase peak was found at a Suc concentration of <20%. The maximum activities of the fractions were routinely 1150 ± 125 and 275 ± 50 nmol ADP mg⁻¹ protein min⁻¹, respectively. When cytoplasmic membranes from bacteroids were analyzed on the Suc gradient, only one peak of ATPase activity appeared at about 20% Suc. By measuring the Cyt content of the preparations by redox spectroscopy, it was estimated that the peribacteroid membrane preparations obtained after differential centrifugation contained maximally 15% cytoplasmic membranes originating from the bacteroids.

The ATPase activity of peribacteroid membrane responded to added ATP in a typical Michaelis-Menten fashion, giving a Vₘₐₓ of 1250 ± 60 nmol ADP mg⁻¹ protein min⁻¹ at 30°C and an apparent Kₘ of 0.52 ± 0.06 mM, which is within the range of Kₘ values reported for other H⁺-pumping plasma membrane ATPases (Sze, 1985). The inhibition constant for vanadate, which competitively inhibited the ATPase activity, was determined from Lineweaver-Burk plots and was 58 ± 4 μM (not shown). Other kinetic properties, such as the moderate cation stimulation and the absence of NO₃⁻ inhibition, did not differ significantly from the data described for peribacteroid membrane preparations from lupin (Domigan et al., 1988). It should be realized that the specific ATPase activity of the pea and lupin peribacteroid membrane preparations is at least 10 to 50 times more active than that of soybean membranes (Blumwald et al., 1985; Domigan et al., 1988; Udvardi and Day, 1989).

**The Proton-Pumping Activity of the Peribacteroid Membrane ATPase**

Energization of the peribacteroid membrane of intact symbiosomes by its ATPase was demonstrated by fluorescent membrane-potential probes (Udvardi and Day, 1989;
Ou Yang et al., 1990; Udvardi et al., 1991). Limited information is available about the generation of a $\Delta \psi$. Fluorescence microscopy with acridine orange with broken protoplasts of infected cells indicated an ATP-dependent proton translocation across the peribacteroid membrane of soybeans (Blumwald et al., 1985), but measurements with isolated symbiosomes with the fluorescent pH probes quinacrine, 9-amino acridine, and acridine orange failed (Udvardi and Day, 1989). Only after dissipation of the $\Delta \psi$ with a permeant anion was a $\Delta \psi$ measuring the $[^{14}C]$-methylamine uptake slowly established (Udvardi et al., 1991). We used the acridine probe ACMA for monitoring a $\Delta \psi$ across the peribacteroid membrane. Like other amines, ACMA accumulates inside membrane vesicles when the internal pH is lower than that of the external medium. The accumulation and protonation of ACMA is associated with a quenching of the probe fluorescence (Schuldiner et al., 1972; Vara and Serrano, 1982; Rotenberg, 1989).

As can be seen in Figure 1, the fluorescence emission of ACMA was decreased after adding MgATP. This indicates that the ATPase generates a $\Delta \psi$ across the peribacteroid membrane vesicles with a lower pH of the internal medium compared with the external medium. MgADP had no effect (not shown). The observed quenching of fluorescence was reversed by the protonophore TTFB, by the inhibitor of the ATPase activity vanadate, and by NH$_4$Cl. NH$_4^+$ dissipates a $\Delta \psi$ by diffusing as NH$_3$ through membranes and reacting inside with H$^+$. Nitrate increased the fluorescence quenching further (Fig. 1). Nitrate dissipates a $\Delta \psi$ by moving into the vesicles in parallel with H$^+$ pumping (Rasi-Caldogno et al., 1985; Sze, 1985; Udvardi and Day, 1989). In the absence of transport systems, NO$_3^-$ collapses the $\Delta \psi$ and increases the $\Delta \psi$ (Rasi-Caldogno et al., 1985), and this was observed (see Fig. 1). This is an indication that pea peribacteroid membrane does not contain a H$^+/\text{NO}_3^-$ symport system as observed for tonoplast vesicles (Blumwald and Poole, 1985). All the ACMA experiments are consistent with the hypothesis that the peribacteroid membrane ATPase generates a significant $\Delta \psi$. The effect of MgATP on the ACMA fluorescence of isolated peribacteroid membrane could also be observed with intact pea symbiosomes (not shown).

For soybean symbiosomes the generation of a $\Delta \psi$ (positive inside) across the peribacteroid membrane was demonstrated (Udvardi and Day, 1989; Udvardi et al., 1991). We were able to confirm these results with isolated peribacteroid membrane vesicles (see Fig. 2). The addition of MgATP caused a red shift of the $A_{530}$ of oxonol VI relative to 608 nm, which is indicative of the formation of a $\Delta \psi$ (Smith et al., 1976; Rasi-Caldogno et al., 1985; Freedman and Novak, 1989). The addition of valinomycin, the protonophore TTFB, and NO$_3^-$, all collapsing the $\Delta \psi$, reversed the spectral shift. The inhibition of ATPase activity by the addition of vanadate (0.4 mM) had the same effect. NH$_4$Cl (10–50 mM) did not collapse the oxonol VI response. This indicates that NH$_4^+$ dissipates the $\Delta \psi$ but not the $\Delta \psi$. In soybean the $\Delta \psi$ is lowered by Cl$^-$, indicative of a significant permeability in relation to the ATPase activity (Udvardi and Day, 1989). This is not the case for pea peribacteroid membrane vesicles.

**Reversibility of Peribacteroid Membrane ATPase**

In principle the P-type ion-motive ATPases are reversible. The slight stimulation (20%) of the ATPase activity by the addition of protonophores to sealed vesicles indicates inhibition by the proton motive force. To prove the coupling between ATP hydrolysis and H$^+$ pumping directly, the reversibility of the peribacteroid membrane ATPase was tested. Peribacteroid membrane vesicles were fused with bacteriorhodopsin-phospholipid vesicles. As shown in Table I, it was possible to demonstrate a protonophore-sensitive light-dependent ATP synthesis. The light dependence and the protonophore-sensitive ATP synthesis excludes the possibility that ATP is formed due to adenylate kinase activity. To exclude the probability that a minor contamination of the peribacteroid membrane preparations with cytoplasmic membrane vesicles derived from bacteroids causes the ATP synthesis via its mitochondrial type of ATP synthase, cytoplasmic membrane vesicles isolated from bacteroids were also used in a reconstitution experiment. When cytoplasmic membrane vesicles were used in an amount corresponding to the maximal contamination observed in peribacteroid membrane preparations (15% on a protein basis), no light-dependent ATP formation was observed (not shown).

The experiments were performed at 22°C. At this temperature the ATPase activity is about 600 nmol Pi released min$^{-1}$ mg$^{-1}$ protein. The specific activity of the ATP synthesis is only 4.8% of the maximal ATPase activity, but this is also found for other systems of bacteriorhodopsin and H$^+$-ATPases (Racker, 1979). The relatively low rate of ATP synthesis compared with the rate of ATP hydrolysis does not mean that the maximal rate of ATP synthesis is only 4.8% of the hydrolysis rate. It should be realized that if the proteins incorporate at random in the phospholipid vesi-
Inhibition of the peribacteroid membrane ATPase by vanadate or the dissipation of the ΔpH by NH₄Cl reversed the inhibition by MgATP. O₂ titration experiments demonstrated that the optimum pO₂ for nitrogenase activity did not change significantly. Therefore, it can be excluded that the inhibition is caused by O₂ inhibition of nitrogenase. At pH 8.4, where the pH of the medium inhibits the nitrogenase activity, one expects a stimulation of the nitrogenase activity when the peribacteroid membrane ATPase is active. As shown in Table II, this was observed. The proton-pumping activity of the peribacteroid membrane ATPase stimulates nitrogenase activity. It is concluded from the experiments that due to the activity of the peribacteroid ATPase, the pH of the peribacteroid space is lowered and this inhibits or stimulates nitrogenase activity of the bacteroids, depending on the pH of the medium.

According to this model, it is possible that in nodules the peribacteroid membrane ATPase activity might inhibit nitrogenase activity. This hypothesis was tested. The air spaces in the root nodules were filled with a vanadate solution by evacuating the root nodules in this solution. The loss in nodular nitrogenase activity due to submerging was relieved by increasing the pO₂ to 80% (v/v). The effect of vanadate was compared with the results of a control experiment with NaCl. As shown in Table III, vanadate stimulates nodular nitrogenase activity. We like to explain this stimulation as an inhibition of the peribacteroid membrane ATPase, thus increasing the pH of the peribacteroid space to a more optimal pH for nitrogen fixation by the bacteroids. We realize that vanadate will also inhibit other ATPases such as the plasma membrane H⁺-ATPase. Further work is needed to clarify the precise mechanism of action of the vanadate stimulation of nodular nitrogenase.

### Table II. Effect of pH and energization of the peribacteroid membrane on the nitrogenase activity of R leguminosarum symbiosomes

<table>
<thead>
<tr>
<th>pH</th>
<th>None</th>
<th>MgATP</th>
<th>MgATP + NH₄Cl</th>
<th>MgATP + Na₃VO₄</th>
<th>NH₄Cl</th>
<th>Na₃VO₄</th>
</tr>
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<tbody>
<tr>
<td>pH 7.4</td>
<td>100 ± 4</td>
<td>39 ± 6</td>
<td>80 ± 4</td>
<td>85 ± 4</td>
<td>94 ± 3</td>
<td>84 ± 3</td>
</tr>
<tr>
<td>pH 8.4</td>
<td>100 ± 4</td>
<td>120 ± 5</td>
<td>102 ± 6</td>
<td>102 ± 6</td>
<td>97 ± 6</td>
<td>97 ± 6</td>
</tr>
</tbody>
</table>

*100% activity at pH 7.4 and 8.4 is 17.3 and 6.1 nmol C₂H₄ formed min⁻¹ mg⁻¹ protein, respectively. Each value represents the mean ± 1 SE of three replicates.

### Table III. Effect of vanadate on nodular nitrogenase activity

<table>
<thead>
<tr>
<th>Addition</th>
<th>Percent of Nodular Nitrogenase Activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mM NaCl</td>
<td>100 ± 15</td>
</tr>
<tr>
<td>15 mM Na₃VO₄</td>
<td>127 ± 16</td>
</tr>
</tbody>
</table>

*100% activity is 32 nmol C₂H₄ formed min⁻¹ g⁻¹ fresh weight. Each value represents the mean ± 1 SE of six replicates.
CONCLUDING REMARKS

It has been shown in this paper that the peribacteroid membrane ATPase of pea is a reversible H⁺ pump and that it generates a $\Delta \Psi$ and a $\Delta p\text{H}$ across the membrane. Ou Yang et al. (1990) and Udvardi and Day (1989) have suggested that the ATP-dependent charge transfer may be essential in maintaining electroneutrality during transport of malate inward across the peribacteroid membrane, as well as cations such as NH₄⁺ outward. We have shown here that an active peribacteroid membrane ATPase inhibits the nitrogenase activity of bacteroids by lowering the pH of the peribacteroid space. Vanadate stimulation of nodular nitrogenase activity indicates that an active peribacteroid membrane ATPase might be involved in regulation of nodular nitrogenase activity. Since the specific activity of the ATPase of pea and lupin peribacteroid membranes is much higher than the soybean analog, and since it has been shown that there is a rapid acidification of the peribacteroid space of pea symbiosomes, which could not be demonstrated for soybean, it is possible that acidification of the peribacteroid space of symbiosomes of a determinate type of nodule (soybean) is not of physiological relevance, whereas it may be part of a regulatory mechanism in an indeterminate type of nodule (pea or lupin).

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