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Review article

Metabolite Exchange Across Symbiosome Membranes

DAVID A. DAY[1]* and MICHAEL K. UDVARDI[2]
[1] *Division of Biochemistry and Molecular Biology, Faculty of Science*
Australian National University, Canberra, ACT 2601, Australia
Tel. (61) 6-249 2870, Fax (61) 6-249 0313
[2] *Institute of Biological Chemistry, Washington State University*
Pulman WA 99164-6340, USA

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Abstract

Symbiosome structure in endocytyobiotic associations and the nature of metabolite transport between symbiotic partners is reviewed. Properties of isolated symbiosomes from nitrogen-fixing legume nodules are described in detail, with an emphasis on the regulation of dicarboxylate transport across the peribacteroid membrane (PBM). The dicarboxylate carrier is tentatively identified as the PBM protein nodulin-26 and protein kinase mediated phosphorylation of this protein is suggested as a key regulatory mechanism in symbiotic nitrogen fixation.

Keywords: symbiotic membranes, metabolite transport, peribacteroid membrane, nitrogen fixation, legumes, rhizobia

Abbreviations: CCCP: carbonyl-cyanide *m*-chlorophenyl hydrazone, nodulin: nodule-enhanced protein, PBM: peribacteroid membrane

* The author to whom correspondence should be sent

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1. Introduction

The term 'symbiosome' was coined to describe the symbiotic compartment within the host cells of endosymbiotic associations – "a membrane-bound compartment containing one or more symbionts and certain metabolic components and located in the cytoplasm of eukaryote cells" (Roth et al., 1988). A generalized scheme of a symbiosome is depicted in Fig. 1; it consists of a host-derived

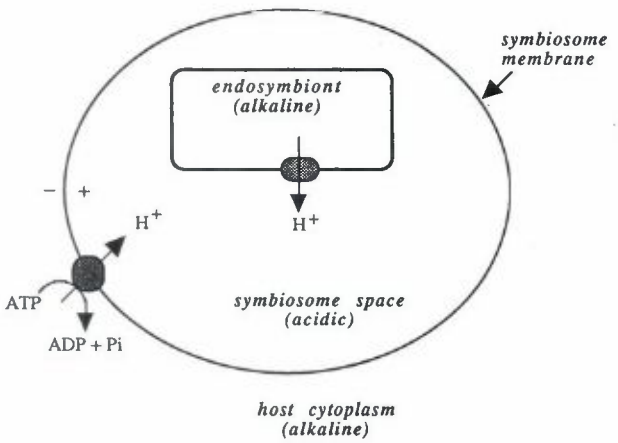

Figure 1. Generalized scheme of a symbiosome consisting of the microsymbiont surrounded by a host-derived membrane (the symbiosome membrane). This structure is common to many endocytobiotic systems (Roth et al., 1988). In some systems, the symbiosome space is acidified by H^+-pumping ATPases or electron transport chains on the symbiosome and/or endosymbiont membrane (Douglas, 1988; Day et al., 1989; Smith and Smith, 1990). Primary transport activities such as these may be common mechanisms for the regulation of nutrient exchange between symbionts.

membrane (the symbiosome membrane) which envelopes a microsymbiont and creates a space termed the symbiosome space (Roth et al., 1988).

As pointed out by Roth et al. (1988), the symbiosome structure is found in an extraordinary range of endocytobiotic systems, both mutualistic and parasitic. The symbiosome membrane forms the interface across which all communication between symbiotic partners must occur and thus has the potential to control the association. In a parasitic association, traffic across the membrane is largely controlled by the invading organism while in a mutualistic association the host retains at least some degree of control (Smith and Smith, 1990). Presumably, during evolution of organelles the host assumed control of the symbiont's own membrane, via genetič transfer, and the symbiosome membrane was lost.

Although the symbiosome terminology has not been universally accepted (Smith and Smith, 1990), it has the merit of drawing common themes in endosymbiosis to the attention of researchers in the field and seems to have been adopted by workers in symbiotic nitrogen fixation (Gresshoff et al., 1990). In this review we discuss metabolite transport across symbiosome membranes and will use the legume-*Rhizobium* system as a detailed example of the regulation of such transport by the host. Although we use the term symbiosome instead of peribacteroid unit, we retain the use of the term peribacteroid membrane (PBM), which we feel is more precise than 'symbiosome membrane' when dealing specifically with the legume symbiosis.

2. General Metabolite Exchange Between Symbiotic Partners

A common structure implies at least some common themes in the nature and regulation of nutrient transport amongst endosymbiotic systems (Smith and Douglas, 1987). At the moment, too few direct studies have been made of metabolite transport across symbiosome membranes to make firm conclusions about this, but some inferences can be drawn from the many indirect studies made with various systems.

A common feature of some (maybe all) endosymbiotic associations is the presence of H^+-translocating enzyme systems on the membranes of both host and symbiont (Smith and Douglas, 1987; Smith and Smith, 1990: see Fig. 1); these enzymes can be either ATPases (especially on the symbiosome membrane), pyrophosphatases, or electron transport chains (especially on bacterial membranes) and their activity results in energisation of the symbiosome membrane and, depending on counter-ion movement and buffering capacity, acidification of the symbiosome space (Mellor and Werner, 1987; Douglas, 1988; Udvardi et al., 1991). This acidification could influence enzyme activity in the symbiosome space, particularly hydrolytic enzymes. This may be important in modifying cell wall composition/deposition in the endosymbiont, and the host in plant associations. However, excessive acidification could adversely affect the endosymbiont (Brewin et al., 1990) and it is expected that the pH of the symbiosome space is controlled. Energization of the symbiosome membrane presumably is important in driving ion transport and maintaining charge balance across the membrane.

Although different systems may have some regulatory features in common, the nature of the species transported across symbiosome membranes is likely to be diverse and to reflect the nature of the association. Table 1 summarizes some demonstrated metabolite exchanges between symbiotic partners; the list

Table 1. Demonstrated metabolite exchange in endosymbiotic associations

Symbiosis	Metabolite transfer
1. Alga – invertebrate[1,2,3]	**From host to alga** phosphate, sulphate, CO_2
	From alga to host
Chlorella – Hydra viridis alga – *Spongilla lacustri* zooxanthellae – coral	maltose, sulphur amino acids sucrose glycerol
2. Bacteria – insect[4] (e.g., cockroaches, leaf hoppers, aphids)	**From host to bacteria** acetate, trace elements, sulphate, uric acid
	From bacteria to host sulphur amino acids, sterols B vitamins
3. Bacteria – marine invertebrate[5] (e.g., tube worms, vent clams)	**From host to bacteria** sulphur
	From bacteria to host sugars
4. Rhizobia – legume[6]	**From host to bacteria** organic acids
	From bacteria to host ammonia

[1] Cook, 1983
[2] Cook, 1980
[3] Wilkerson, 1980
[4] Ishikawa, 1989
[5] Childress et al., 1987
[6] Dilworth and Glenn, 1985

is by no means exhaustive (see Smith and Douglas, 1987, for more examples) but serves to illustrate the types of nutrients exchanged. Most endosymbionts depend on their host for micronutrients and inorganic compounds such as phosphate (Smith and Douglas, 1987) but transport of these compounds into the symbiont has rarely been demonstrated directly. For example, in the *Rhizobium*-legume system, large quantities of iron may be supplied to the bacteroid which then presumably exports them to the plant but these transports have not been directly measured.

One of the primary nutritional transfers between host and symbiont is the transport of reduced nitrogen from the microsymbiont to the host. For example, in bacteria-insect and algal-invertebrate associations, the symbionts apparently supply amino acids to their hosts, while in legume nodules, ammonia is transported to the plant cytoplasm. Carbohydrates or organic acids are

sometimes, but not always, transferred in the opposite direction – from host to symbiont – but the nature of the compound transported again varies between symbioses. This variation in the nature of nutrients transported across symbiotic membranes suggests that transport mechanisms also vary, but there may be common mechanisms for the regulation of these transporters. The following section describes a regulatory mechanism which appears to operate in nitrogen-fixing legume symbioses.

3. Symbiosomes From Legume Nodules

Following the pioneering work of Robertson et al. (1978) and Verma et al. (1978), who first demonstrated that intact symbiosomes could be isolated from legume nodules, techniques have been developed for the rapid isolation of pure symbiosomes on a larger scale (Price et al., 1987; Day et al., 1989; Herrada et al., 1989), allowing direct measurements of nutrient exchange across the PBM to be made; these studies are leading to the identification of specific transport proteins. The enormous numbers of symbiosomes in legume nodules (Bergersen and Goodchild, 1973) and the relative ease of obtaining large quantities of nodules have helped to make these studies possible, in contrast to the situation with many other associations. However, nucleic acid and antibody probes developed with legumes may prove to be of use in other systems.

Structure of legume symbiosomes

The structure of symbiosomes in legumes varies with the plant species; the PBM may enclose either a single bacteroid (as in lupin and alfalfa) or several bacteroids (as in soybean [Fig. 2] and french bean). Most transport studies to date have been conducted with the latter type because their size and density allow them to be readily separated from free bacteroids and plant organelles, and intact and ruptured symbiosomes are easily distinguished by light microscopy (Day et al., 1989). The high density of these symbiosomes also allows them to be rapidly sedimented during transport assays. Further, the large difference in the volume of the symbiosome compared to a free bacteroid in these species allows the integrity of the symbiosome to be monitored during the transport assay (Udvardi and Day, 1990; Ou Yang et al., 1990). Studies with isolated single-bacteroid symbiosomes (Miller et al., 1988; Appels and Haaker, 1991) have been less convincing because the proportion of intact to ruptured symbiosomes has not been determined precisely and free bacteroids may take up compounds which cannot cross the PBM (Udvardi et al., 1988b and 1990).

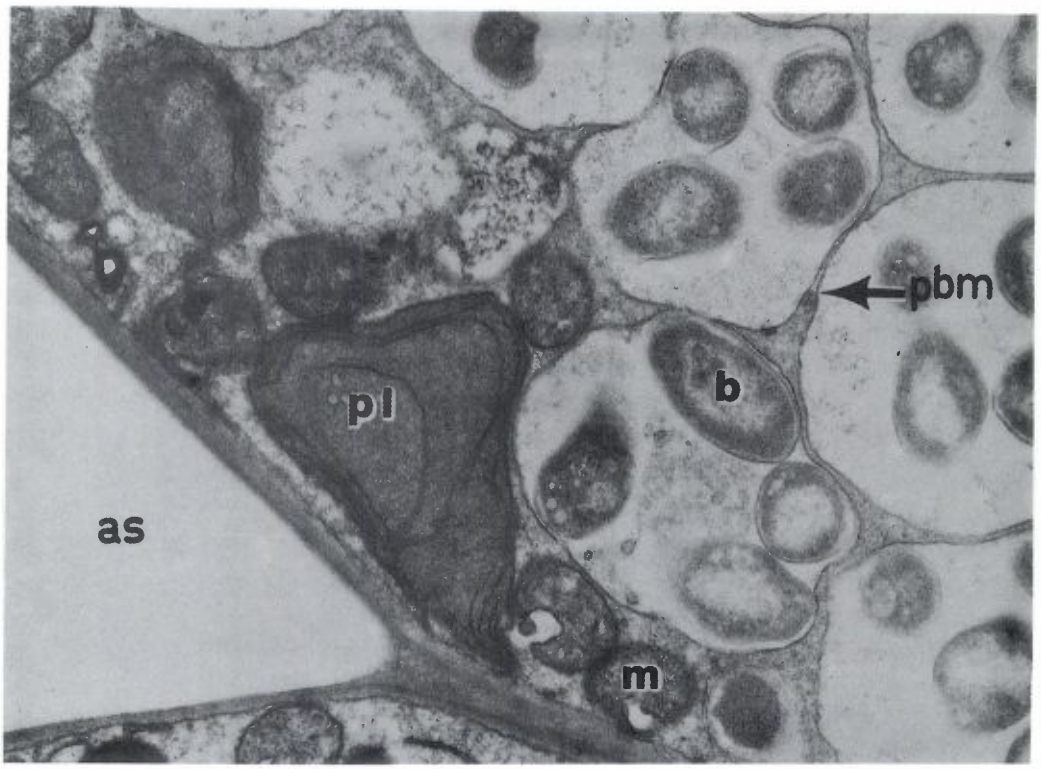

Figure 2. Electron micrograph of an infected cell from a soybean nodule showing the prominent symbiosomes. as: intercellular air space; b: bacteroid; m: mitochondrion; pbm: peribacteroid membrane; pl: plastid. Magnification ×20,000.

The structure of soybean symbiosomes can be seen in Fig. 2; the plant-derived PBM encloses a large peribacteroid space whose volume varies with the concentration of external osmoticum (Day et al., 1989; L-J. Ou Yang and D.A. Day, unpublished results) and which contains several bacteroids. Sometimes electron-dense inclusions can be seen in the peribacteroid space which contains a number of specific proteins including α-mannosidase and other hydrolytic enzymes (Mellor and Werner, 1987) as well as a protease inhibitor (Garbers et al., 1988). However, care must be taken in distinguishing between periplasmic and peribacteroid space contents in this type of analysis, with the most reliable approach being careful rupture of intact symbiosomes (Streeter, 1990).

Transport properties of legume symbiosomes

Measurements of metabolite transport into legume symbiosomes have been conducted with three species to date: soybean (see Day et al., 1990, for a review), siratro (L-J. Ou Yang and D.A. Day, unpublished results) and french bean (Herrada et al., 1989). In all cases, very similar results have been obtained, with the exception that french bean symbiosomes (and bacteroids) are permeable to glucose (Herrada et al., 1989). These results are summarized in Fig. 3. It should be noted, however, that all of these nodules studied produce ureides for export to other plant parts and properties of symbiotic membranes from amide-producing nodules may not be the same.

Two transport activities have been definitely identified on the soybean PBM: an H^+-translocating ATPase (Blumwald et al., 1985; Udvardi and Day, 1989; Udvardi et al., 1991) and a dicarboxylate transporter (Udvardi et al., 1988a; see Fig. 3). The dicarboxylate carrier can catalyse uptake of malate and succinate into isolated symbiosomes at rates sufficient to meet the energy demands of nitrogenase in isolated bacteroids (Day et al., 1989). In addition to these

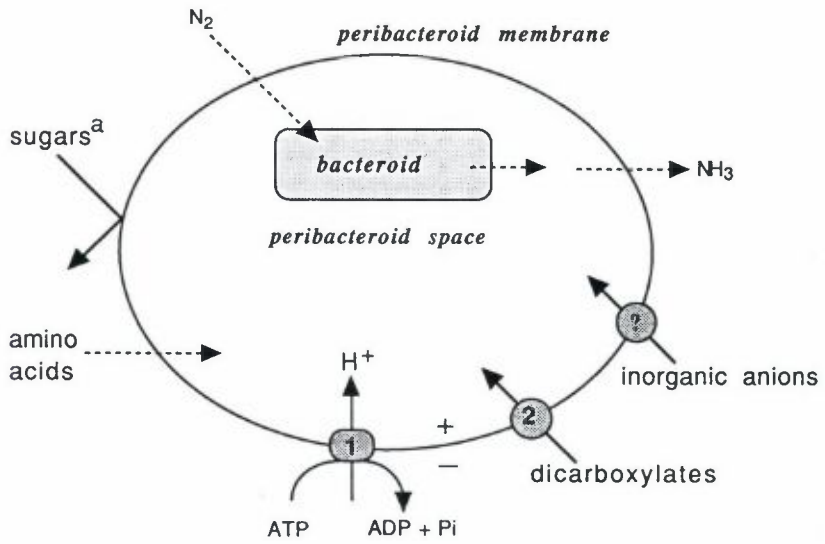

Figure 3. Summary scheme of metabolite transport across the peribacteroid membrane of isolated legume symbiosomes. Broken lines indicate passive diffusion, solid lines indicate carrier-mediated support. 1: peribacteroid membrane ATPase; 2: peribacteroid membrane dicarboxylate carrier; ?: putative channel for inorganic anions. The scheme is based on data for soybean, siratro and french bean symbiosomes. [a]Note that in french bean symbiosomes, the peribacteroid membrane is permeable to glucose but not other sugars tested (Herrada et al., 1989).

transport activities, it is apparent that the PBM is permeable to a range of inorganic anions (Udvardi and Day, 1989; Udvardi et al., 1991). The transport of these has not been characterized yet in any detail, but it is worth noting that antibodies raised against band 3 protein from red blood cells also react with PBM proteins in soybean, particularly a polypeptide of approximately 50 kDa (D.A. Day, L. Rosenberg and R.T. Leonard, unpublished results). Band 3 protein catalyses chloride/bicarbonate exchange in red blood cells and therefore the 50 kDa PBM protein mentioned above may also be an anion transporter.

Apart from glucose uptake in french bean symbiosomes (see above), the PBM appears to be impermeable to a range of sugars and symbiosomes suspended in different sugar concentrations behave as osmometers (Ou Yang and Day, unpublished results). Uptake studies with a number of amino acids have been conducted, but no evidence of carriers for these compounds on the PBM has been produced to date in any of the three plants studied (Udvardi et al, 1988b, 1990; Herrada et al., 1989 and unpublished results). Nonetheless, diffusion rates of some amino acids across the PBM are sufficient to contribute to bacteroid protein synthesis, since labelling of bacteroid proteins can be detected upon incubation of pea (Katinakis et al., 1988) and soybean (Udvardi et al., 1990) symbiosomes with [^{35}S]methioinine. In pea but not soybean symbiosomes, bacteroids may secrete proteins into the peribacteroid space.

The ammonia produced by reduction of atmospheric nitrogen appears to diffuse from the bacteroid and the symbiosome as NH_3 (Streeter, 1989; Udvardi and Day, 1990). Efflux rates can be rapid but clearly will be dependent on the pH of the compartments traversed, especially that of the symbiosome space which will vary *in vivo* according to supply of ATP and permeant anions, and on the rate of assimilation of ammonia in the plant cytoplasm.

4. Regulation of Metabolite Transport in Soybean Symbiosomes

PBM energization

ATPase activity has been detected on the PBM from lupin (Domigan et al., 1988), soybean (Blumwald et al., 1985; Bassarab et al., 1986; Udvardi and Day, 1989) and siratro nodules (Ou Yang and Day, unpublished results). In most of these, the activity resembles that of the plasmamembrane (P-type) enzyme in terms of pH response and inhibitor sensitivity, but Bassarab et al. (1986) suggested that an additional tonoplast-like (V-type) enzyme was also located on the PBM of soybean. We have made a detailed investigation of this question in soybean and siratro and, on the basis of both biochemical and immunological results (L-J. Ou Yang, D.A. Day and H. Sze, unpublished

data), concluded that both tissues contain only a single, P-type ATPase on their PBM.

Formation of an electrical potential ($\Delta\Psi$) across soybean peribacteroid membranes upon addition of ATP has been detected using oxonol fluorescence quenching (Udvardi and Day, 1989; Udvardi et al., 1991); protonophores reverse this quenching, showing that the ATPase is converted to a ΔpH which can be measured using [^{14}C]methylamine uptake (Udvardi et al., 1991; see Fig. 4). Energization of the PBM has the potential to regulate the rate of ion uptake by symbiosomes and presumably is also important in maintaining charge balance across the PBM. By influencing the pH of the peribacteroid space, PBM-ATPase activity may also influence the rate of movement of ammonia and inorganic carbon into and out of the symbiosome by changing their ionic state.

In the presence of 1 mM ATP and 10 mM KNO_3, a pH gradient of 1–1.5 pH units can be measured (Fig. 4); thus if the plant cytoplasm has a pH of, say, 7.5, that of the peribacteroid space would be about 6.0. *In vivo*, this will vary according to ATP and anion concentrations in the various compartments.

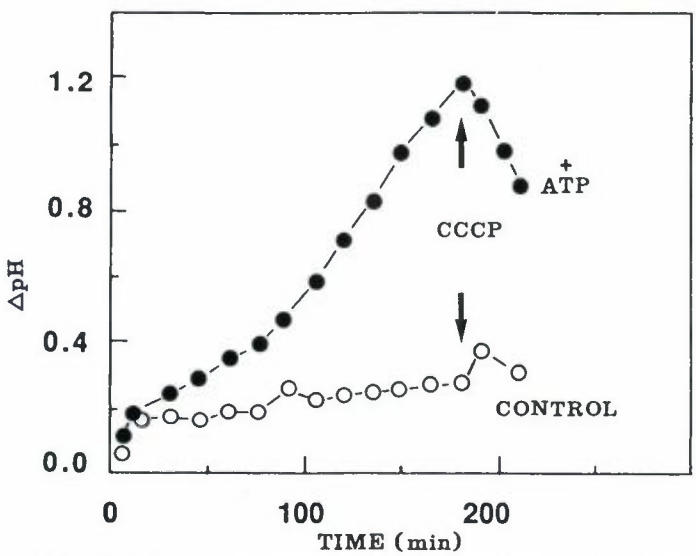

Figure 4. Formation of ΔpH across the peribacteroid membrane of soybean symbiosomes. Isolated symbiosomes were incubated in isotonic medium containing 10 mM KNO_3 to provide a permeant anion and 10 μM [^{14}C]methylamine. Samples were taken at the times shown and [^{14}C]methylamine uptake measured (Udvardi et al., 1991). Where indicated, 5 μM of the protonophore CCCP was added. The ΔpH was calculated from the distribution of methylamine across the peribacteroid membrane, using the Henderson-Hasselbach equation, in the presence (•) or absence (o) of 1 mM ATP.

Changes in ΔpH are very slow in isolated symbiosomes (Fig. 4), suggesting that the peribacteroid space is heavily buffered; therefore changes in the contents of this space will also affect its pH *in vivo*. The bacteroid respiratory chain seems to contribute only slightly to the acidity of the peribacteroid space but bacteroid metabolism may help buffer dramatic changes in its pH (Udvardi et al., 1991).

Regulation of the PBM dicarboxylate transporter

The pH response of malate uptake into soybean symbiosomes indicates that it is the univalent malate anion that is transported across the PBM (Udvardi et al., 1988a) and decreases in $\Delta\Psi$ have been measured following addition of high concentrations of malate and succinate (Day et al., 1990; Udvardi et al., 1991). This does not necessarily imply that transport across the PBM is active in the strictest sense of the term. Although malate and succinate are accumulated against a concentration gradient in isolated symbiosomes (Udvardi et al., 1988a and 1990), this could be due to active transport across the bacteroid membrane and even some metabolism within the bacteroid (despite the short times over which we measure uptake). Our current working model for the PBM dicarboxylate carrier is that is facilitates the uniport of malate down a concentration and charge gradient.

Preliminary results with ATP and bacteroid inhibitors indicated that energization of both the PBM and the bacteroid membrane are required to obtain maximum rates of malate uptake into isolated symbiosomes, with the latter substantially more important than the former (Ou Yang et al., 1990). Obviously the bacteroid provides a strong sink for the uptake of dicarboxylates and *in vivo* bacteroid metabolic rates, especially respiration rates, will play a role in the rate of substrate delivery by the plant.

In general, the effect of added ATP on malate uptake by isolated symbiosomes is small and rather variable (Fig. 5), despite the fact that ATP-dependent PBM energization is consistently observed. However, ATP may influence the PBM in other ways. For example, the PBM contains a protein kinase which phosphorylates PBM proteins (Bassarab and Werner, 1987; Weaver et al., 1991). The major protein phosphorylated on the PBM is nodulin-26 (Weaver et al., 1991), a nodule-enhanced protein which has been cloned from soybeans (Fortin et al., 1987). We have shown that in intact symbiosomes phosphorylation of a 26 kDa PBM protein can be reversed by incubation with an exogenous alkaline phosphatase (Ou Yang et al., 1991). Phosphatase treatment of symbiosomes also inhibits malate uptake dramatically and subsequent addition of ATP recovers the rate (Fig. 5; Ou Yang et al., 1991). These results

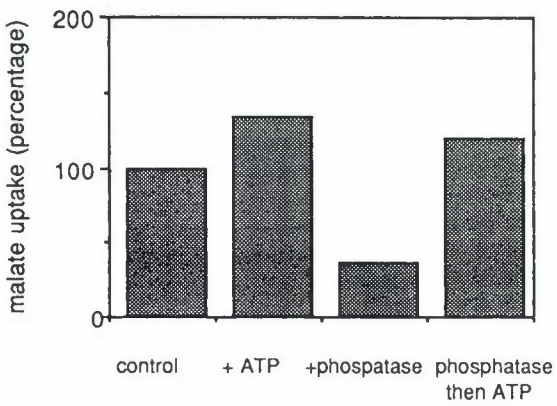

Figure 5. Effect of peribacteroid membrane phosphorylation on malate uptake by soybean symbiosomes. Isolated symbiosomes were divided into two batches, one of which was treated with 40 units of calf intestine alkaline phosphatase for 30 min; both batches were then re-isolated on Percoll gradients and [^{14}C]malate uptake measured in either the presence or absence of 1 mM ATP and 0.5 mM $CaCl_2$. In parallel experiments, autoradiography of peribacteroid membrane proteins separated on SDS-PAGE showed that a single major polypeptide of $\approx$ 26 kDa was phosphorylated in the presence of [γ-^{32}P]ATP (Ou Yang et al., 1991).

show that the rate of dicarboxylate transport across the PBM is isolated symbiosomes can be controlled by phosphorylation/dephosphorylation of PBM proteins, which has important implications for the regulation of the rate of nitrogen fixation in nodules.

The above results suggest very strongly, but do not prove, that nodulin-26 is the protein via which phosphorylation exerts its control on malate transport and that nodulin-26 may be the dicarboxylate carrier itself. Preliminary studies of nodulin-26 expressed in *E. coli* (Verma, 1990) support this suggestion. Further, the deduced amino acid sequence of nodulin-26 shows homology with other putative transport proteins: the major intrinsic protein (MIP) of bovine lens (Sandal and Marcker, 1988), the glycerol-facilitator protein of *E. coli* (Baker and Saier, 1990), the *Drosophila bib* gene product (Rao et al., 1990) and tonoplast intrinsic protein (TIP) of seeds (Johnson et al., 1990). MIP and TIP are also phosphorylated by protein kinases (Lampe and Johnson, 1989; Johnson and Chrispeels, 1991) and it is probable that this is a common regulatory mechanism in this family of transport proteins.

Weaver et al. (1991) have shown that phosphorylation of nodulin-26 is stimulated by calcium and more recently (pers. commun.) that the PBM possesses an endogeneous phosphatase. Since calcium concentrations in infected cells are

likely to be determined by plant factors, and since phosphorylation of nodulin-26 occurs on cytoplasmically exposed portions of the protein (Weaver et al., 1991; Ou Yang et al., 1991), this regulatory mechanism provides the plant with a means by which to control the symbiosis. It remains to be seen whether analogous mechanisms exist in other symbioses.

5. Concluding Remarks

A description of metabolite transport across an endosymbiotic interface has been made possible by the isolation of large quantities of intact symbiosomes from nitrogen-fixing legume nodules and the cloning of genes coding for proteins on the peribacteroid membrane (PBM). Two possible mechanisms for the regulation of this transport have been identified: energization of the PBM via a H^+-ATPase and phosphorylation of PBM proteins via a calcium-stimulated protein kinase. Both of these mechanisms are likely to be under host control via the concentration of ATP, calcium and permeant anions in the cytoplasm of nodule-infected cells. Above all, the results obtained with different legumes (see above) have demonstrated that it is the PBM which determines what compounds can reach the bacteroid and, to a large extent, the rate at which they are transported. Since there are some properties common to peribacteroid membranes from legumes and the symbiosome membrane of other endocytobiotic systems (e.g., ATPases), similar regulatory mechanisms may exist in other systems. It is hoped that molecular tools and insights generated from the study of legume-*Bradyrhizobium* symbioses may be useful in advancing our understanding of other symbiosome membranes.

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