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Mg^{2+} Dependent Pyrophosphatase, A Tonoplast Enzyme in the Peribacteroid Membrane of *Glycine max* Root Nodules

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Abstract

Purified peribacteroid membranes (PBM) from soybean root nodules, harvested 26–29 days post infection, contain a specifically Mg^{2+} dependent pyrophosphatase (PP_iase). Its activity is only slightly inhibited (0–10%) by ATPase-inhibitors such as azide, DES, DCCD and NO_3^-, but is strongly inhibited by KF (68%). The pH-optimum is about pH 9. Hydrolysis of inorganic pyrophosphate (PP_i) is sensitive to Mg^{2+} and, in acid pH conditions, to molybdate. Molybdate sensitivity is due to acid phosphatase found in the peribacteroid space. The apparent $K_m$for the hydrolysis of PP_i is about 124 ± 21 μM (pH 8.5) and the apparent K_m for the PBM associated ATPase is about 340 ± 42 μM (pH 6.0). Peribacteroid membrane associated PP_iase and ATPase were separated by gel filtration.

Keywords: acid phosphatase, ATPase, *Glycine max*, peribacteroid membrane, pyrophosphatase, root nodule

Abbreviations: DCCD — N-N′-dicyclohexylcarbodiimide, DES — diethylstilbestrol, EDTA — ethylendiaminetetraacetic acid, MES — 2-N-morpholinoethanesulfonic acid, PBM — peribacteroid membrane, PBS — peribacteroid space, P_i — inorganic phosphate, PNP-P — p-nitrophenyl phosphate, PP_iase — inorganic pyrophosphatase, PP_i — inorganic pyrophosphate, TCA — trichloroacetic acid, TRIS — tris-(hydroxymethyl)aminomethane

1. Introduction

Root nodules are formed after successful infection of *Glycine max* root cells by *Bradyrhizobium japonicum*. Infected cells are densely packed with bacteroids of *B. japonicum*. A structural characteristic of infected cells is the occurrence of the peribacteroid membrane (PBM) surrounding the procaryote in all effective, i.e. nitrogen-fixing, symbioses. This membrane envelope is of plant origin (Verma et al., 1978; Robertson and Lyttleton, 1982) but is built under the genetic control of the micro-symbiont (Werner et al., 1988). The PBM is a barrier between the symbiotic partners. One of its functions is to mask the bacteria and to prevent their degradation, since absence or early loss leads to a plant defense response such as phytoalexin production (Werner et al., 1985) or disintegration of the bacteroids (Werner et al., 1980; Werner et al., 1984). Another function is to allow transport and communication processes. Transport of organic acids across the PBM is facilitated by specific carrier-proteins (Udvardi et al., 1988). Differences in protein and lipid composition of the PBM and other plant membrane systems have been reported (Fortin et al., 1985; Mellor et al., 1985; Robertson et al., 1978), yet little is known about enzymatic properties of the PBM. Until now, a H^+-translocating ATPase activity (Blumwald et al., 1985; Bassarab et al., 1985), a Ca^{2+}-dependent protein kinase (Bassarab and Werner, 1987) and a dicarbonic acid carrier (Udvardi et al., 198) have been localized in the PBM. Here evidence is presented that a PP_iase, often associated with tonoplasts, is also located in the PBM of soybean nodules. PBM enclosed bacteroids are called 'symbiosomes' as proposed by G. Stacey (personal communication).

2. Material and Methods

Growth of tissue

Glycine max L. Merr. cv. Maple Arrow (Ottawa Research Station Canada) was grown under controlled conditions. Ten days old seedlings were inoculated with known titers of *Bradyrhizobium japonicum* 61-A-101 (Nitragin & Co., Milwaukee) as described previously (Werner et al., 1975). Nodules were harvested 26–29 days post inoculation and used immediately.

Cell fractionation and PBM isolation

Homogenisation and membrane preparation was done as described by Mellor et al. (1985) with the following changes. All solutions contained 1 mM PMSF (phenylmethyl-sulfonylfluoride) to inhibit endogenous protease activity. By sedimentation centrifugation in a continuous 30% to 60% sucrose density gradient (26 ml) topped with 5 ml 20% sucrose, symbiosomes

(bacteroids still enclosed in the PMB) banded between 1.23 and 1.25 g ml^{-1}. Symbiosomes were removed from the first sucrose gradient and adjusted with buffer A (5 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) to about 20% sucrose and pelleted by centrifugation of 6000×g for 30 min. Symbiosome-pellets were resuspended in buffer A containing 6% sucrose and pressed 5 times through a hypodermic needle (0.6 mm×25 mm) to free the peribacteroid membrane (PBM). The hypoosmotically treated symbiosome fraction was then layered on top of 36% sucrose in buffer A and centrifuged for 30 min and 50000×g. The interface containing the PBM was processed as before. These changes in the method increased the yield from approximately 20–25 μg PBM protein per gram nodule fresh weight, which is close to the figure given by Robertson et al. (1978) obtained with a similar technique, to 70–80 μg PBM-protein per gram nodule fresh weight.

PBM solubilization

Aliquots of PBM were solubilized as described by Rea and Poole (1986) with minor modifications. PBM pellets were resuspended in a solution containing 10% w/w glycerol; 3.0 mM $MgSO_4$; 1 mM EDTA; 40 mM Tris-Mes, pH 8.0, 2 mM DTT; and 0.5 mg phosphatidylcholin×ml^{-1}. The protein to Triton X-100 ratio was adjusted to 1:10, the resuspended PBM pellet was incubated on ice for 30 min and the suspension was then centrifuged at 200,000×g for 60 min. The supernatant was designated solubilized PBM.

Gel filtration

About 1–2 mg of solubilized PBM protein were loaded on a Sepharose CL-6-B column (40×15 cm) equilibrated with the same buffer used for PBM resuspension except that Triton X-100 concentration was 0.3% (w/w). Proteins were eluted with the same buffer. The flow rate of the column was 250 μl×min^{-1}; 750 μl fractions were collected. Aliquots of 250 μl were then used for ATPase, for PP_iase-assays and for protein determination.

Enzyme assays

ATPase (E.C. 3.6.1.3.) activity was measured as the rate of P_i liberation. The reaction took place routineously in 125 μl total volume containing 3 mM Na_2ATP, 3 mM $MgSO_4$, and 200 μM Na_2MoO_4 in 40 mM Tris-Mes pH 6.0 or 40 mM Tris-Mes pH 8.0. At the end of incubation time P_i was measured in 20 μl aliquots using a modification of the method published by Altmann et al. (1971). Linearity of P_i estimation is given within a range of 0.5–10 nmol P_i per assay.

The PP_iase (E.C. 3.6.1.1.) assay was performed as described above except that Tris-Mes buffer pH 8.5 was used and the substrate was 3 mM Na_4PP_i. All P_i estimations were corrected against non-enzymatic substrate breakdown, and membrane and solution blanks by performing P_i-estimations in the substrate and in the enzyme solution under assay conditions.

Acid phosphatase was routinely measured in a medium containing 40 mM Tris-Mes pH 5.5 and 3 mM PNP-P. The final volume was 0.5 ml. Free p-nitrophenol was determined as in Bassarab et al. (1984).

All enzyme assays were performed at 20°C and started by adding 5–20 μg protein per assay. Apparent K_m-values were estimated according to Lineweaver-Burk. Enzyme activities are given in mU per mg protein and min. One U is defined as the enzyme activity which turns over one μmol of substrate.

Protein determination

Protein was determined by the method of Lowry et al. (1951) on TCA-precipitated protein to prevent interference with phenolic substances, which are common in legumes (Harborne and Simmonds, 1964; Khanna et al., 1969). Protein measurement in the fractions obtained by gel filtration was done with the Bio-Rad® reagent.

Chemicals

All chemicals used were from either Sigma (München, FRG), Serva (Heidelberg, FRG) or Boehringer (Mannheim, FRG).

3. Results and Discussion

The PBM of soybean nodules contains an alkaline inorganic PP_iase. The activity is linear for at least 40 min and is proportional to the protein concentration. The specific activity is about 21.2 ± 3.4 mU/mg protein min at 20°C and pH 8.5 under the standard assay conditions described in Material and Methods. This is about one-fourth of the specific ATPase activity associated to the PBM (Bassarab and Werner, 1986). The specific PP_iase activity in the PBM of soybean root nodules is significantly lower than e.g. in tonoplasts of *Avena sativa* with activities of about 70 mU/mg protein min at 25°C (recalculated from Pope and Leigh, 1987). In red beet vacuoles the specific PP_iase activity is about 130 mU/mg protein min at 30°C (recalculated from Walker and Leigh, 1981).

Potassium stimulation

PP_iase is known to be stimulated by KCl to different extents, ranging from about 7.8 fold (Rea and Poole, 1985) to about 1.8 fold (Walker and Leigh, 1981). In both cases, red beet vacuoles have been used. Under standard assay conditions in the presence of 3 mM $MgSO_4$ we found only about 40% stimulation of PP_iase activity at pH 8.5. However, if the $MgSO_4$ concentration was lowered to 0.5 mM, 50 mM KCl stimulated PP_iase 3–4 fold. If no $MgSO_4$ was added, stimulation was 10–12 fold compared to controls without KCl. Obviously the ionic "background" in the assay affects the KCl stimulation of PP_iase.

pH-dependency of PP_iase and K_m-values

Figure 1 shows the pH-dependency of PP_i hydrolysis in the presence and absence of the acid phosphatase inhibitor molybdate. As described by Karlsson (1975) and by Wagner and Mulready (1983) for tonoplasts an alkaline optimum around pH 9 was found for the PP_iase in the PBM (Fig. 1). The inhibitory effect of molybdate under acid assay conditions is due to inhibition of an acid phosphatase known to be associated to different vacuoles (Boller and Kende, 1979; Leigh and Walker, 1980). It is also found in the peribacteroid space (this work) and possibly contaminates the PBM preparation. The amount of contaminating acid phosphatase can be reduced by an additional centrifugation step of the PBM preparation in buffer A, without effect on the specific PP_iase activity. Some of the characteristics of the PBS located acid phosphatase, which are common to other acid phosphatases, are summarized. The pH optimum is about 5.5. The apparent K_m-value with PNP-P is 0.26 mM at pH 5.5 and 2.39 mM at pH 8.0. The specific activity in the peribacteroid space is 85 mU/mg protein min at pH 5.5. Molybdate (200 μm) inhibits the acid phosphatase at pH 5.5 by 64%. PBM associated PP_iase is not effected by molybdate at all. At pH 5.5 in the absence of Mg^{2+} the PBS associated acid phosphatase hydrolizes different substrates with the following specific activities: phosphoenolpyruvate, 189 mU/mg protein min; PP_i, 177 mU/mg protein min; ATP, 152 mU/mg protein min; GTP, 132 mU/mg protein min and glucose-6-phosphate, 87 mU/mg protein min.

Figure 1 shows that there is hardly any inhibition by molybdate at pH values above 7.5, indicating that PP_iase is not affected by molybdate. The apparent K_m-value for the ATP-ase at pH 6.0 is 351 ± 4.3 μM, which is similar to data of Domigan et al. (1988). The apparent K_m-value for the PP_iase is 124 ± 21.3 μM if estimated by a Lineweaver-Burk blot.

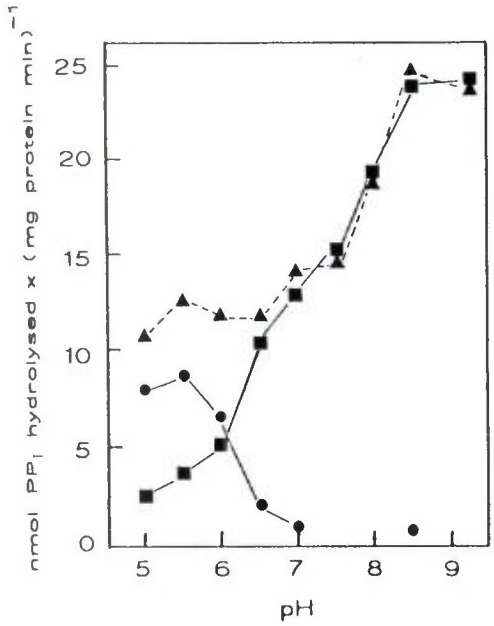

Figure 1. pH dependence of PP_i-hydrolysis by the PBM-associated pyrophosphatase (▲) activity in the absence of 200 μM Na_2MoO_4; (■) activity in the presence of 200 μM Na_2MoO_4; (•) Na_2MoO_4 sensitive PP_iase activity.

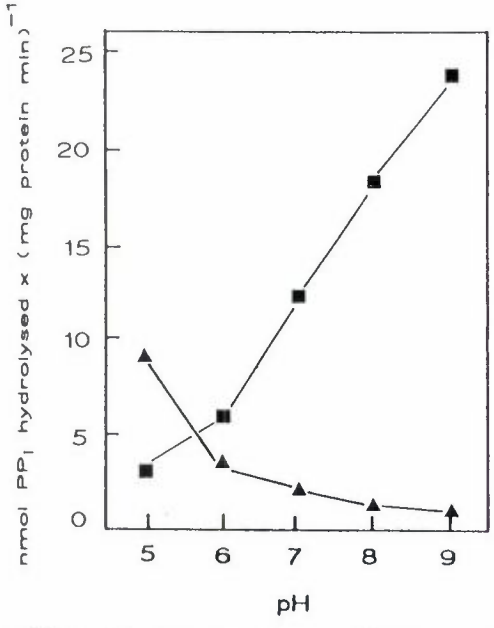

Figure 2. PP_iase activity (■) in the presence of 3 mM $MgSO_4$ and 200 μM Na_2MoO_4; (▲) control without $MgSO_4$ and Na_2MoO_4.

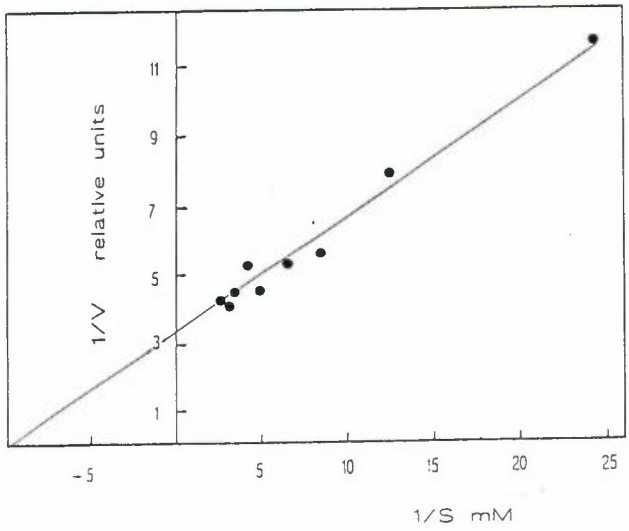

Figure 3. PBM-PP_iase activity in a Lineweaver-Burk plot. K_m app. 124 ± 21 μM (n = 3).

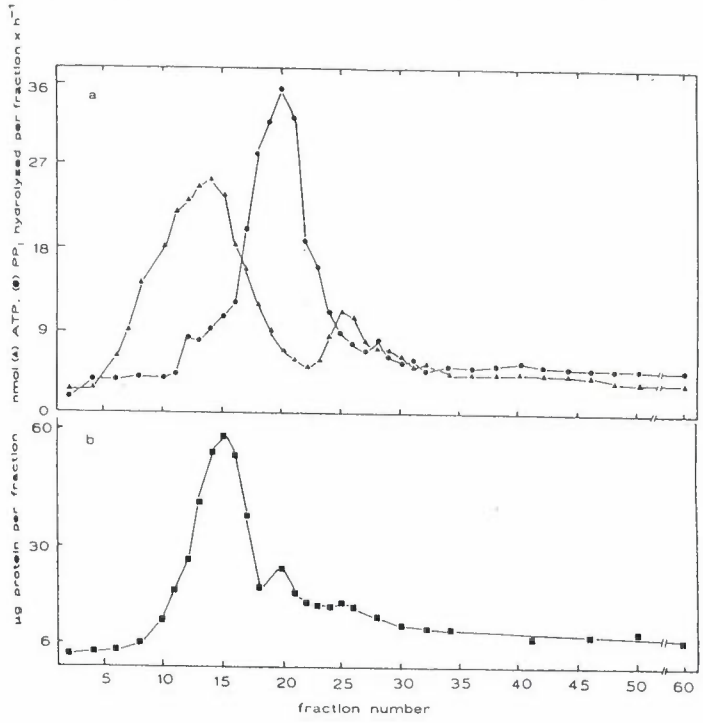

Figure 4. A. Elution profile of (▲) ATPase and (•) PP_iase activity of solubilized PBM after separation on Sephadex CL-6-B. B. Protein content of the fractions.

Table 1. Influence of divalent cations on the PBM associated ATPase and PP_iase

Salt added	% activity			
	ATPase		PP_iase	
	pH 6.0[e]	pH 8.5[e]	pH 8.5[e]	pH 8.5[f]
none	11.2	15.7	11.8	12.3
$MgCl_2$	100.0[a]	100.0[b]	100.0[c]	100.0[d]
$MnCl_2$	88.6	61.5	4.6	7.7
$CaCl_2$	8.4	44.7	1.0	1.2
$ZnCl_2$	23.1 0	0	1.4	

The 100% activities given in mU/mg protein min are: a = 78, 4, b = 64.7, c = 23.2 and d = 24.8

[e]Salt concentration 3 mM; substrate concentration 2 mM

[f]Salt concentration 1.5 mM; substrate concentration 1 mM

Assay conditions as described in Materials and Methods except that the PBM was resuspended in the appropriate buffer containing 1 mM EDTA.

Data are averages from two experiments.

Table 2. Inhibition of PBM-associated PP_iase and ATPase by various inhibitors

Inhibitor	Concentration	% Inhibition	
		PP_iase	ATPase
NaN_3	2.0 mM	7.1±1.4	8.2±2.1
DES	0.2 mM	17.5±6.9	58.2±9.4
DCCD	0.2 mM	4.0±1.3	18.7±2.1
KF	1.0 mM	68.1±2.1	6.0±2.9
KNO_3	50.0 mM	0	21.3±3.4
$NaVO_3$	0.1 mM	5.6±1.2	41.7±4.2
$NaVO_3$	0.2 mM	11.4±2.3	65.8±4.8

Assay conditions as described in Materials and Methods

n = 3. Both activities were measured at pH 8.0.

Requirement of divalent cations

PP_iase specifically requires Mg^{2+} for its activity; other divalent cations are very poor substitutes (Table 2). PBM associated ATPase activity is far

less specific in respect to the divalent cation. As described by Domigan et al. (1988) Mg^{2+} too maintains high ATPase activity (Table 1). For comparison see also Walker and Leigh (1981). Mg^{2+} stimulates ATPase ca. 15 fold better than PP_iase. Data from Table 1 further indicate that 3 mM Ca^{2+} and Zn^{2+} inhibit PP_iase compared to controls with no salt added. Within the optimum pH range Mg^{2+} stimulates PP_iase 8.4 fold, which is in line with data from Wagner and Mulready (1983) and Rea and Poole (1986). The highest PP_iase activity is achieved at a PP_i to Mg^{2+} ratio of 1:1. Figure 2 shows that PBM-associated PP_iase depends strictly on Mg^{2+}, in the absence of Mg^{2+} only the acid phosphatase activity can be detected (Fig. 2), which is not stimulated by Mg^{2+} at all.

Inhibitor studies

Additional evidence that ATPase activity and PP_iase are not due to the same enzyme has been achieved by a set of inhibitor experiments. Both activities were titrated against various concentrations of different inhibitors. Results are summarized in Table 2. PP_i hydrolysis is hardly affected by any of the tested ATPase inhibitors, but strongly inhibited (68%) by 1 mM KF which is a potent inhibitor of PP_iase (Karlsson, 1975; Wang et al., 1986). ATPase is only slightly inhibited (6%) at 1 mM KF. With 0.2 mM DES, ATPase activity is inhibited by 58%, PP_iase activity only by 17%. Significant differences are also found for nitrate and vanadate. Nitrate, a known inhibitor of tonoplast ATPases (Smith et al., 1984), inhibits PBM-ATPase by about 21% (Bassarab et al., 1986; Day et al., 1988), but does not affect PP_iase activity at all. Vanadate, prepared from sodium vanadate according to Gallagher and Leonard (1982), a potent inhibitor of the ATPase in the PBM (Blumwald et al., 1985; Bassarab et al., 1986; Domigan et al., 1988), inhibits PP_iase only by 6%, whereas at the same concentration ATPase is inhibited by 42%. Therefore we assume that PBM-PP_iase does not bind inorganic phosphate covalently during the enzymatic cleavage of PP_i. On the basis of these inhibitor studies, PBM-PP_iase is distinct from PBM-ATPase.

Separation of PP_iase and ATPase

For further confirmation of a PP_iase present in the PBM, both activities were separated by gel filtration as described in Material and Methods. The elution profile of the enzyme activities is shown in Fig 4a. ATPase and PP_iase represent two distinct activity peaks. No significant ATPase activity is found in the PP_iase peak-fraction and vice versa (for comparison see Rea and Poole, 1986).

The results demonstrate for the first time a PP_iase in the PBM of soybean nodules. As far as we know this activity has not been shown in plasma membrane preparations but is found to be associated to tonoplasts (Wang et al., 1986; Wagner and Mulready, 1983). The argument that PP_iase activity comes from cross contamination can be ruled out at this stage because the method of PBM isolation (Bassarab and Werner, 1987) utilizes the large density differences between the plant endomembranes, ranging from 1.11 for tonoplast vesicles (Mandala et al., 1982) and 1.23 to 1.26 $g \times ml^{-1}$ for the PBM enclosed symbiotic procaryote (Bassarab et al., 1986; Robertson et al., 1978; for review Mellor and Werner, 1986). Substantial contamination by bacterial membranes can also be ruled out, because hardly any azide sensitive ATPase activity is found in the PBM fraction (Bassarab et al., 1986). Also no plant or bacterial cytoplasmatic markers such as alcohol-dehydrogenase, leghemoglobin, or alanine-dehydrogenase are found in the PBM or peribacteroid space (PBS) fraction. Therefore PP_iase activity can not be due to contamination by soluble PP_iase. Another control is the absence of any significant membrane material in the 1.23–1.26 $g \times ml^{-1}$ fraction in preparations from uninfected root tissue.

We have shown a soluble acid phosphatase in the PBS, but this activity, as shown in Figs. 1 and 2, is not dependent on Mg^{2+}, it is inhibited by molybdate, and it has an acid pH optimum of 5.5, whereas PBM associated PP_iase depends on Mg^{2+}, it is molybdate insensitive, and it has an alkaline pH optimum. The various steps of the PBM-purification indicate, that the PP_iase is truely membrane bound.

Since it is still a point of discussion whether the PBM is more plasma membrane like or more tonoplast like, the finding of a PP_iase in the PBM is particularly important. We consider the PBM as a symbiotic structure built by a direct membrane flow from ER via Golgi to the PBM. The PBM is optimized and specialized for its symbiotic needs and bears characteristics of the plasma membrane, e.g. vanadate sensitive ATPase (Blumwald et al., 1985) which is also inhibited by nitrate (Bassarab et al., 1986; Day et al., 1988), as well as characteristics of the tonoplast, e.g. a dicarboxylic acid carrier (Udvardi et al., 1988) and an alkaline PP_iase. It is doubtful whether the vanadate-sensitive ATPase is a plasma membrane ATPase, since its 100 kD subunit (Kasamo, 1986) should be found in SDS-PAGE of PBM protein. But neither by Brewin et al. (1985) and Fortin et al. (1985), nor by Bassarab et al. (1987) a 100 kD subunit was reported as prominent on SDS gels. Additional data which favour that the PBM encloses a vacuole like struc-

ture, are the hydrolytic enzymes as proteases, α-mannosidases (Mellor et al., 1984; Kinnback et al., 1987) and acid phosphatase(s) (this paper) which are located in the peribacteroid space.

If PP_i has a special function in the symbiosis is not known at present, but it might be involved in the H^+-translocation (membrane energization) or in providing P_i for bacteroid differentiation. More details about the physiological characteristics and the regulation of the PBM synthesis will help to understand the function of *Rhizobium* legume metabolite exchange.

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