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Protein phosphorylation stimulates the rate of malate uptake across the peribacteroid membrane of soybean nodules

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Incubation of intact isolated symbiosomes with [γ-^{32}P]ATP, followed by isolation of the peribacteroid membrane and polypeptide analysis, showed that a single major polypeptide at 26 kDa was labelled. Antibodies raised against nodulin 26 reacted with a similar sized polypeptide. Incubation of the symbiosomes with alkaline phosphatase removed the label from this polypeptide. Pre-incubation with ATP stimulated malate accumulation by isolated symbiosomes, but only slightly (10–30%). Pre-treatment of symbiosomes with alkaline phosphatase inhibited malate uptake substantially and this inhibition was completely relieved by addition of ATP. The ATP stimulation of malate uptake was not affected by ATPase inhibitors.

It is suggested that the rate of malate uptake across the peribacteroid membrane is controlled by phosphorylation of nodulin 26.

Nitrogen fixation; Peribacteroid membrane; Symbiosis; Protein kinase

1. INTRODUCTION

Nitrogen-fixing bacteroids in legume nodules are surrounded by a membrane of host origin, the peribacteroid membrane (PBM), which effectively excludes the microsymbiont from the plant cytoplasm and controls metabolic exchange between the symbiotic partners [1,2]. The PBM contains several nodule-specific proteins (nodulins) [3,4] and also possesses an electrogenic ATPase and a dicarboxylate transport system [2,5]. The relationship between these nodulins and the demonstrated transport activity is not known, but nodulin 26 [3] shows sequence homology with known transport proteins of other membranes [6–8].

Weaver et al. [9] have shown recently that nodulin 26 is phosphorylated by a calcium-dependent protein kinase on the PBM. We have shown that ATP stimulates dicarboxylate uptake by isolated symbiosomes [10] but the stimulation was not marked and we assumed that it was a result of PBM energization via the ATPase. In the present study, we have examined ATP effects on PBM transport processes in more detail and demonstrate that phosphorylation/dephosphorylation of nodulin 26 is accompanied by substantial changes in the rate of malate uptake by isolated symbiosomes.

Abbreviations: CCCP, carbonyl-cyanide *m*-chlorophenyl hydrazone; nodulin, nodule-specific protein; PBM, peribacteroid membrane; symbiosome, the peribacteroid unit consisting of bacteroids surrounded by the peribacteroid membrane

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2. MATERIALS AND METHODS

Soybeans (*Glycine max* (L.) Merr. cv. Bragg) were inoculated with *Bradyrhizobium japonicum* USDA110 and grown in a naturally illuminated glasshouse as described previously [1]. Nodules were harvested 6–8 weeks after inoculation. L-[U-^{14}C]malic acid (1.85 GBq per mmol) and [γ-^{32}P]ATP (1.11 TBq per mmol) were purchased from Amersham International (Amersham, UK). Silicon oil was purchased from Wacker Chemie (Munich, Germany). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Intact symbiosomes were isolated by Percoll density gradient centrifugation as described by Day et al. [1] and resuspended in a wash buffer containing 0.35 M mannitol, 3 mM $MgSO_4$ and 25 mM MES/BTP buffer, pH 7.0. There was no evidence of contamination by other membranes [10]. Protein was estimated according to Peterson [11].

Measurements of [^{14}C]malate uptake employed the technique of silicon oil filtration-centrifugation [5]. AR-200 silicon oil (density 1.04 g/l) was used undiluted. ^3H_2O and [U-^{14}C]sucrose were used to estimate the total and external water volumes of pelleted particles, respectively. Symbiosome volumes were typically estimated as 4–5 ml per mg protein, while bacteroid volumes were 1–2 ml per mg protein. (It should be noted that the protein content of the PBM itself is insignificant compared to that of the enclosed bacteroids. Therefore,direct comparisons between symbiosomes and bacteroids can be made on the basis of protein estimates.) This difference in volume between symbiosomes and bacteroids indicates that little damage to the PBM occurred during pelleting of the symbiosomes through the silicon oil. Unless stated otherwise, reactions were carried out 22 ± 1°C and pH 7.0. For dephosphorylation of the PBM prior to uptake measurements, symbiosomes were incubated with 40 U of alkaline phosphatase for 10 min (see below) and then re-isolated on Percoll gradients.

Phosphorylation of PBM proteins was achieved by incubating intact symbiosomes (approximately 1 mg protein in wash buffer) with 0.5 mM [γ-^{32}P]ATP (5 MBq per μmol) for 30 min in the presence of 0.25 mM $CaCl_2$, 0.2 mM vanadate (to inhibit the PBM ATPase as well as protein phosphatases) and 0.1 mM molybdate (to inhibit phosphatases) in a total volume of 1.5 ml. The PBM was then ruptured by vortexing and isolated as described in [1]. When dephosphorylation was desired, the symbiosomes were pelleted in a microfuge, resuspended in wash buffer and incubated with 40 U of alkaline phosphatase

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Table I

Effect of PBM phosphorylation on malate uptake by soybean symbiosomes

Assay conditions	Rate of malate uptake[a]	
	Untreated symbiosomes	Phosphatase treated symbiosomes
Control	100	55
+ ATP	135	n.d.
+ ATP and $CaCl_2$	155	116
+ Phosphatase	55	37
+ KCN	51	n.d.
+ CCCP	48	n.d.

[^{14}C]malate uptake was measured over 1 min as described in Materials and Methods, using 0.3 mM malate as substrate, in the presence of 0.2 mM sodium vanadate (to inhibit ATPase). Where indicated, symbiosomes were pre-incubated (10 min) with 1 mM ATP, 0.5 mM $CaCl_2$, 48 U of alkaline phosphatase (from calf intestine), 0.5 mM KCN or 5 μM CCCP. Symbiosomes were washed on Percoll gradients after phosphatase treatment, to remove excess enzyme.

[a]Rates are expressed as percentages of the control rate (7.7 nmol·min^{-1}mg^{-1} protein) obtained with untreated symbiosomes. Results from a typical experiments are shown; similar results were obtained in 3 separate experiments
n.d., not determined

(calf intestine, Boehringer-Mannheim) for 20 min prior to PBM isolation. The PBM proteins were then solubilized and subjected to SDS-PAGE and autoradiography as described by Udvardi et al. [12]. Western blotting was performed as described by Weaver et al. [9] and membranes were probed with antibodies raised against the nodulin 26-derived peptide, CK-15 [9].

3. RESULTS

Preincubation of symbiosomes with ATP alone resulted in a small and variable stimulation of the rate of malate uptake (10–35%; see Table I and [10]). Addition of $CaCl_2$ increased this stimulation (Table I). ATP stimulation was insensitive to the ATPase inhibitor vanadate [13] but was inhibited by the bacteroid respiratory poison KCN and the protonophore CCCP (Table I). This suggests that while energization of the bacteroid membrane and maintenance of bacteroid metabolism are required for rapid uptake of malate by symbiosomes [10], PBM energization is not so important (at least in these short-term experiments). Nonetheless, in experiments testing protein phosphorylation (see below), vanadate was added to selectively inhibit the PBM ATPase and thus avoid confusion between effects mediated by the ATPase and those mediated by PBM protein kinase.

Treatment of symbiosomes with alkaline phosphatase, followed by re-isolation (to remove phosphatase), severely inhibited subsequent malate uptake (Table I). Addition of ATP and $CaCl_2$ completely relieved this inhibition (Table I). Michaelis–Menten analysis of the response of malate uptake rates to the phosphatase and

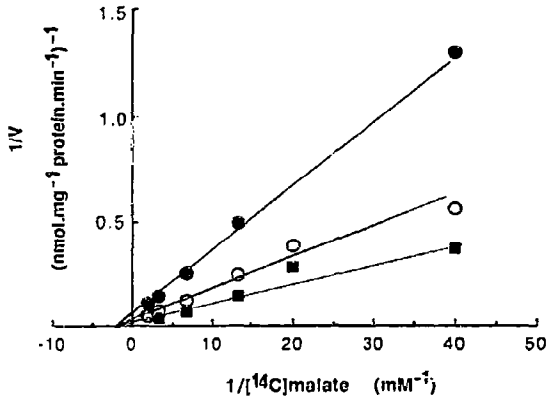

Fig. 1. Effect of ATP and phosphatase on the kinetics of [^{14}C]malate uptake by isolated soybean symbiosomes. [^{14}C]malate uptake was measured for 1 min as described in Table I, with varying malate concentrations. ○, control; ■, + 1 mM ATP and 0.25 mM $CaCl_2$; ●, symbiosomes pretreated with alkaline phosphatase.

ATP treatments (Fig. 1) indicated that the V_{max} was altered, not the affinity of the carrier for substrate.

Phosphorylation of PBM proteins under the same conditions as those described in Table I was monitored by incubating isolated, intact symbiosomes with [γ-^{32}P]ATP and then isolating PBM vesicles. The latter were subjected to SDS-PAGE followed by autoradiography. In accord with the results of Weaver et al. [9] with isolated PBM, we observed a major phosphorylated protein band (Fig. 2A, lane 1) with an apparent molecular weight of 25 kDa, which corresponded to a band identified on Western blots with an antibody against nodulin 26 (Fig. 2B, lane 2). Treatment of symbiosomes with phosphatase after incubation with [^{32}P]ATP removed most of the label (Fig. 2A, lane 2). Subsequent re-incubation with [^{32}P]ATP resulted in labelling of the same protein (Fig. 2A, lane 3). Label was also observed at the origin of the gel and some minor labelled protein bands at approximately 20 kDa could also be seen (Fig. 2A). The label at the origin probably represents aggregation of the highly hydrophobic nodulin 26 which is sometimes observed upon heating in SDS (Weaver and Roberts, unpublished results). The appearance of the labelled bands at a lower molecular weight is varable and may be the result of a minor proteolytic product of nodulin 26.

4. DISCUSSION

The results presented here confirm those of Weaver et al. [9] and show that nodulin 26 is phosphorylated in the presence of ATP and $CaCl_2$. Since we used intact symbiosomes and because it is unlikely that the added phosphatase and ATP can cross the PBM, the site of phosphorylation is most likely to be on the outer surface of the PBM, which in vivo would face the host cytoplasm. This is logical since the cytosolic compartment is the principal location of calcium fluxes that would modulate the phosphorylation of nodulin 26. Additio-

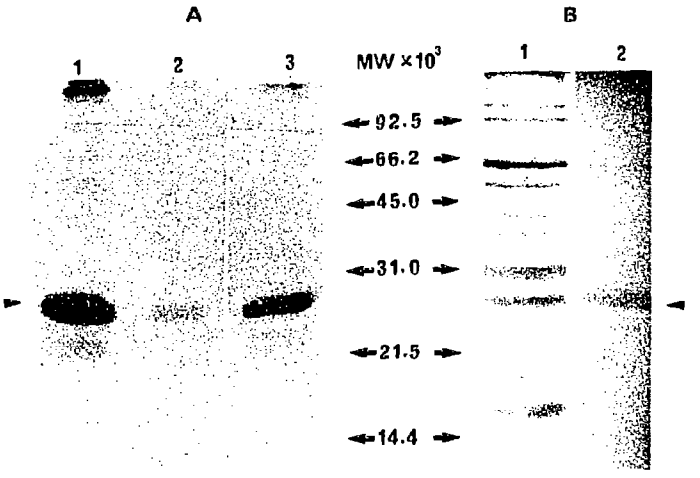

Fig. 2. Phosphorylation of PBM proteins in soybean symbiosomes. Isolated symbiosomes were incubated with [γ-^{32}P]ATP as described in Materials and Methods. The PBM was subsequently ruptured and isolated and subjected to SDS-PAGE. (A) autoradiograph showing phosphorylated bands (lane 1, control; lane 2, symbiosomes were treated with alkaline phosphatase after incubation with [^{32}P]ATP; lane 3, as for lane 2 but incubated again with [^{32}P]ATP after phosphatase treatment; 96 μg protein loaded in each lane). (B) lane 1, silver stained polypeptides (36 μg protein loaded); lane 2, Western blot probed with antibodies raised against the CK-15 region of nodulin 26 (36 μg protein).

nally, based on the proposed topology of nodulin 26 [14], the sites for nodulin 26 phosphorylation should be exposed to the cytosolic compartment.

Our results show further that the phosphorylation status of nodulin 26 is correlated with the rate of malate uptake across the PBM. Since the PBM dicarboxylate carrier restricts the rate of succinate and malate uptake into bacteroids [1,2], this has important implications for the regulation of symbiotic nitrogen fixation. Weaver et al. [9] showed that phosphorylation also occurs in intact nodules; the small and variable nature of ATP effects on malate uptake by freshly isolated symbiosomes, and the large inhibition by phosphatase treatment (Table I), indicate that a substantial portion of the nodulin 26 molecules on the PBM are phosphorylated when symbiosomes are isolated. PBM protein phosphorylation is calcium-dependent [9] and further studies of the calcium homeostasis in infected cells and the mechanisms by which calcium fluxes are mediated are required for insight into the regulation of PBM protein phosphorylation in vivo. In addition, studies of the regulation of PBM phosphatases are also needed. In preliminary experiments, we have detected an endogenous phosphatase that can dephosphorylate nodulin 26 (Weaver and Roberts, unpublished observations). Clearly, since dicarboxylates are likely to be the major source of carbon supplied to bacteroids [15,16], control of their rate of transport across the PBM is likely to be important for the overall regulation of nitrogenase activity, and phosphorylation–dephosphorylation of PBM proteins may be an important mechanism by which such control is imposed.

Although the results presented here suggest that nodulin 26 is the PBM dicarboxylate carrier, they do not constitute proof. However, it should be noted that nodulin 26 has sequence homology with channel proteins from other organisms, including the Major Intrinsic Protein (MIP) from bovine lens [7,8]. This protein has been shown to be the target of phosphorylation by multiple protein kinases [17,18]. Another nodulin 26 homolog, the tonoplast intrinsic protein [19], has also recently been reported to be the target of calcium-dependent phosphorylation [20]. Phosphorylation may be a common regulatory feature of these transport proteins.

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