ELSEVIER

Journal of Chromatography B, 711 (1998) 139–149

JOURNAL OF CHROMATOGRAPHY B

Comparison of Mg^{2+}-dependent ATP hydrolase activities of pea nodule symbiosomes and of pea root plasmalemma, obtained by an aqueous polymer two-phase system

Patricia Rojas-Ojeda[a], Luis E. Hernández[a,b], Nicholas J. Brewin[b], Ramón Carpena-Ruiz[a,*]

[a]*Departamento de Química Agrícola, Geología y Geoquímica, Universidad Autónoma de Madrid, Cantoblanco 28049 Madrid, Spain*
[b]*John Innes Centre, Norwich Research Park, Colney Lane, Norwich NR4 7UH, UK*

Abstract

The characteristics of the Mg^{2+}-dependent ATPase activity from the peribacteroid membrane of pea symbiosomes was compared with that from pea root plasma membranes. Enzyme inhibitors, optimum reaction pH, substrate specificity and antibody recognition were the main parameters examined. Both the symbiosomes and the root plasma membrane were purified with an aqueous polymer two-phase system (APS). The final concentration of the APS for the purification of symbiosomes were: 6.3% w/w dextran T500, 6.3% w/w poly(ethylene glycol) 3350, 5 m*M* KH_2PO_4–K_2HPO_4, 5 m*M* KCl, 0.33 *M* sucrose, (pH 7.85); for the root plasma membrane was: 6.2% (w/w) dextran T500, 6.2% poly(ethylene glycol) 3350, 330 m*M* sucrose, 5 m*M* K_2HPO_4 and 4 m*M* KCl (pH 7.8). The lack of contamination of pea symbiosomes with endoplasmic reticulum, mitochondria, broken bacteroids and/or tonoplast vesicles was established. Similarly, the aqueous two-phase system used here provided a fairly enriched root plasma membrane with low cross-contamination from other sources. Both symbiosomal and root plasma membrane ATPase activities were highly specific to ATP. The symbiosome ATPase apparently corresponds to an E_1E_2–ATPase mechanism, similar to that found at the plasma membrane. The similarity between these two ATPases was further supported by immuno-analysis. Mg^{2+}-ATPase of pea symbiosome and root plasma membranes were very similar, by all parameters tested.

Keywords: Aqueous two-phase systems; ATPase; Enzymes

1. Introduction

Nitrogen fixation in nodules of legume plants infected by *Rhizobiaceae* bacteria takes place in a symbiont organelle, the symbiosome, formed in the last step of nodule maturation, when the bacteria reach the infected cell through an infection thread [1]. Bacteroids are isolated from the cytosol by the peribacteroid membrane (PBM), which forms the primary interface between the bacterium and the host plant cell [2]. In the first stages of symbiosome development the PBM is thought to be derived from the plant cell plasma membrane, via endocytosis [3]; but several phases have been distinguished in the PBM development, each one involving differences in composition and in function, as reviewed recently by Udvardi and Day [see Ref. [4]].

The PBM controls the transport of metabolites between the host cytosol and the bacteroid, and therefore has a key role in the regulation of symbiotic nitrogen fixation. A transport system for dicarboxylic acids towards the bacteroid [5,6], and

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00110-8

for ammonia from the bacteroid towards the plant cytosol have been identified in the last few years [7]. Szafran and Haaker [8] proved the presence of a H^+-ATPase in the PBM; which generates an electrochemical gradient between the symbiosomal fluid space and the host cytosol [9], being positive and more acidic inside the symbiosome. These differences would facilitate the exchange dicarboxylate/ammonium; and, on the other hand, this exchange could contribute to balance the pH inside the symbiosome [4].

Blumwald et al. [10] characterized the symbiosomal ATPase obtained from soybean root nodules as K^+ stimulated and VO_4^{3-} inhibited, similar to the properties of the plasma membrane ATPase. Bassarab et al. [11] indicated the presence of two types of enzyme, one being a minor Golgi-type ATPase and the other a major plasmalemma-like H^+-ATPase, according to their optimum reaction pH. Other authors have found similar profiles of the symbiosomal ATPase to that reported in [10], i.e. with a maximum enzyme activity at about pH 6.5 [9].

In the present work, we have studied the characteristics of the PBM Mg^{2+}-dependent ATPase of pea symbiosomes and the activity from vesicles of pea root plasma membranes. In this sense, we have first established a method of purification of plasmalemma microvesicules by an APS [12,13]. However, due to the limited yields of plasma membrane from the nodule cells [[14,15], and our own experience], we obtained plasmalemma vesicles from uninfected root cells. After this optimization, enzyme markers, optimum reaction pH, substrate specificity and antibody recognition were used to ascertain the similarities between the Mg^{2+}-ATP hydrolytic activity of intact symbiosomes (which behave as 'inside out' plasmalemma vesicles) and of RPM purified vesicles.

2. Experimental

2.1. Plant material

Pea seeds (*Pisum sativum*), cv. Argona, were germinated on moistened paper for 4 days at 25°C. Three seedlings were transferred to plastic pots containing vermiculite, inoculated with *Rhizobium leguminosarum* biovar. *viciae* 3841 and grown under glass (25°C day and 18°C night). Each pot was watered by capillarity with the following nutrient solution (m*M*): $CaSO_4$ (0.5), $CaCl_2$ (1.5), KCl (1), K_2SO_4 (0.5), KH_2PO_4 (2), $MgSO_4$(1), NaCl (0.1), plus micronutrients (mg ml^{-1}): Fe (2.5), Mn (1), Zn (0.4), B (0.25), Cu (0.2), and Mo (0.02). After 6 weeks, nine pots were sampled from each replicate and approximately 8 g of nodules collected. Similar procedure, but without inoculation, was carried out to obtain uninfected roots.

2.2. Symbiosome extraction and purification

The procedure for symbiosome extraction described by Hernández et al. [13] was used. Fresh nodules were homogenised gently with a chilled pestle and mortar in 15 ml extraction buffer (350 m*M* mannitol, 10 m*M* EGTA, 10 m*M* $MgSO_4$, 5 m*M* DTT, 1% PVP-40, 20 m*M* ascorbic acid, 25 m*M* MES, 10 m*M* NaF, made to pH 7.0 with KOH), plus 30 μl of fresh anti-protease cocktail diluted in methanol (2.5 mg ml^{-1} 4-amidinophenyl-methanesulfonyl fluoride, 0.8 mg ml^{-1} phosphoramidon and 0.4 mg ml^{-1} pepstatin). These and all subsequent procedures were done at 4°C. The homogenate was sieved through a nylon cloth (240 μm), centrifuged at 10 000 *g* for 15 min. The supernatant was further centrifuged at 100 000 *g* for 30 min, the subsequent pellet resuspended in 1.5 ml ATPase buffer (5 m*M* PIPES, 350 m*M* mannitol, 5 m*M* EDTA, 5 m*M* EGTA, 10 m*M* NaF, made to pH 6.5 with NaOH), constituting the nodule microsomal fraction (NMF). To purify the symbiosomes, the resultant pellet obtained at 10 000 *g* of nodule homogenate was resuspended in 4 ml washing buffer (350 m*M* mannitol, 3 m*M* $MgSO_4$, 10 m*M* EGTA, 25 m*M* MES, 10 m*M* NaF, made to pH 7.0 with BTP), referred to as nodule symbiosomal fraction (NSF). The aqueous polymer two-phase system (APS) was used (final concentration: 6.3% w/w dextran T500, 6.3% w/w poly(ethylene glycol) 3350, 5 m*M* KH_2PO_4/K_2HPO_4, 5 m*M* KCl, 0.33 *M* sucrose, pH 7.8), by mixing 3 g of NSF to 9 g of preweighed APS components to give a final weight of 12 g. The mixture was shaken gently and centrifuged at 1000 *g* for 10 min (first stage). Symbio-

somes, which appeared stacked at the interface, were collected with a Pasteur pipette and the procedure repeated twice with new APS (stages 2 and 3). The interface and the first stage nodule lower phase (NLP) were diluted with washing buffer. Symbiosomes and NLP components were precipitated at 10 000 *g* for 15 min, and the resulting pellets resuspended in 1.5 ml ATPase buffer and stored at −75°C for further analysis.

2.3. Extraction and purification of plasma membrane fractions

Root plasma membranes (RPM) were prepared from roots free of nodules (20–40 g), homogenized in 30 ml extraction buffer (50 m*M* HEPES, 500 m*M* sucrose, 1 m*M* DTT, 5 m*M* ascorbic acid, 0.6% Polyclar AT PVPP, at pH 7.5), plus 60 ml freshly added anti-protease cocktail. The homogenate was sieved and the filtrate subjected to a differential centrifugation, first at 10 000 *g* for 15 min (pellet discarded), and then at 100 000 *g* for 30 min. The resultant pellet was resuspended in 4 ml resuspension buffer (330 m*M* sucrose, 10 m*M* NaF, 5 m*M* K_2HPO_4/KH_2PO_4, at pH 7.8) constituting the root microsomal fraction (RMF). These and all subsequent procedures were done at 4°C, except where stated otherwise.

The aqueous polymer two-phase system for plasma membrane purification was optimised using a 'polymer series', as described above [12]. The APS technique utilises mixtures of polymers, dextran T500 and poly(ethylene glycol) PEG 3350 prepared as aqueous solutions on a weight per weight basis (see Section 3). Resulting fractions, the upper (root plasmalemma vesicles RPM) and the lower phase (root cell endomembranes REM) were diluted with resuspension buffer and centrifuged at 100 000 *g* for 30 min. Pellets were resuspended in 1.5 ml ATPase buffer (5 m*M* PIPES, 330 m*M* sucrose, 5 m*M* EGTA, 5m*M* EDTA, 10m*M* NaF made to pH 6.5 with NaOH).

2.4. Nucleoside polyphosphate hydrolytic and another enzyme marker activities

Samples were diluted (final content of 5 to 50 mg protein) in 500 ml of the assay medium, composed of 3 m*M* $MgSO_4$, 0.01% (v/v) Triton X-100, 100 m*M* $Na_2MoO_4 \cdot 2H_2O$ and 40 m*M* Tris made to pH 6.5 with MES, unless stated otherwise, and preincubated for 2 min at 37°C. The reaction was started by adding the phosphoryl-nucleoside substrate-usually ATP-(3 m*M*). After incubation for 15 min at 37°C, the reaction was stopped with 1 ml of a 'stopping reagent' (0.5% w/v SDS, 0.5% w/v $NH_4MoO_4 \cdot 7H_2O$ and 2% v/v H_2SO_4), according to Ref. [16]. The colour of the phosphomolybdate complex was developed by adding 20 ml of 10% (w/v) ascorbic acid, allowed to develop for 10 min and the absorbance read at 750 nm.

The determination of cytochrome *c* oxidase activity reported by [17] was used with minor modifications [13]. The NAD(P)H-cytochrome *c* reductase activity was determined after Ref. [18].

2.5. SDS–PAGE of membrane proteins

Membrane proteins were precipitated with chloroform to avoid their lysis during sample handling in a screw-cap Eppendorf tube. Briefly, aliquots containing 0.2 ml of sample (diluted to appropriate protein concentration) were thoroughly mixed in sequence with 0.8 ml methanol, 0.4 ml chloroform and 0.4 ml water. The emulsion was centrifuged at 5000 *g* for 5 min. At this stage, proteins were located at the interface; this and the solvent phase were washed twice with 0.4 ml methanol–water (1:2, v/v) to remove excess salt and sucrose. Methanol (0.4 ml) was added to disrupt aqueous-solvent partitioning, and protein was pelleted at 5000 *g* for 5 min. The supernatant was discharged and washed twice with 50 m*M* HEPES/KOH buffer (pH 7.5). The protein pellet was resuspended in 0.2 ml of the buffer described above and diluted with Laemmli sample buffer (final concentration: 400 m*M* Tris-HCl, 40% v/v glycerol, 4% w/v SDS, 4 m*M* bromophenol blue and 20% v/v β-mercaptoethanol, pH 6.8).

Proteins were separated in denaturing conditions by polyacrylamide gel electrophoresis (10% acrylamide) as described in [19].

2.6. Western-blot immunoanalysis

Protein polyacrylamide gels were semi-dry blotted onto a nitrocellulose membrane (Schleicher and

Schuell, Dassel, Germany). Both gel and membrane were placed between nine sheets of 3MM paper (Whatman, UK) soaked in blotting buffer (48 m*M* Tris, 39 m*M* glycine, 1.3 m*M* SDS, 20% v/v methanol, pH 8.4) in a semi-dry electroblotter (Sartorius, Goettingen, Germany) for 1 h at 200 mA continuous current.

For immunoanalysis, the membrane was soaked in deionized H_2O and in TBS (50 m*M* Tris, 200 m*M* NaCl, adjusted to pH 7.4 with HCl) for 5 min. After 2 h of blocking in TBS plus 3% (w/v) bovine serum albumin, the membrane was incubated overnight at 4°C with the primary polyclonal antibodies diluted 1000 to 2000 fold. Polyclonal antiserum which recognised H^+-ATPase from plasma membrane and tonoplast were obtained from Prof. R. Serrano and from Dr. R. Ratajczak, respectively. Immunostaining was visualised using an IgG goat-anti-rabbit second antibody conjugated with peroxidase (Sigma, St. Louis, MO USA). Peroxidase activity was assayed by adding a solution of 3 mg ml^{-1} 4-chloro-1-naphthol in methanol plus 5 volumes of TBS and 0.01% (v/v) H_2O_2.

2.7. Protein measurement

The Bio Rad Coomassie-blue assay reagent was used, with thyroglobulin as standard [20].

2.8. Data analysis

Results are the means of at least eight replicate samples. When required, significance of data was determined by using an analysis of variance (ANOVA) integrated in the SAS 6.0 statistical analysis software package. The significance level was fixed for $P<0.05$.

3. Results

3.1. Isolation and purification of plasmalemma microvesicles of pea root tissues by an aqueous two-phase system of polymers

In order to compare the properties of both ATP hydrolase activities from RPM vesicles and from intact symbiosomes, it was first necessary to optimise the APS for the obtention of RPM vesicles in same way as we previously done for symbiosomes [13]. RPM vesicles were isolated by loading 3 ml of the microsomal fraction (RMF) onto a 'polymer-series' with variable percentage of dextran T500 and PEG 3350 (5.5–6.6% w/w; and, 330 m*M* sucrose, 5 m*M* K_2HPO_4 and 4 m*M* KCl at pH 7.8). After a purification procedure following Larsson et al. [12], we compared protein content and ATPase activity of the obtained fractions (upper phase, containing RPM vesicles) (Table 1). According to these data and in order to reach high enzyme activity and yield and to avoid enzyme inactivations we selected the following APS composition: 6.2% T500 dextran and 6.2% 3350 PEG maintaining the rest of components.

Both, the upper (RPM) and lower phases (root endomembranes fraction, REM) were diluted with resuspension buffer and centrifuged at 100 000 *g* for 30 min. The pellets were resuspended in 1.5 ml of ATPase buffer and stored at −75°C.

3.2. Marker enzyme activities of pea root plasmalemma vesicles and pea nodule symbiosomes

Table 2 shows marker enzyme activities of root membrane fractions. Root microsomal fraction (RMF), root plasma membrane (RPM) and root endomembranes (REM) fractions were analysed. The enzyme activity associated with soluble acid phosphatases (molybdate-sensitive ATPase) was similar in all fractions, indicating the absence of this kind of contamination. Vanadate-sensitive ATPase (marker for plasma membrane) was largest in RPM (almost 60% inhibition), whereas in RMF and REM plasma membrane-derived vesicles represented a much lesser proportion. Tonoplast marker (nitrate-sensitive

Table 1
Polymer series prepared to determine the optimum concentration of dextran T-500 and PEG for the aqueous polymer two-phase system for plasma membrane purification

Polymer (%)		ATPase activity (μmol Pi mg^{-1} prot. h^{-1})	Protein (mg ml^{-1})
Dextran	PEG		
6.0	6.0	22.89	4.86
6.2	6.2	28.00	4.50
6.4	6.4	31.00	2.22
6.6	6.6	20.00	3.42

Table 2
Marker enzyme activities of root membranes

Fraction	ATPase activities[a]						Cyt *c* oxidase	NADH cyt *c* reductase	Protein
	Basal	+Molybdate	+Vanadate	+Potassium	+Nitrate	+Azide			
RMF	18.5±0.9 (100)	20.1±0.7 (108)	15.5±1.7 (84)	18.0±2.4 (97)	17.7±1.2 (95)	13.3±2.0 (72)	0.69±0.08	0.09±0.03	0.12±0.01
RPM	28.2±1.4 (100)	28.3±1.4 (100)	12.0±0.7 (43)	29.0±1.9 (103)	32.3±1.6 (115)	22.9±1.5 (81)	N.D.	N.D.	0.01±N.D.
REM	18.5±2.4 (100)	18.6±2.7 (101)	16.7±1.6 (90)	16.5±1.6 (89)	13.3±1.3 (72)	9.2±0.8 (50)	0.38±0.06	N.D.	0.10±0.01

Marker enzyme activities in root microsomal fraction (RMF), root plasma membrane (RPM) and root endomembranes (REM). Soluble phosphatase activity (+100 μ*M* $Na_2MoO_4 \cdot 2H_2O$), P-type ATPase activity (+300 μ*M* Na_3VO_4), stimulation by potassium (+50 m*M* KCl), V-type ATPase activity (+50 m*M* KNO_3), and F_0F_1-type ATPase activity (+100 μ*M* NaN_3) were included in the reaction media buffer of the basal ATPase assay. Cytochrome *c* oxidase and NADH-cytochrome *c* reductase activities were determined as mitochondria and ER markers respectively ($n=8$).

[a]ATPase activity was expressed as μmol P_i mg^{-1} protein h^{-1}, cyt *c* oxidase and NADH cyt *c* reductase as μmol cyt *c* mg^{-1} protein h^{-1}, and content of protein expressed as mg g^{-1} fresh weight root. Values in parentheses represent the activity of ATPase markers vs basal activity. N.D.=not detected.

ATPase) was largest in REM (approximately 30% inhibition), but little or no contamination was observed in RPM. Similarly, azide-sensitive ATPase (marker of mitochondria) was much lower in RPM than in REM, in agreement with data of cytochrome *c* oxidase (also a specific marker for mitochondria, which was not detected in RPM. NADH-cytochrome *c* reductase (an ER marker) was only detected in RMF, probably due to the limited sensitivity of the method used. Finally, the presence of K^+ in the reaction medium did not modify the Mg^{2+}-ATPase activity of any of the fractions tested. On the basis of their relative protein concentrations, it is concluded that RPM was enriched 12 times from the RMF, and contained little contamination from other membrane sources.

Table 3 shows marker enzyme activities from nodule membrane fractions. Nodule symbiosomal (NSF), symbiosome (SY) and nodule lower-phase (NLP) fractions were analysed. Molybdate-sensitive ATPase activity (acid-phosphatases) was similar in all fractions. Vanadate-sensitive ATPase activity (plasma membrane type ATPase) was largest in SY (almost 50% inhibition), whereas in NSF and NLP this inhibition was less evident, indicating a lesser presence of P-type ATPase. Nitrate-sensitive ATPase activity (tonoplast-like ATPase) represented approximately 20% in SY, and no effect was observed in other fractions. Azide-sensitive ATPase activity (mitochondria) was low in SY (ca. 20% inhibition), in agreement with data of cytochrome *c* oxidase (also a specific marker for mitochondria and broken

Table 3
Marker enzyme activities of nodule membranes

Fraction	ATPase activities[a]						Cyt *c* oxidase	NADH cyt *c* reductase	Protein
	Basal	+Molybdate	+Vanadate	+Potassium	+Nitrate	+Azide			
NSF	8.0±1.3 (100)	7.9±1.3 (99)	7.1±0.9 (89)	7.9±1.4 (100)	7.3±1.5 (92)	7.8±1.3 (98)	1.6±0.4	0.9±0.2	2.5±0.6
SY	16.0±1.4 (100)	16.3±1.1 (102)	9.0±0.8 (56)	15.8±0.9 (98)	12.0±0.8 (75)	12.5±1.2 (78)	0.7±0.1	0.2±0.1	1.1±0.5
NLP	2.3±0.7 (100)	1.8±0.4 (76)	2.2±0.4 (93)	2.3±0.7 (100)	2.5±0.5 (105)	N.D. (0)	2.5±0.6	0.8±0.1	0.4±0.1

Marker enzyme activities in nodule symbiosomal fraction (NSF), symbiosomes (SY) and nodule lower phase partitioned components (NLP). Soluble phosphatase activity (+100 μ*M* $Na_2MoO_4 \cdot 2H_2O$), P-type ATPase activity (+300 μ*M* Na_3VO_4), stimulation by potassium (+50 m*M* KCl), V-type ATPase activity (+50 m*M* KNO_3), and F_0F_1-type ATPase activity (+100 μ*M* NaN_3) were included in the reaction media buffer of the basal ATPase assay. Cytochrome *c* oxidase and NADH-cytochrome *c* reductase activities were determined as mitochondria and ER markers respectively ($n=8$).

[a]ATPase activity was expressed as μmol P_i mg^{-1} protein h^{-1}, cyt *c* oxidase and NADH cyt *c* reductase as μmol cyt *c* mg^{-1} protein h^{-1}, and content of protein expressed as mg g^{-1} fresh weight nodule. Values in parentheses represent the activity of ATPase markers vs basal activity. N.D.=not detected.

symbiosomes). NLP contained the largest cytochrome *c* oxidase activity and the presence of azide inhibited completely the Mg^{2+}-ATP hydrolysis. These results may indicate that either mitochondria or broken bacteroids (or both) were effectively separated from the symbiosomes and were mostly found in the lower phase of the APS. NADH-cytochrome *c* reductase activity (an ER marker) was detected in NLP at similar levels to those observed in NSF, but activity in SY was much lower. These results are essentially in agreement with our previous data [13]. The symbiosome fraction was relatively free of cross-contamination by other membrane or organelle sources, which contained most of the protein content determined in NSF (ca. 50%).

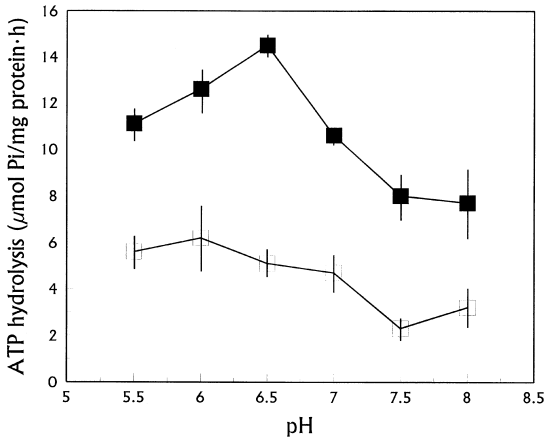

Fig. 1. Influence of the reaction media pH on pea root plasma membrane (■) and symbiosomes (□) ATP hydrolysis.

3.3. Effect of reaction medium pH on Mg^{2+}-dependent ATP hydrolytic activity

The Mg^{2+}-ATPase activity of RPM increased concomitant with pH up to 6.5, where it reached the maximum. Above this point, the hydrolase activity decreased sharply as pH increased further (Fig. 1). On the other hand, symbiosome Mg^{2+}-ATPase was higher at acid pH (5.5 to 6.5) decreasing subsequently (Fig. 1). Therefore, symbiosome ATPase resembled that of RPM, but was less affected than the later by the reaction medium pH.

3.4. Substrate specifity of pea root plasma membrane and pea nodule symbiosome ATPase activity

Several phosphorylated substrates were tested to determine the specifity of the Mg^{2+}-ATP hydrolase activity of RPM and symbiosomes. The largest hydrolysis (measured as inorganic phosphate production) was obtained in both fractions when ATP was used as substrate (Tables 4 and 5). Adenosine di-phosphate (ADP) and inosine tri-phosphate (ITP) were hydrolysed only ca. 20%, compared to that of

Table 4
Substrate specifity of RPM hydrolase

Substrate	Hydrolytic activity[a]		% Inhibition
	Basal	+300 μ*M* Na_3VO_4	
ATP	14.9±1.3 (100)	6.4±0.5	57
CTP	1.8±0.4 (12)	1.1±0.2	39
ITP	2.5±0.9 (17)	2.0±0.4	20
GTP	1.0±0.2 (6)	0.8±0.1	20
ADP	2.7±1.2 (18)	2.4±0.7	13
IDP	N.D.	N.D.	–
AMP	N.D.	N.D.	–
Glucose-6P	N.D.	N.D.	–

Hydrolysis of phosphorylated substrates by pea root plasma membrane. P-type ATPase activity was determined in the absence and in the presence of vanadate.

[a]ATPase activity was expressed as μmol P_i mg^{-1} protein h^{-1}. Values in parentheses represent the percentage of substrate hydrolysis relative to that of ATP.

N.D.=not detected.

Table 5
Substrate specifity of symbiosome hydrolase

Substrate	Hydrolytic activity[a]		% Inhibition
	Basal	+300 μ*M* Na_3VO_4	
ATP	11.5±1.3 (100)	5.7±1.3	50
CTP	N.D.	N.D.	–
ITP	2.6±0.9 (22)	2.4±1.5	5
GTP	1.2±0.3 (10)	0.9±0.2	24
ADP	2.4±1.1 (21)	1.7±0.8	29
IDP	N.D.	N.D.	–
AMP	N.D.	N.D.	–
Glucose-6P	N.D.	N.D.	–

Hydrolysis of phosphorylated substrates by pea nodule symbiosomes. P-type ATPase activity was determined in the absence and in the presence of vanadate.
[a]ATPase activity was expressed as μmol P_i mg^{-1} protein h^{-1}. Values in parentheses represent the percentage of substrate hydrolysis relative to that of ATP.
N.D.=not detected.

ATP, by the RPM and symbiosome enzyme. There was also a very low hydrolase activity (approximately 10%) when cytosine tri-phosphate (CTP) and guanosine tri-phosphate (GTP) were tested with RPM. Similar activity was determined for GTP by the symbiosome hydrolase, and no activity was detected when CTP was used as substrate. The rest of the phosphorylated substrates tested, i.e. inosine di-phosphate (IDP), adenosine mono-phosphate (AMP) and glucose-6 phosphate, were not hydrolysed by RPM nor by symbiosomes.

3.5. Effect of inhibitors on Mg^{2+}-dependent ATP hydrolysis

The hydrolysis of ATP was determined in the presence of several known inhibitors of plant plasma membrane ATPases. Increasing concentration of vanadate caused a sharp inhibition of RPM and symbiosome ATP hydrolysis (Fig. 2A and Fig. 3A). In symbiosomes this effect was less evident; above 50 μ*M* VO_4^{3-} an inhibition of ca. 50% was achieved, but it did not increase at higher concentrations of vanadate (Fig. 3A). ATP hydrolysis decreased in RPM up to 300 μ*M* VO_4^{3-} (ca. 60%), after which it did not decrease further at larger concentrations of vanadate (Fig. 2A).

The presence of azide and nitrate did not provoke substantial modifications in the hydrolysis of ATP by

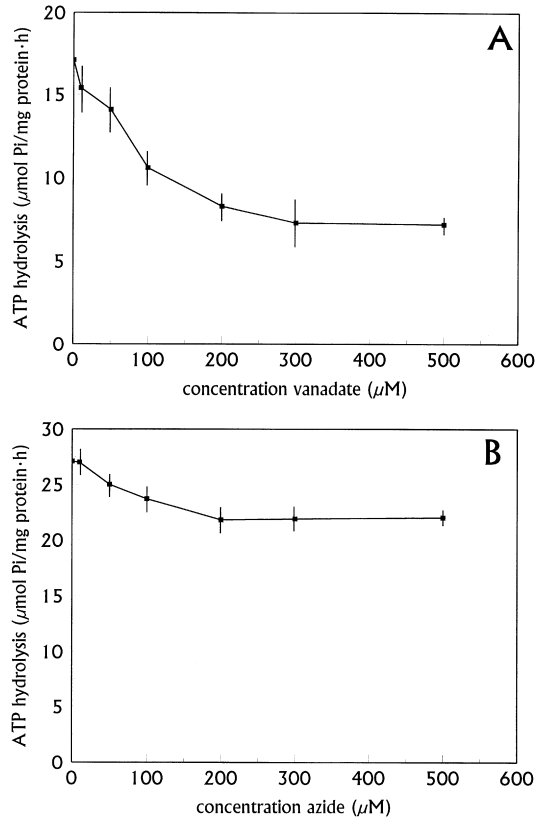

Fig. 2. Modification of pea root plasma membrane Mg^{2+}-ATPase activity by increasing concentrations of vanadate (A) and azide (B).

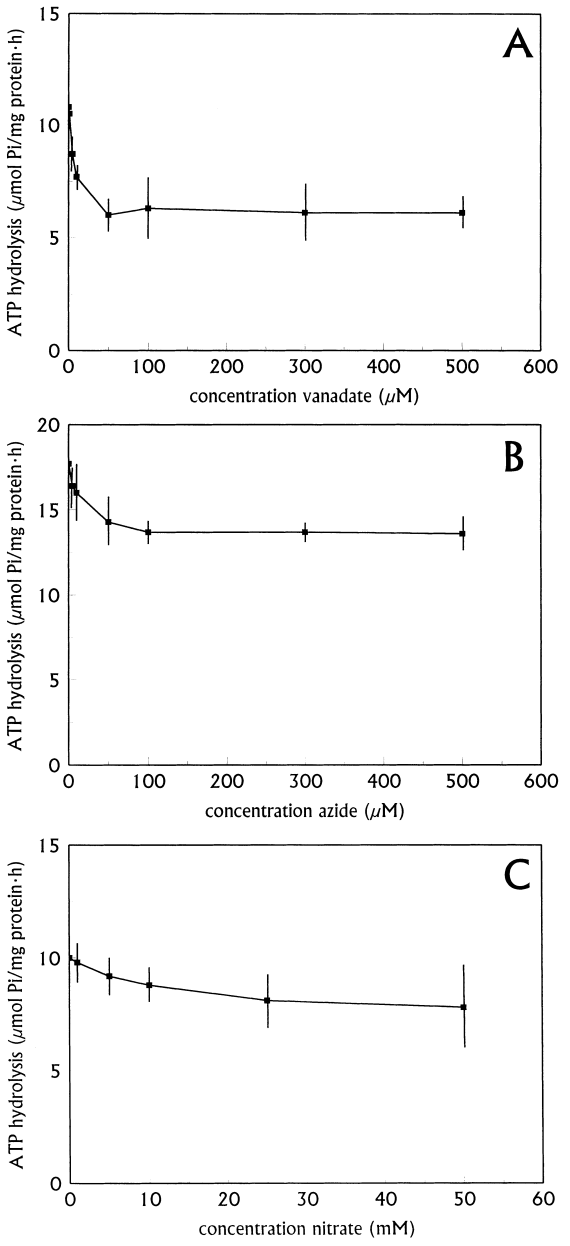

Fig. 3. Modification of pea nodule symbiosome Mg^{2+}-ATPase activity by increasing concentrations of vanadate (A), azide (B) and nitrate (C).

the RPM vesicles (Fig. 3B) nor by the symbiosomes (Fig. 3B and Fig. 3C), in agreement with the results described above.

The apparent kinetic parameters (K_m and V_{max}) of the ATP hydrolysis by RPM vesicles and the symbiosomes were determined in the presence and absence of vanadate (300 μ*M*). ATP hydrolysis by RPM vesicles and symbiosomes showed typical Michaelis–Menten kinetics at increasing ATP concentrations (0.25–4 m*M*, Fig. 4). Addition of VO_4^{3-} caused the inhibition of ATP hydrolysis, reaching similar values as described above. The determination of the kinetic parameters showed that K_m values were similar in presence and absence of VO_4^{3-} (there were no significant differences at $P<0.05$), but V_{max} was significantly lower when VO_4^{3-} was added (Table 6).

3.6. Western-immunoanalysis of pea root and nodule membrane fractions

Symbiosomes, nodule microsomal fraction (NMF), RMF, RPM, REM and free living bacteroids

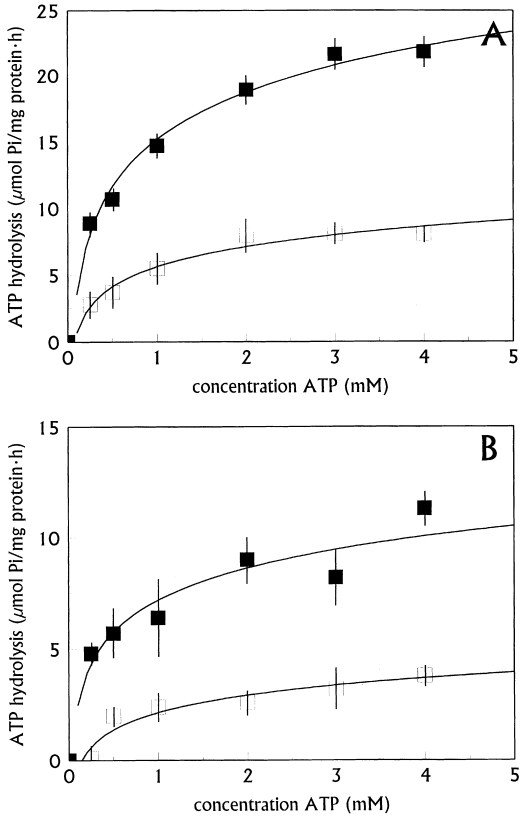

Fig. 4. Mg^{2+}-ATPase inhibition by vanadate (300 μ*M*) in increasing concentrations of ATP of pea root plasma membrane (A) and pea nodule symbiosomes (B). Basal activity (■) and plus VO_4^{3-} (□).

Table 6
Kinetic parameters of ATPase activity from RPM vesicles and symbiosomes

Marker	K_m		V_{max}	
	RPM	SY	RPM	SY
Basal	0.4±0.1 a	0.3±0.1 a	22.0±1.2 a	9.0±0.6 a
$+Na_3VO_4$	0.5±0.2 a	0.4±0.2 a	8.4±1.5 b	3.4±0.5 b

Apparent K_m (m*M*) and V_{max} (μmol P_i mg^{-1} protein h^{-1}) values for pea root plasma membrane (RPM) vesicles and symbiosomes (SY) Mg^{2+}-ATPase activity in the absence and presence of vanadate (300 μ*M*).
Different letters indicate significant differences at $P<0.05$.

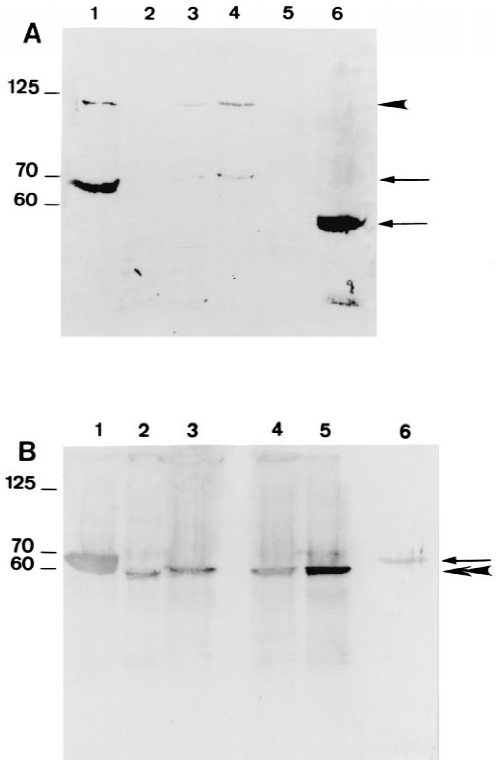

Fig. 5. Western-immunoblot analysis of pea root and nodule membrane fractions using polyclonal antiserum raised against plasma membrane H^+-ATPase (A) and anti-vacuolar H^+-ATPase (B). Lanes: 1, symbiosomes; 2, nodule microsomal fraction; 3, root microsomal fraction; 4, root plasma membranes; 5, root endomembranes; and 6, free-living bacteria homogenate. Positions of putative P-type and V-type ATPase antigens are marked by solid arrowhead and double-arrowhead respectively. Positions of irrelevant antigen bands are marked with thin arrows.

were subjected to protein PAGE separation and western-blotting immuno-analysis with rabbit antiserum prepared against purified plant plasma membrane H^+-ATPase and vacuolar H^+-ATPase (Fig. 5). The results indicate that a faint band of ca. 100 kDa was recognised in the symbiosome and RPM fractions by polyclonal anti-plasma membrane ATPase (Lanes 1 and 4, solid arrowhead, Fig. 5A). Other strong antigen bands of lower molecular weight (less than 70 kDa, thin arrows) were also detected, but these bands also reacted with preimmune serum (data not shown), and were therefore presumed to be irrelevant.

When the protein blot was probed with polyclonal anti-vacuolar ATPase, an antigen band characteristic of the 60 kDa subunit was visualized in NMF, RMF, RPM and REM (Lanes 2–5, solid double-arrowhead, Fig. 5B, respectively). The presence of some V-type ATPase in the RPM could be due to cross-contamination but it was, in any case, a minor component compared with the REM. In Lanes 1 and 6, there was a band of high intensity of a slightly larger molecular weight (ca. 65 kDa, thin arrow), which also reacted with preinmune serum (data not shown).

4. Discussion

The lack of contamination of pea symbiosomes with ER, mitochondria, broken bacteroids and/or tonoplast vesicles was established (Table 3), in agreement with our previous work [13]. Similarly, the aqueous two-phase system used here provided a fairly enriched RPM fraction with low cross-contamination from other sources (Table 2).

The effect of pH upon H^+-ATPase of RPM has been shown by different authors to have a pH optimum of between 6.0 and 6.5 [6,10,21], in agreement with our results, where pea RPM ATPase showed a clear optimum at pH 6.5 (Fig. 1). In contrast to this unanimity, the PBM H^+-ATPase has been reported to have a pH optimum of 5.25 [22], 6.3 [10], 6.5 [6,9] or 6.0 in the presence of K^+ and 8.0 without K^+ [11]. Our results indicate that the PBM ATPase had a similar activity at pH values ranging from 5.5 to 7.0, with a slight increase at pH 6.0 (Fig. 1). Over pH 7.0 a reduction in activity was observed, similar to that found in RPM ATPase.

Vanadate has been described as a powerful inhib-

itor of P-type ATPases [16,23]. On the basis of vanadate inhibition, our results with PBM ATPase (Table 3) agree with those of other workers [9,10,24] and indicate that the symbiosome ATPase resembles an E_1E_2-ATPase mechanism (i.e. a P-type ATPase), similar to that found at the plasma membrane. The apparent kinetic parameters indicated that VO_4^{3-} inhibition was of a non-competitive class (Table 6), meaning that vanadate binds to the enzyme in a site that affects catalytic activity rather than substrate binding. This was completely in agreement with data presented for PBM ATPase of lupin symbiosomes [22]. The RPM Mg^{2+}-ATPase behaved identically, and it seems that vanadate blocks the hydrolysis of the phosphorylated intermediate, but not the formation of the substrate–enzyme complex, inhibiting the transition from the ATPase E_1 conformation to the E_2 state [25,26].

The similarities between pea PBM–ATPase and RPM–ATPase were further supported by the immuno-analysis with the polyclonal antiserum against plant plasma membrane H^+-ATPase (Lanes 1 and 4, solid arrowhead, Fig. 5A). In both fractions, a band of 100 kDa was recognised, characteristic of plant plasmalemma H^+-ATPase.

The presence of increasing concentration of azide did not alter the ATPase activity of RPM and PBM (Fig. 2B and Fig. 3B). These results are in agreement with those of Domingan et al. [22], who found no significant contamination of F_1F_0-ATPase, when measuring H^+-ATPase activity of lupin PBM. Similarly, Udvardi and Day [9] and Christiansen et al. [24] reported no change of PBM H^+-ATPase activity from soybean and pea nodules after addition of 100 μM azide.

PBM pea symbiosome Mg^{2+}-ATPase was not affected by the presence of NO_3^- in the reaction medium (Table 3, Fig. 3C), in agreement with results of siratro [6], soybean [9], and lupin [22] PBM ATPase activity. Christiansen et al. [24] reported that ATPase activity of isolated PBM from pea nodules was not affected by its exposure to a concentration of NO_3^- similar to the one used here. A similar pattern was also observed in pea RPM (Table 2, data not shown). This suggests that a vacuolar-type ATPase activity is not present in the pea PBM. These results are strongly supported by the immunoanalysis data using a polyclonal antibody against vacuolar type ATPase. The antibody strongly recognises the 60 kDa band of the hetero-trimeric vacuolar ATPase. A similar band was clearly observed in all plant material but the symbiosomes (solid double-arrowhead, Fig. 5B).

Potassium stimulation of PBM H^+-ATPase activity has been found by a number of workers [10,11,22]. We did not observe K^+ stimulation of the ATPase activity (Table 3). These results are in agreement with our previous work [13]. Similarly, RPM ATPase did not respond to the presence K^+. The mechanism of stimulation of H^+-ATPase by K^+ is not well understood, because it differs between plant species and growing conditions. Consequently, a number of different roles for K^+ have been described [21].

Pea PBM–ATPase was highly specific for ATP (Table 5). These results were in agreement with those reported for lupin PBM, where other phosphorylated substrates were not hydrolysed [22,27]. Similarly, RPM ATPase reacted specifically with ATP, other substrates being much less hydrolysed, in agreement with results reported for rice RPM [28]. The presence of vanadate in the reaction medium caused an inhibition of ATP hydrolysis between 50 to 60% in RPM and symbiosomes (Tables 4 and 5). The hydrolysis of CTP by RPM was inhibited to a lower extent (ca. 40%, Table 4). Hydrolysis of other substrates was inhibited less than 30%, indicating that no phosphorylated intermediate, characteristic of plasma membrane H^+-ATPase, was formed when these phosphorylated substrates were assayed with RPM and symbiosomes (Tables 4 and 5).

In conclusion, biochemical and immunochemical analysis suggest that pea PBM Mg^{2+}-ATPase was very similar to that found in pea RPM. Therefore, infected host cells probably extrude protons towards the symbiosome fluid. It is possible that a strict control over the pH environment (slightly acidic) in the symbiosome compartment is needed for an efficient N_2-fixation [8]. It is clear that the presence of a higher concentration of H^+ could influence the efficiency of biological N_2-fixation by modifying the activity of acid proteases, the equilibrium between ammonia and ammonium ions or the transport of dicarboxylates in the symbiosome. Further work is needed to understand how these regulatory systems might operate [4].

5. Abbreviations

APS	aqueous two-phase system
DTT	dithiothreitol
EDTA	ethylene diaminetetraacetic acid
EGTA	ethyleneglycol-*bis*-(β-aminoethyl ether) *N*,*N*,*N′*,*N′*-tetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
MES	2-(morpholino)ethanesulfonic acid
PIPES	1,4-piperazine-di-ethanesulfonic acid
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
PBM	peribacteroid membrane
RPM	root plasma membrane
TBS	Tris saline buffer.

Acknowledgements

This work was funded by the Spanish DGCyT through the project PB95-0217-C02-02 and by the EU Biotech Programme through project CHRX CR940699. Authors are indebted to Dr B.K. Drøbak (JIC, Norwich, UK) for general discussion, and for technical advice and assistance. Polyclonal antiserum prepared against plasma membrane H^+-ATPase and tonoplast H^+-ATPase were the kind gift of Prof. R. Serrano (Universidad Politécnica, Valencia, Spain) and of Dr R. Ratajczak (Technische Hochschule, Darmstadt, Germany), respectively. We also gratefully thank Dr P. Bonay (CBM-UAM, Madrid, Spain) for his technical support, essential to produce this work.

References

[1] N.J. Brewin, Ann. Review Cell Biol. 7 (1991) 191.
[2] L.E. Roth, G. Stacey, Eur. J. Cell Biol. 49 (1989) 13.
[3] J.G. Robertson, B. Wells, N.J. Brewin, E. Wood, C.D. Knight, J.A. Downie, J. Cell Sci. Suppl. 2 (1985) 317.
[4] M.K. Udvardi, D.A. Day, Ann. Rev. Plant Physiol. Plant Mol. Biol. 48 (1997) 493.
[5] D.A. Day, G.D. Price, M.K. Udvardi, Aust. J. Plant Physiol. 16 (1989) 69.
[6] L.J. Ouyang, D.A. Day, Plant Physiol. Biochem. 30 (1992) 613.
[7] S.D. Tyerman, L.W. Whitehead, D.A. Day, Nature 378 (1995) 629.
[8] M.M. Szafran, H. Haaker, Plant Physiol. 108 (1995) 1227.
[9] M.K. Udvardi, D.A. Day, Plant Physiol. 90 (1989) 982.
[10] E. Blumwald, M.G. Fortin, P.A. Rea, D.P.S. Verma, R.J. Poole, Plant Physiol. 78 (1985) 665.
[11] S. Bassarab, R.B. Mellor, D. Werner, Endocyt. Cell Res. 3 (1986) 189.
[12] C. Larsson, S. Widell, P. Kjelbom, Methods Enzymol. 148 (1987) 558.
[13] L.E. Hernández, P. Rojas-Ojeda, D.T. Cooke, R.O. Carpena-Ruiz, J. Chromatogr. B 680 (1996) 171.
[14] S. Bassarab, S.U. Schenk, D. Werner, Bot. Acta 102 (1989) 196.
[15] J.G. Robertson, P. Lyttleton, S. Bullivant, G.F. Grayston, J. Cell Sci. 30 (1978) 129.
[16] R. Serrano, Biochim. Biophys. Acta 947 (1988) 1.
[17] S.J. Cooperstein, A. Lazarow, J. Biol. Chem. 189 (1951) 665.
[18] J.M. Lord, T. Kagawa, T.S. Moore, H. Beevers, J. Cell Biol. 57 (1973) 659.
[19] U.K. Laemmli, Nature 227 (1970) 680.
[20] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[21] J.P. Grouzis, R. Gibrat, J. Rigaud, A. Ageorges, C. Grignon, Plant Physiol. 93 (1990) 1175.
[22] N.M. Domingan, K.J.F. Farnden, J.G. Robertson, B.C. Monk, Arch. Biochem. Biophys. 264 (1988) 564.
[23] S.R. Gallagher, R.T. Leonard, Plant Physiol. 70 (1982) 1335.
[24] J.H. Christiansen, L. Rosendahl, S. Widell, J. Plant Physiol. 147 (1995) 175.
[25] R. Scalla, A. Amory, J. Rigaud, A. Goffeau, Eur. J. Biochem. 132 (1983) 525.
[26] F. Vara, R. Serrano, J. Biol. Chem. 258 (1983) 5334.
[27] I. Andreev, P. Dubrovo, V. Kryslova, I.N. Andreeva, V. Koren'kov, E.M. Sorokin, S.F. Izmailov, J. Plant Physiol. 151 (1997) 563.
[28] R. Ros, A. Sanz, J. Segura, I. Picazo, Physiol. Plant. 83 (1991) 75.