Symbiosis, 19 (1995) 141–154
Balaban, Philadelphia/Rehovot

Transport of Fixed Nitrogen Across Symbiotic Membranes of Legume Nodules

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Received May 27, 1995; Accepted August 9, 1995

Abstract

Symbiotic nitrogen fixation requires an exchange of reduced carbon to the endosymbiont for fixed nitrogen to the host plant. In this review, the general properties of isolated symbiosomes and transport across the peribacteroid membrane are discussed, with attention focused on the efflux of fixed nitrogen. In *Bradyrhizobium* symbiosomes, no evidence has been obtained for amino acid transporters on the peribacteroid membrane, even though bacteroids possess active uptake systems. Studies with free-living *Bradyrhizobium japonicum* suggest that the presence of bacteroid transporters reflects an amino acid poor peribacteroid space. Evidence for the presence of a monovalent cation channel on the peribacteroid membrane of soybean symbiosomes, capable of transporting ammonium out of the symbiosome, is presented and discussed. This channel is voltage-gated and outwardly rectified, properties well-suited to ensure rapid efflux of NH_4^+ to the

Presented at the 10th International Congress on Nitrogen Fixation, May 28 – June 3, 1995, St. Petersburg, Russia

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0334-5114/95/$05.50

plant cytosol upon energization of the peribacteroid membrane. We suggest that, in soybean at least, the simple scheme of malate into the symbiosome and ammonium out represents the major exchange of carbon and nitrogen between bacteroid and plant.

Keywords: *Bradyrhizobium*, legumes, nitrogen fixation, ammonium channel

1. Introduction

Nitrogen-fixing bacteroids within legume nodules are surrounded by a membrane of plant origin – the peribacteroid membrane (PBM) – which effectively excludes the bacteroid from the host cytosol and which controls the movement of metabolites between the two symbiotic partners (Day and Udvardi, 1992). The PBM and its enclosed bacteroids together make up the symbiosome, the basic nitrogen-fixing unit of the nodule infected cell (Roth et al, 1988). The essential metabolic exchange between bacteroid and plant is reduced carbon into the bacteroid for fixed nitrogen to the plant. While there is general agreement that dicarboxylates are the major source of carbon supplied to the bacteroid (see Day and Copeland, 1991; Streeter, 1991; Vance and Heichel 1991, for reviews), the form in which nitrogen is supplied to the plant remains controversial (Long and Staskawicz, 1993).

The most detailed studies of metabolite transport across the PBM have been performed with symbiosomes isolated from soybean, siratro and french bean nodules (Day et al., 1989; Herrada et al., 1989; OuYang and Day, 1992). The transport properties of these symbiosomes are remarkably similar, although minor differences exist (eg., glucose uptake by french bean symbiosomes: Herrada et al. 1989). Common properties include:

(1) a dicarboxylate transporter on the PBM, with highest affinity for malate and succinate;
(2) a distinct dicarboxylate carrier on the bacteroid membrane, catalysing H^+ symport with divalent malate;
(3) a single P-type ATPase on the PBM which pumps H^+ and energises the membrane;
(4) impermeability to sugars, with the exception of glucose in french bean (see above);
(5) lack of PBM amino acid transporters;
(6) permeability to inorganic anions such as nitrate and chloride (presumably via channels).

Longer-term studies with microaerobic soybean and pea symbiosomes (Rosendahl et al., 1992) largely confirm the short-term studies described above, but

also show that when metabolism is allowed to proceed during feeding of organic acids under nitrogen-fixing conditions, efflux of metabolites, especially amino acids, also occurs, as it does in isolated bacteroids (Kouchi et al., 1991). These results raise the possibility of a link between provision of dicarboxylates and efflux of fixed nitrogen in symbiosomes, as suggested first by Kahn et al. (1985). This is explored further below.

2. Isolated symbiosomes

Isolated symbiosomes have proved valuable tools for the study of metabolite transfer across the PBM, particularly in the identification of transport mechanisms. However, certain precautions must be observed with this approach and in this context the multi-bacteroid symbiosomes of *Bradyrhizobium* symbioses, as in siratro (Fig. 1), have an advantage. It is most

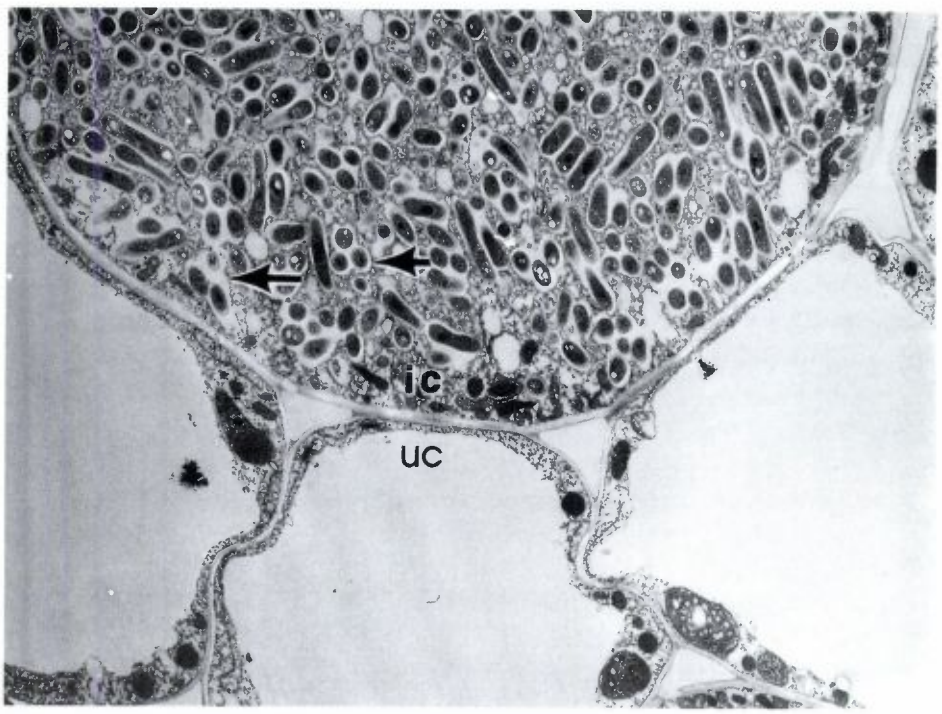

Figure 1. Electron micrograph of cells within the infected zone of a siratro nodule, showing multi-bacteroid symbiosomes (arrow); ic: infected cell; uc: uninfected cell.

important that the purity and integrity of symbiosome preparations are ascertained to avoid misleading results, since even minor contamination by free bacteroids can lead to erroneous conclusions (Udvardi et al., 1988b and Herrada et al., 1993). One advantage of using large symbiosomes is that their high density allows them to be readily and quickly purified by centrifugation on Percoll gradients, avoiding contamination by plant organelles which can confuse transport studies. The integrity of large symbiosomes is also easily checked. The particles can be distinguished from free bacteroids by light microscopy (Day et al., 1989) and PBM integrity can be monitored during uptake experiments by measuring their volume which is much larger than that of free bacteroids (OuYang et al., 1990). The integrity of the PBM in soybean and french bean symbiosome preparations can also be determined by the rate of glutamate uptake. The PBM is impermeable to glutamate in these systems, while bacteroids possess an active transporter (Udvardi et al., 1988b; Herrada et al., 1989), and PBM rupture leads to an increase in the rate of glutamate uptake by the symbiosome preparation (Herrada et al., 1993). The density of large symbiosomes also allows them to be separated from any contaminating free bacteroids if the centrifugal filtration technique (Palmieri and Klingenberg, 1979) is used to measure uptake.

3. Amino acid transport across symbiotic membrane

Strong cases have been made for hypotheses which link nitrogen efflux to carbon supply via obligatory exchanges across symbiotic membranes. These hypotheses include:

(1) a malate/aspartate shuttle, in which malate and glutamate enter and 2-oxoglutarate and aspartate leave the bacteroid (Kahn et al., 1985);
(2) a proline shuttle linking supply of reductant to the bacteroid with purine synthesis and the pentose phosphate pathway in the plant (Kohl et al., 1988);
(3) a malonamate shuttle proposed to take advantage of abundant malonate in some legume nodules (Kim and Chae, 1990; Schramm, 1992).

The malate/aspartate shuttle is an attractive scheme but appears unlikely to operate in view of the impermeability of the PBM to glutamate, at least in soybean, french bean and siratro symbiosomes (see above). Likewise, no transport mechanism for proline has been identified on the PBM of soybean or siratro, although this amino acid diffuses into symbiosomes at a significant rate (Udvardi et al., 1990; OuYang and Day, 1992). Malonamate transport across the PBM has not been measured directly to our knowledge, but malonate

uptake across the PBM, which is also required in this scheme (Kim and Chae, 1990), can occur via the dicarboxylate transporter (OuYang et al., 1990). However, malonate uptake across the PBM is severely inhibited by malate and while the malonate concentration of nodule homogenates is very high, so is that of malate (Streeter, 1991). Malonate uptake by bacteroids appears to be restricted to passive diffusion across the bacteroid membrane (Werner et al., 1982). We therefore consider all of these schemes to be unlikely mechanisms for the major flux of fixed nitrogen to the plant.

Exchange of an amino acid for dicarboxylates between the bacteroid and plant does not necessarily require operation of a complicated shuttle. For example, it has been reported (Kouchi et al., 1991) that both alanine and aspartate, which can be readily formed in the bacteroid from malate (McDermott et al 1989), efflux rapidly from isolated soybean bacteroids, and some efflux of these amino acids has been seen also in long term experiments with isolated symbiosomes (Rosendahl et al., 1992). We have, therefore, re-investigated the transport of these amino acids in soybean symbiosomes and bacteroids.

Bacteroids isolated from various legumes have a number of active transport mechanism for the uptake of amino acids, notably aspartate and glutamate (see Rosendahl et al., 1991 for a review). We (L. Whitehead, S. Young, L. Schuller and D. Day, unpublished results) have found that when the bacteroids are loaded with ^{14}C-aspartate, the amino acid remains in the bacteroid until certain other compounds are added to the external medium. Under these conditions, bacteroid aspartate will exchange for external cold aspartate, glutamate and malate. Glutamate uptake and exchange also occurs readily across bacteroid membranes (Fig. 2: see also Jin et al., 1990; Salminen and Streeter, 1987; Udvardi et al., 1988b). However, no such transport of aspartate or glutamate occurs across the PBM. Likewise, we have been unsuccessful in identifying a mechanism for alanine transport across the PBM (L. Whitehead, S. Young, L. Schuller and D. A. Day, unpublished results).

Why do bacteroids maintain active transport mechanisms for amino acids when the PBM appears to restrict availability of their substrates? This may reflect the regulatory pathways which control synthesis of amino acid transport proteins in rhizobia. We have found that in free-living *Bradyrhizobium japonicum* a glutamate transporter is present when the cells are grown in the absence of glutamate but not when glutamate is available at high concentrations in the growth medium (Fig. 2A). Similar results have been obtained with *Rhizobium trifolii* (Jin et al., 1990).. It is noteworthy that the kinetics of glutamate uptake by free-living *B japonicum* cells grown in NH_4^+ Cl as nitrogen source are very similar to those observed with isolated bacteroids (Fig. 2). These results suggest that the presence of a glutamate transporter in

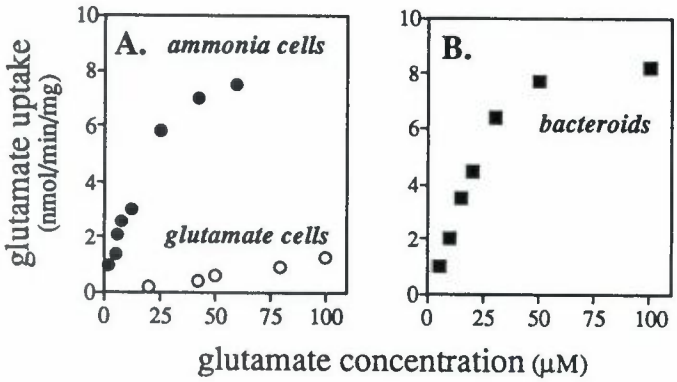

Figure 2. Glutamate uptake by (A)*Bradyrhizobium japonicum* cells, grown in the presence of ammonium chloride (filled circle: ammonia cells) or glutamate (empty circle: glutamate cells) as nitrogen source, and (B) bacteroids isolated from soybean root nodules (filled square). Bacteroids were isolated from soybean nodules as described by Day et al. (1989) and cells were grown according to Howitt et al. (1985). ^{14}C-glutamate uptake was measured as described by Howitt et al. (1985) for cells and Udvardi et al. (1988b) for bacteroids.

bacteroids reflects the nitrogen environment of the microsymbiont, and support the experiments with intact symbiosomes; that is, the bacteroids are in a scavenging mode with respect to amino acids. Some amino acids diffuse across the PBM at significant rates (OuYang et al, 1990) and this, coupled with the active transporters on the bacteroid membrane, may allow some uptake of amino acids as supplementary nitrogen sources for the bacteroid (see Fig. 3). Certainly, labelling of bacteroid proteins occurs when isolated intact symbiosomes are incubated over an hour with ^{14}C-methionine which also appears to just diffuse across the PBM (Katanakis et al., 1988; Udvardi et al., 1990).

4. Ammonium transport across the PBM

The direct product of nitrogen fixation in the bacteroid is ammonia. Since the ammonium assimilatory enzymes in the bacteroid are repressed in the symbiotic state, NH_3 will diffuse rapidly into the PBS, driven by its concentration gradient and the expected ΔpH across the bacteroid membrane. The acidic pH in the PBS, the result of proton pumping by both the PBM ATPase and the bacteroid respiratory chain, will ensure that the bulk of NH_3 is converted to the NH_4^+. Repression of the bacteroid ammonium carrier (Brown and Dilworth, 1975; Howitt et al., 1985), prevents re-uptake of NH_4^+ which is, therefore, available to the plant. However, a transporter is required on the

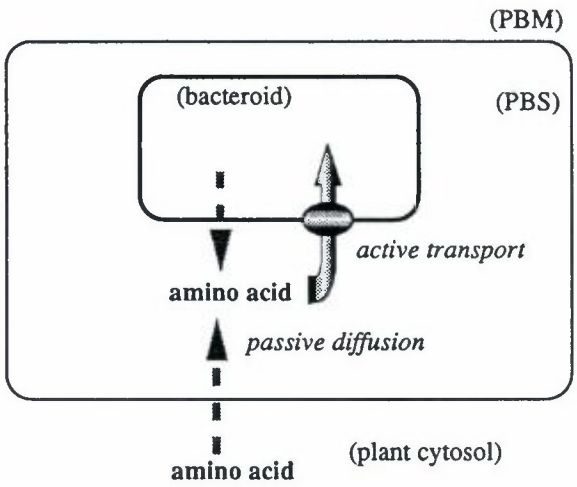

Figure 3. Scheme of amino acid transport into legume symbiosomes. Broken arrows indicate passive diffusion; the bold arrow indicates active uptake.

PBM for the rapid movement of this ion. Previous studies, using the ammonium analogue, methylammonium, failed to find any evidence for such a carrier (Udvardi and Day, 1990) but this technique has limitations, especially when looking at efflux rather than uptake.

Given the lack of amino acid transporters on the PBM, we have re-investigated the movement of NH_4^+ in isolated symbiosomes using the patch clamp technique. The relatively large size of symbiosomes from mature soybean nodules (2–5 μm in diameter) allows gigaohm seals to be formed when small pipettes (0.3–0.8 μm tips) are placed against intact symbiosomes bathed in the appropriate mixture of salts (Tyerman et al., 1995). Since it is difficult to maintain the symbiosomes attached to the pipette without rupture (monitored by microscopy), most measurements to date have been performed with detached patches of an inside out configuration (ie., the side of the PBM usually facing the PBS faced the external medium). In all such patches, time-dependent currents (from the PBS face to the cytosolic face) were recorded when either K^+ or NH_4^+ were present in the external medium and when the membrane potential was made more negative (cytosolic side with respect to the PBS side: Fig. 4). In other words, when an electrical potential was induced across the membrane, similar to that which would be formed by the PBM ATPase, NH_4^+ ions flowed from the external medium into the pipette: this is equivalent to NH_4^+ flowing out of the symbiosome.

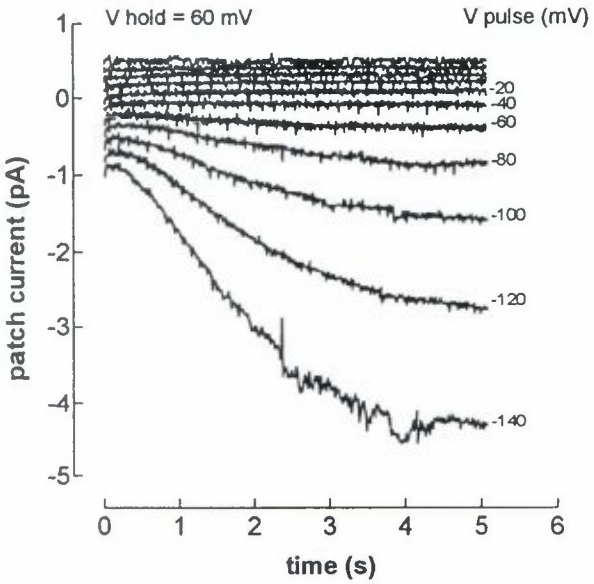

Figure 4. Patch-clamping of isolated soybean symbiosomes. Time-dependent inward NH_4^+ currents (external medium into pipette) across inside-out detached patches of peribacteroid membrane from soybean symbiosomes. Superimposed current-time curves, recorded in response to voltage clamp pulses are shown. The patch pipette contained 150 mM KCl, 10 mM $CaCl_2$ and 25 mM HEPES buffer (pH 7.2). The bath contained 20 mM ammonium chloride, pH 7.2. The holding potential was +60 mV and negative pulses up to –140 mV were applied. These membrane voltages were cytoplasmic with respect to the symbiosome face so that inward currents (downward deflections) represent movement of positive charge from the peribacteroid space to cytoplasm. Similar currents were detected when potassium replaced ammonium.

The kinetics of current activation and deactivation indicated that a voltage-gated channel was responsible for the currents (Tyerman et al., 1995). These currents were rectified, so that no movement of cations out of the pipette was detected when the external medium was made more negative (Fig. 5). An electrical potential is known to be generated across the PBM of isolated symbiosomes by the H^+ pumping ATPase, which is positive on the interior (Blumwald et al., 1985; Udvardi and Day 1989). *In vivo*, this would ensure that NH_4^+ efflux would occur upon ATP hydrolysis. Rectification would ensure that movement of NH_4^+ back into the symbiosome did not occur. In other words, the movement of monovalent cations such as NH_4^+ would be unidirectional – out of the peribacteroid space – and driven by the electrical potential.

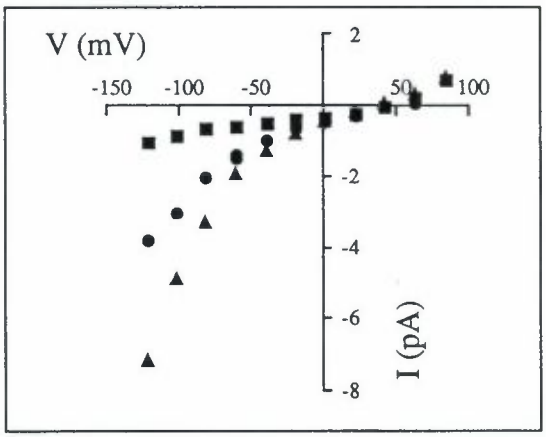

Figure 5. Current-voltage curves of time-dependent current across inside-out patches of peribacteroid membrane. 30 mM chloride salts of choline (filled square), potassium (filled circle) and ammonium (filled triangle) were used in the external medium. The membrane voltage is cytoplasmic with respect to the peribacteroid space so that the current flows effectively out of the symbiosome.

Typical current responses of a PBM patch to changing membrane voltage are shown in Fig. 5. The channel is selective for small monovalent cations, with K^+ and NH_4^+ being the most active, and does not transport divalent cations, larger cations such as choline (Fig. 5), or anions. At physiological concentrations (10–20 mM), the channel shows a preference for ammonium over potassium (Tyerman et al., 1995): at high concentrations, this preference disappears. In some patches, the conductance of the channel showed saturation kinetics with an apparent Km of about 30 mM for NH_4^+. This is similar to the estimated concentration of NH_4^+ inside symbiosomes during nitrogen fixation (Streeter, 1989). The channel was strongly inhibited by calcium ions in the external medium, with a Ki of about 20 μM, perhaps indicating a potential regulatory mechanism for controlling NH_4^+ flux. The conductance of this channel is extremely small (subpicosiemen in amplitude) and discrete channel opening could not be detected readily. Non-stationary noise analysis of the currents recorded suggests that the channel is present in the PBM at very high density – 800–1000 channels per μm^2 (Tyerman et al., 1995) – consistent with the time-dependent current flows observed.

Although the channel described above has properties that one would expect of a transporter delivering ammonium to the plant, its operation during nitrogen fixation remains to be demonstrated. Experiments with symbiosomes isolated under micro-aerobic conditions should confirm this. Certainly the magnitude of

the current flows across the patch, extrapolated to the whole symbiosome, are quite adequate to account for estimates of nitrogen fixation rates in symbiosomes *in vivo* (Bergersen 1994). The degree to which membrane energisation by the ATPase will be required to drive these fluxes will depend on the magnitude of the resting potential of the PBM, which is currently unknown. It is also possible that some diffusion of NH_3 could occur across the PBM *in vivo*, depending on the pH of the peribacteroid space (Udvardi and Day 1990).

Several questions regarding the putative ammonium channel remain to be answered. For example, the channel is not specific for ammonium and the concentration of other monovalent cations on either side of the membrane will influence the flux of ammonium through the channel. Likewise, the concentration of calcium in the peribacteroid space will be critical. Bacteroids may contain large quantities of calcium but how much of this is free to move into the peribacteroid space remains to be seen. In this context it should be noted that our few experiments with intact symbiosomes attached to the patch clamp pipette indicate that ion currents out of the symbiosome are detectable (Tyerman et al., 1995). Providing that the peribacteroid space is acidic, then it is inevitable that NH_3 will enter the that space and be converted NH_4^+ ions; as long as the PBM is energised, the channel described will open and NH_4^+ will diffuse into the plant cytosol down its electrochemical gradient. Rapid assimilation by the plant glutamine synthase will ensure that this gradient is large.

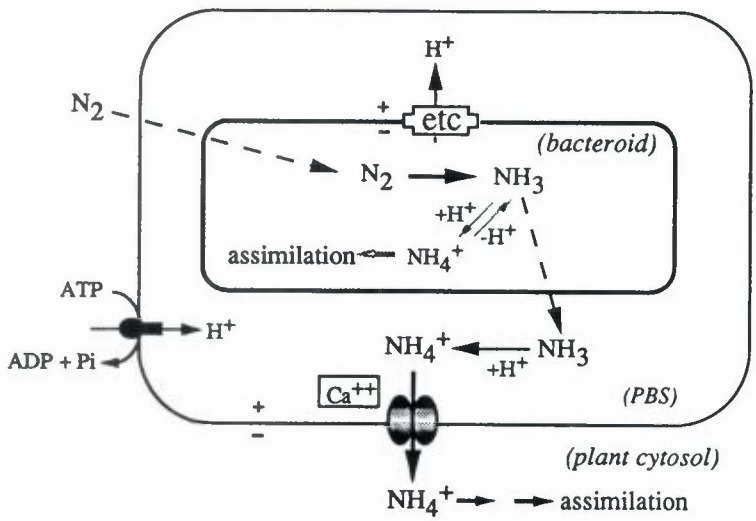

Figure 6. Scheme of ammonium efflux from the bacteroid to the plant cytosol in soybean nodules. See text for details. Etc = electron transport chain.

The possible integration of the NH_4^+ channel into nitrogen metabolism of the symbiosome is shown in Fig. 6.

The NH_4^+/K^+ channel that we have discovered on the PBM is different from other cation channels so far described for plant membranes, including the inwardly rectified potassium channel on the plasma membrane (Schroeder et al, 1994), which also transports ammonium ions, and the high affinity ammonium transporter from *Arabidopsis* (Ninnemann et al., 1994). The high concentration of the PBM channel may have some advantages for the regulation of ammonium flux to the plant. For a small particle like the symbiosome, a large number of low conductance channels would allow finer control of fluxes both in the long term, via protein synthesis, and in the short term via the percentage of channels in the open configuration. It is also interesting to note that ammonium regulates the expression of plant glutamine synthase (Miao et al., 1991). It is possible that expression of other plant genes are controlled in this fashion and NH_4^+ flux through the PBM channel may, therefore, be involved in the regulation of symbiotic exchange in more than one way.

5. A model for carbon/nitrogen exchange across the peribacteroid membrane

Based on the evidence reviewed here, the simplest scheme of carbon and nitrogen exchange between bacteroid and plant is malate and N_2 into the bacteroid for CO_2 and NH_4^+ out to the plant (Fig. 7). It should be pointed out that this does not preclude other metabolic exchanges across the PBM occurring; however, we believe that the scheme shown in Fig. 7 would be the most efficient.

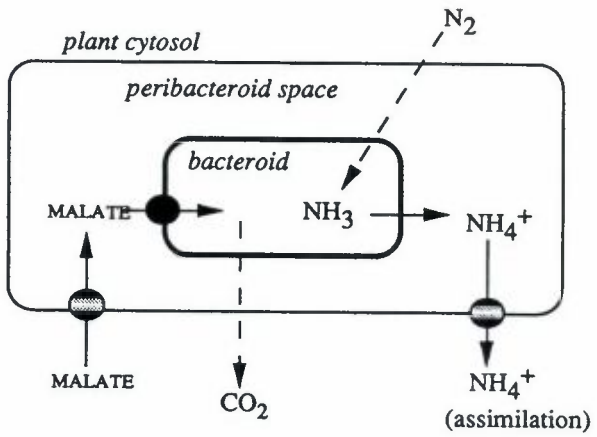

Figure 7. Simplest scheme for carbon/nitrogen exchange across the peribacteroid membrane of soybean nodules. Dashed lines represent diffusion.

The mechanisms required for this exchange are present on the symbiotic membranes of soybean nodules. Dicarboxylate uptake occurs via two carriers, one on the PBM which catalyses electrophoretic movement into the PBS and a proton symporter on the bacteroid membrane for uptake into the bacteroid. Malate (or succinate) can be completely oxidised in the bacteroid via malic enzyme and associated enzymes (Day and Copeland, 1991). Ammonia formed in the bacteroid by nitrogenase will diffuse into the PBS where it will be converted to NH_4^+ which then enters the plant cytosol via the ammonium channel on the PBM. This exchange is coordinated via the membrane potentials of the PBM and bacteroid membrane, and the associated pH gradients (Fig. 6). Regulation of the exchange may occur via the concentration of calcium on both sides of the PBM. For example, in the plant cytosol Ca is required for activity of the PBM protein kinase which phosphorylates the ion channel nodulin 26 (Weaver et al, 1991, 1994) while in the PBS Ca inhibits the activity of the ammonium channel (Tyerman et al., 1995). Since bacteroids appear to possess rather large quantities of Ca, it is possible that release of this cation by the microsymbiont may control both carbon input and nitrogen delivery. The ATP concentration in the plant cytosol will also be important for the regulation of carbon/nitrogen exchange across the PBM, since it acts as a substrate for both the ATPase and the protein kinase of the PBM.

Acknowledgments

The research described in this paper was supported by grants from the Australian Research Council to DAD and SDT.

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