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# A dicarboxylate transporter on the peribacteroid membrane of soybean nodules

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Using preparations of peribacteroid membrane (PBM)-enclosed bacteroids from soybean root nodules, we show here that the PBM possesses a dicarboxylate transporter capable of mediating a rapid flux of dicarboxylate anions, such as malate and succinate, to the bacteroids inside the nodule. The transporter has a higher affinity for the monovalent malate anion than for the succinate anion ($K_m = 2$ and 15 $\mu$M, respectively) although the $V_{max}$ for malate$^-$ appears to be lower than for succinate$^-$ ($V_{max} = 11$ and 30 nmol·min$^{-1}$·mg protein$^{-1}$, respectively).

Nitrogen fixation; Peribacteroid membrane; Dicarboxylate transporter; (*Glycine max* (L.), *Bradyrhizobium japonicum*)

## 1. INTRODUCTION

*(Brady)rhizobium* bacteroids within infected cells of legume root nodules are enclosed by a membrane of plant origin known as the peribacteroid membrane (PBM). The PBM, which effectively excludes the bacteroids from the host cell cytoplasm, is an essential feature of these nitrogen-fixing symbioses and has the potential to regulate nutrient exchanges between bacteroid and host.

The primary carbon source entering legume root nodules is sucrose which is translocated from the shoot. Nodule metabolism subsequently generates significant amounts of the monosaccharides glucose and fructose and several organic acids including malate and succinate [1,2]. Smaller amounts of fermentation products such as ethanol and acetaldehyde are also produced [3]. Whether or not these or other compounds contribute significantly to the energy requirements of bacteroid nitrogen fixation depends, among other things, on the rate at which each crosses the intervening PBM. Several indirect approaches have suggested that the major course of carbon for bacteroids derives from dicarboxylic acids in the infected cell cytoplasm [4–6]. More recently, respiration studies with isolated PBM-enclosed bacteroids (peribacteroid units, PBUs) in our laboratory have shown that the PBM is permeable to malate and succinate but poorly permeable to oxoglutarate, glutamate, pyruvate and arabinose [7]. These results, together with a consideration of the chemical nature of dicarboxylic acids such as malate and succinate, which are essentially anionic at physiological pH, suggested the presence of a dicarboxylate anion carrier on the PBM. Until now, however, no direct measurements of metabolite transport across the PBM have been made. Here we present direct evidence for a dicarboxylate anion transporter on the PBM of soybean root nodules.

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*Abbreviations:* PBM, peribacteroid membrane; PBUs, peribacteroid units; BM, bacteroid inner membrane; M, malate



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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Seeds of soybean (*Glycine max* L. cv. Bragg) were inoculated with *Bradyrhizobium japonicum* USDA110 and grown in pots in a glasshouse, as described [7]. Phthalonic acid was a gift from Drs I.B. Dry and J.T. Wiskich (University of Adelaide, South Australia) and α-cyano-4-hydroxycinnamic acid was purchased from Aldrich (WI). Other chemicals were purchased from Sigma (St Louis, MO).

### 2.2. *Preparation of PBUs and bacteroids*

PBUs were prepared from soybean (*Glycine max*) root nodules induced by *B. japonicum* USDA110 as described [7] with the following alterations. Glycerol was omitted, and mannitol increased to 350 mM, in all solutions. PBUs were collected as a band at the 60%/80% interface on a Percoll step gradient after centrifugation for 15 min at 5000 rpm in a Sorvall HB-4 swing-out rotor. PBU enrichment was achieved by dispersing the PBUs gently into 20 ml wash buffer and pelleting onto an 80% Percoll cushion by centrifugation for 15 s at 5000 rpm in the same rotor. This band was resuspended in wash buffer prior to uptake studies. Bacteroids were prepared from intact PBUs simply by vortexing vigorously for 2–3 min thus avoiding the possibility of osmotic shock. Rupture was monitored by light miscroscopy.

### 2.3. *Transport studies*

Measurements of L-[U-$^{14}$C]malate and [2,3-$^{14}$C]succinate (both from Amersham International) uptake were made using the technique of silicon oil filtration centrifugation [8]. $^{3}H_2O$ (Amersham) and [$^{14}$C]dextran (New England Nuclear) were used to estimate total and external water volumes [8]. Uptake reactions were performed at 22 ± 1°C and pH 7.0 and terminated after 30 s unless stated otherwise. Where pH values were varied this was achieved by resuspending 0.1 ml PBUs in 1 ml wash buffer containing either 100 mM Mes (pH 5.5, 6.0, 6.5) or 100 mM Mops (pH 7.0, 7.5).

### 2.4. *Protein estimation*

Protein concentration was estimated by the method of Lowry et al. [9].

## 3. RESULTS AND DISCUSSION

Initial experiments established that the rates of malate and succinate uptake by both PBUs and bacteroids were constant during the first few minutes. Fig.1 shows such a time course for malate uptake by bacteroids. Subsequently, all reactions were termintaed after 30 s which is clearly in the linear phase of uptake.

Fig.2 illustrates the relative kinetics of malate uptake by PBUs and bacteroids. The saturation kinetics displayed by both membrane systems are consistent with carrier mediated mechanisms. The apparent $K_m$ for the PBUs was substantially

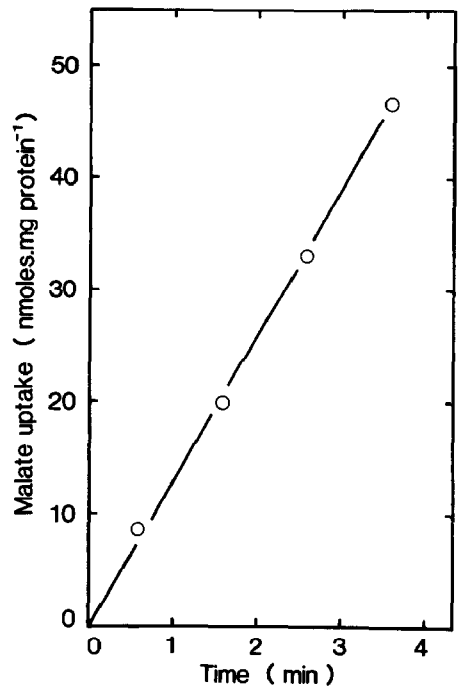


Fig.1. Time course of malate uptake by bacteroids. External malate concentration was 1 mM. Data points are the means of quadruplicates. Linear regression analysis of the points gave $r = 0.99$.

greater than that for the bacteroids (table 1). The difference in $V_{max}$ between the two systems was not so marked. Table 1 summarizes several different experiments in which the kinetic parameters for both malate and succinate uptake across the PBM and the bacteroid inner membrane (BM) were determined. The PBM system also had a lower affinity for succinate than did that on the BM but the $V_{max}$ for the two systems was not significantly different. In experiments not shown, succinate and malate were found to inhibit each other's uptake.

The data shown in fig.2 and table 1 are suggestive of a carrier for succinate and malate on the PBM but caution must be exercised when interpreting such data obtained with a double membrane system. A simple diffusion barrier on the PBM could have the effect of increasing the apparent $K_m$ for a carrier on the BM. The possible involvement of malate and succinate metabolism within the bacteroid complicates the issue further.

We therefore searched for compounds which might distinguish between the PBM and BM systems by differential inhibition. Fig.3 shows the

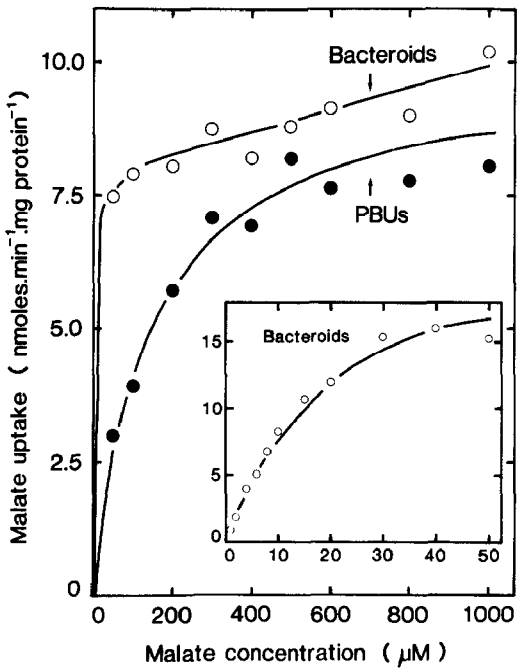


Fig.2. A typical experiment showing the concentration dependence of the rate of malate uptake by PBUs and bacteroids. Data points are the means of duplicates. ●, PBUs; ○, bacteroids. Note that the data shown in the inset are from an experiment separate from that depicted in the main figure, in which bacteroid uptake at lower malate concentrations was measured. Lineweaver-Burk plots of the data gave correlation coefficients of $r = 0.99$ for both PBUs and bacteroids.

effects of two such inhibitors, phthalonic acid and α-cyano-4-hydroxy-cinnamic acid, both of which are known inhibitors of mitochondrial organic acid transporters [10–13], on the uptake of malate by PBUs and bacteroids. At the concentrations used, both of these compounds were potent inhibitors of uptake by PBUs but had no effect on malate uptake by bacteroids.

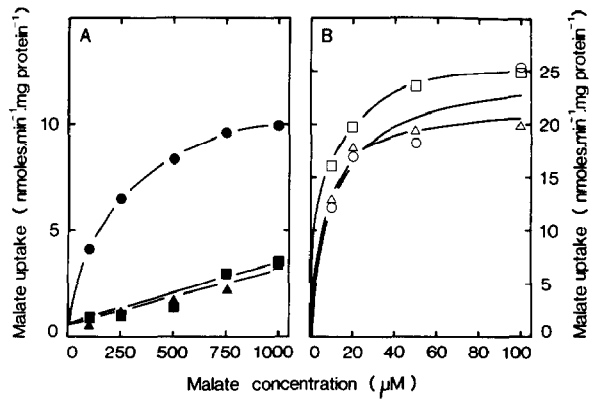


Fig.3. Effect of inhibitors on PBU (A) and bacteroid (B) malate uptake. Data points are the means of duplicates. ●, ○, controls; ▲, △, plus 1 mM α-cyano-4-hydroxycinnamic acid; ■, □, plus 5 mM phthalonic acid. Inhibitors were added 5 min prior to malate. Lineweaver-Burk plots gave the following correlation coefficients: ●, $r = 0.99$; ○, $r = 0.96$; △, $r = 0.94$; □, $r = 0.99$. Linear regression of the lines through ■ and ▲ gave $r = 0.98$ and $r = 0.96$, respectively. The positive intercept in A reflects slight contamination by free bacteroids in the PBU preparations [7].

The selectivity of these inhibitors for malate uptake across the PBM leaves little doubt that a separate transport mechanism, presumably a proteinaceous carrier, exists on this membrane. Such a transporter had been predicted from indirect measurements of selective permeability of PBUs to a range of metabolites [7] but this is the first direct demonstration.

Malate exists as an equilibrium of 3 species in aqueous solution. This can be represented as follows:

$$MH_2 \overset{pK_{a1}\,3.4}{\rightleftharpoons} MH^- + H^+ \overset{pK_{a2}\,5.11}{\rightleftharpoons} M^{2-} + 2H^+$$

These equilibria can be shifted by changes in pH. Fig.4 illustrates an experiment in which this

Table 1

Kinetic parameters for malate and succinate transport across the PBM and BM. Values ± standard errors were obtained from at least five separate experiments in each case

| Substrates | PBUs | | Bacteroids | |
|---|---|---|---|---|
| | $K_m$ (μM) | $V_{max}$ (nmol · min$^{-1}$ · mg protein$^{-1}$) | $K_m$ (μM) | $V_{max}$ (nmol · min$^{-1}$ · mg protein$^{-1}$) |
| Malate | 156 ± 27 | 11 ± 1 | 9 ± 2 | 24 ± 1 |
| Succinate | 391 ± 83 | 33 ± 7 | 13 ± 4 | 23 ± 6 |

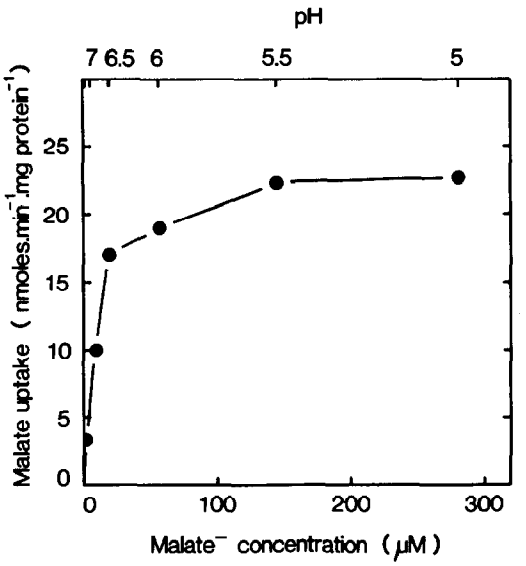


Fig.4. Effect of monovalent malate anion concentration on malate uptake by PBUs. The monovalent malate anion concentration was varied by changing the external pH (top horizontal axis) in the presence of a constant total malate concentration of 500 μM. The monovalent malate anion concentration was calculated using $pK_{a2}$ = 5.11 and $pK_{a1}$ = 3.40. Data points are the means of duplicates. Linear regression analysis of a Lineweaver-Burk plot gave a correlation coefficient of $r$ = 0.99.

was done, and shows the effect of monovalent malate anion concentration on the rate of malate uptake by PBUs. The monovalent malate anion ($MH^-$) concentration was altered by varying pH while maintaining the total malate concentration ($MH_2 + MH^- + M^{2-}$) constant at 500 μM. The data conform to Michaelis-Menten kinetics. There was no meaningful relationship between the rate of malate uptake and the concentration of either diacid malate ($MH_2$) or divalent malate anion ($M^{2-}$) (not shown). Taken together, these results strongly suggest that the true substrates for the PBM carrier are monovalent dicarboxylate anions. When this is taken into account, the $K_m$ values for malate and succinate shown in table 1 adjust to 2 and 15 μM, respectively. Thus the PBM carrier has a higher affinity for malate than succinate.

Given current estimates of malate and succinate concentration in the nodule cytosol (4.2 and 0.7 μmol/g fresh wt [2]; ~ 4.5 and 0.8 mM, respectively), it would appear from our experiments that the dicarboxylate transporter on the PBM is normally saturated with substrate. The results also indicate that under these conditions the transporter-mediated flux of dicarboxylates through the PBM is sufficient to support a maximal, or near maximal, rate of uptake by the corresponding bacteroid transporter and that malate is likely to the the preferred substrate.

It is interesting to speculate on the origin of the PBM dicarboxylate carrier. The vacuolar marker, α-mannosidase, has been found in the peribacteroid space [14] suggesting perhaps a biogenic relationship between the PBU and vacuole. Indeed malate transporters have been described for the tonoplast of some plant cells [15,16] but their characteristics are somewhat different from those described here. On the other hand, the plasmalemma and the PBM share some common properties [17]. We are not aware, however, of any report of a dicarboxylate carrier on the plasmalemma. An interesting possibility is that the PBM dicarboxylate carrier is a nodulin (or nodule-specific protein) induced as a result of *Bradyrhizobium* infection of the root [18]. Since dicarboxylic acids appear to be essential for bacteroid nitrogen fixation [4–6] and the transporter on the PBM facilitates the rapid flux of dicarboxylates to the bacteroid, the evolutionary implications of this final possibility are great.

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