Plant Physiol. (1995) 109: 285–292

The Tonoplast H^+-ATPase of *Acer pseudoplatanus* Is a Vacuolar-Type ATPase That Operates with a Phosphoenzyme Intermediate

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The tonoplast H^+-ATPase of *Acer pseudoplatanus* has been purified from isolated vacuoles. After solubilization, the purification procedure included size-exclusion and ion-exchange chromatography. The H^+-ATPase consists of at least eight subunits, of 95, 66, 56, 54, 40, 38, 31, and 16 kD, that did not cross-react with polyclonal antibodies raised to the plasmalemma ATPase of *Arabidopsis thaliana*. The 66-kD polypeptide cross-reacted with monoclonal antibodies raised to the 70-kD subunit of the vacuolar H^+-ATPase of oat roots. The functional molecular size of the tonoplast H^+-ATPase, analyzed in situ by radiation inactivation, was found to be around 400 kD. The 66-kD subunit of the tonoplast H^+-ATPase was rapidly phosphorylated by [γ-^{32}P]ATP in vitro. The complete loss of radioactivity in the 66-kD subunit after a short pulse-chase experiment with unlabeled ATP reflected a rapid turnover, which characterizes a phosphorylated intermediate. Phosphoenzyme formed from ATP is an acylphosphate-type compound as shown by its sensitivity to hydroxylamine and alkaline pH. These results lead us to suggest that the tonoplast H^+-ATPase of *A. pseudoplatanus* is a vacuolar-type ATPase that could operate with a plasmalemma-type ATPase catalytic mechanism.

The ion motive ATPases discovered to date are divided into three major categories designated as "P," "F," and "V" (Pedersen and Carafoli, 1987a). The P-type ATPases have been identified in fungi, eubacteria, plant, and animal plasma membranes. They are inhibited by vanadate and form an acylphosphate intermediate during ATP hydrolysis (Goffeau and Slayman, 1981). The P-type ATPases are usually composed of a single catalytic polypeptide of about 100 kD and probably function as dimers in the direction of ATP hydrolysis (Nelson and Taiz, 1989).

Members of the second category, F-type ATPases, are present in the plasma membrane of respiring bacteria and on the inner membrane of mitochondria and chloroplasts, where they function as ATP synthases. They are inhibited by DCCD and azide. F-ATPases have a molecular mass of about 500 kD and a complex structure consisting of two multisubunit components, F1 and F0. F1 is a water-soluble complex made up of five subunits (α to ϵ), with the catalytic site on the β subunit. F0 is a membrane component made up of several subunits including multiple copies of an 8-kD proteolipid involved in H^+ translocation.

Members of the third category, V-type ATPases, are located in organelles connected with the vacuolar system of eukaryotic cells, including the vacuoles of plants, lysosomes, endosomes, clathrin-coated vesicles, chromaffin granules, and Golgi vesicles (Sze, 1985; Boller and Wiemken, 1986; Mellman et al., 1986). V-type ATPases are inhibited by DCCD, nitrate, and bafilomycin but are insensitive to azide and vanadate. They are usually large multimeric enzymes, with molecular masses of about 400 to 750 kD, constituted of a hydrophilic catalytic complex and an integral membrane moiety (Forgac, 1992; Sarafian et al., 1992). The hydrophilic moiety is composed of two major polypeptides of about 70 (A) and 60 kD (B) along with several smaller peptides. The membrane moiety is constituted of six copies of a proteolipid of 16 kD (Nelson and Taiz, 1989). V-ATPases couple the energy derived from ATP hydrolysis to proton transport. They usually operate without a phosphoenzyme intermediate and have been shown to undergo single-site catalysis similar to the F-ATPases (Uchida et al., 1988).

Recent works concerning the plasma membrane ATPase of several archaebacteria will probably lead to an extension of the classification of ATPases. Indeed, the archaebacterial ATPases resemble the eukaryotic V-ATPases more than the eubacterial F-ATPases (Gogarten et al., 1989a). For instance, (a) the ATPases of *Halobacterium halobium* and *Sulfolobus acidocaldarius* are insensitive to azide and inhibited by nitrate (Lübben et al., 1987; Mukohata et al., 1987); and (b) the sequences of the two major subunits, 64 and 54 kD, of the *S. acidocaldarius* ATPase were found to be 50% identical with the subunits A and B, respectively, of the eukaryotic vacuolar ATPase (Gogarten et al., 1989a). Intriguing results were also obtained with the archaebacteria *Methanococcus thermolithotrophicus*, whose plasma membrane

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; F-type, ATP synthases in mitochondria, chloroplasts, and bacteria; G6PDH, Glc-6-P dehydrogenase; P-type, plasmalemma-type; TDAB, tetradecyltrimethylammonium bromide; V-type, vacuolar-type.

ATPase is a V-type ATPase, the sequence of which contains the highly conserved aspartate residue of the P-type ATPase phosphorylation domain. Thus, despite belonging to the V-type class of proton pumps, the ATPase of *M. thermolithotrophicus*, like that of *Methanococcus voltae*, may have a phosphorylated intermediate (Gogarten et al., 1989b). Moreover, it has recently been reported that the tonoplast of barley contains a vanadate-sensitive ATPase involved in glutathione *S*-conjugate uptake (Martinoia et al., 1993) that is remarkably similar to the glutathione *S*-conjugate export pump in the canalicular membrane of mammalian liver (Kobayashi et al., 1990; Akerboom et al., 1991; Ishikawa, 1992)

We previously reported on the properties of the tonoplastic H^+-ATPase of *Acer pseudoplatanus* cells (Pugin et al., 1987, 1991; Pugin and Montrichard, 1988). The properties of ATP hydrolysis and ATP-dependent H^+-transport are similar to those of other known vacuolar ATPases (Nelson and Taiz, 1989). The enzyme has a pH optimum of 7.3, and it is stimulated by chloride and is inhibited by nitrate, thiol reagents, and DCCD. Nevertheless, the tonoplast H^+-ATPase of *A. pseudoplatanus* differs from most other vacuolar H^+-ATPases in its sensitivity (50% inhibitory concentration < 10 μM) to low concentrations of vanadate (Montrichard et al., 1989). This result led us to question the type of this tonoplast ATPase, which could be either a P-type ATPase or V-type ATPase with a P-type catalytic mechanism. This paper reports on the molecular mass, the purification, and the polypeptide composition of the vanadate-sensitive tonoplast ATPase of *A. pseudoplatanus*. We also demonstrate the formation of a phosphoenzyme intermediate during ATP hydrolysis.

MATERIALS AND METHODS

Plant Material

The strain of *Acer pseudoplatanus* cells that we used came from a culture of cambial tissue obtained by Lamport (1964). Cells were cultivated in shaken liquid medium (150 rpm, 26°C, constant lighting) and were routinely subcultured every 8 d as previously described (Pugin et al., 1988).

Tonoplast Preparation

Vacuoles were prepared from protoplasts as previously reported (Pugin et al., 1986). Isolated vacuoles were submitted to an osmotic shock in 30 mM Tris-Mes buffer (pH 7.3), 1 mM DTT, 5 mM $MgSO_4$, 1 mM PMSF, 5 μg mL^{-1} aprotinin, 1 μg mL^{-1} pepstatin, 2 μg mL^{-1} leupeptin, 0.25 M mannitol, final concentration. Tonoplasts were pelleted by centrifugation at 150,000*g* for 1 h (Beckman 70 Ti rotor). The supernatant was discarded, and the pellet was resuspended in the same buffer. A second centrifugation allowed the elimination of the soluble unspecific phosphatase activity. The pellet was homogenized in 3 mM Tris-Mes buffer (pH 7.0), 20% (w/v) glycerol, 1 mM DTT, 1 mM EDTA, 0.25 M mannitol and was stored at −80°C.

Radiation Inactivation

Target-size analysis by radiation inactivation is a well-established method to study structure-function relationships in biologically active macromolecules without prior purification or solubilization (Kepner and Macey, 1968; Jung, 1984). This method is well suited to hydrophobic proteins because with the usual methods for molecular mass determination, detergents used to solubilize these proteins lead to overestimated values. The decrease of the biological activity due to radiation exposure to γ rays is an exponential function of the absorbed dose and is related to target size or molecular mass. The loss in tonoplast ATPase activity was compared with that of the G6PDH that was used as an internal standard (Mcintyre and Churchill, 1985). A 5-mL mixture of tonoplast in glycerol buffer (500 μg tonoplast protein mL^{-1}) with G6PDH type XXIII from *Leuconostoc mesenteroides* (M_r = 104,000, Sigma) was distributed in 10 Eppendorf microcentrifuge tubes (0.5 mL per tube). The samples were flushed with nitrogen gas and placed (standing in a circle) on an aluminium rack immersed in a thermos full of dry ice. The thermos was placed in front of a ^{60}Co source (1500 Ci). The thermos was turned (2 rpm) to ensure isodoses for 10 samples. The irradiation lasted for 18 d. One sample was removed every 48 h and kept in dry ice. At the end of the experiment, the samples were thawed for ATPase and G6PDH assays. Enzyme activities did not change when samples were kept in dry ice for the duration of the experiment. The data were analyzed with the single-target, single-hit model of radiation inactivation (Kepner and Macey, 1968; Jung, 1984; Mcintyre and Churchill, 1985).

Membrane Solubilization

Tonoplast preparations were solubilized by the dropwise addition of Triton X-100 with constant stirring at 4°C. After a 30-min period of incubation, the mixture was centrifuged (150,000*g*, 45 min, 4°C, Beckman 70 Ti rotor). The supernatant was collected for ATPase purification.

Gel-Filtration Chromatography

A column (100 × 2.6 cm) of Sephacryl S-400 HR (Pharmacia) was equilibrated with a 5 mM Tris-Mes buffer (pH 7.3), 20% (w/v) glycerol, 0.2% (w/v) Triton X-100, 1 mM EDTA, 4 mM DTT, 4 mM $MgCl_2$, 0.2 mg mL^{-1} phospholipids. The phospholipids used in these experiments were mixed soybean phospholipids (L-α-phosphatidylcholine, type IV from Sigma) prepared as previously described (Manolson et al., 1985). Around 5 mg of solubilized tonoplast proteins were loaded on the column and eluted at a flow rate of 12 mL h^{-1} (4°C). Fractions of 4 mL were collected and assayed for ATPase activity using 150-μL aliquots.

Ion-Exchange Chromatography

The enzyme solution from Sephacryl S-400 HR was first concentrated using centrifugal microconcentrators-100 (Amicon, Beverly, MA). Then, partially purified enzyme (1.5 mL) was injected onto a 0.5 × 5 cm Mono Q column

(Pharmacia) equilibrated with a 5 mM Tris-Cl buffer (pH 6.0), 20% (w/v) glycerol, 0.2% (w/v) Triton X-100, 1 mM EDTA, 4 mM DTT, 4 mM $MgCl_2$, 0.2 mg mL^{-1} phospholipids. Chromatography was developed with a 0 to 1 M KCl gradient. The buffer was pumped at 4°C at a flow rate of 0.5 mL min^{-1}. Fractions were collected every 2 min. ATPase activity was measured with 150-μL aliquots.

Cationic Detergent/PAGE at Acid pH

Gel polymerization was obtained as previously described (Amory et al., 1980). The 10% running gel solution was made by sequential addition of 6.7 mL of 30% (w/v) acrylamide and 0.8% (w/v) *N,N'*-methylenebisacrylamide, 8.9 mL of distilled water, 1 mL of 1.5 M KH_2PO_4 adjusted to pH 2.0 with H_3PO_4, 1.5 mL of 1% (w/v) freshly dissolved ascorbic acid, 0.15 mL of 0.03% (w/v) freshly prepared $FeSO_4 \cdot 7H_2O$. After a 2-min vacuum treatment along with vigorous magnetic stirring, 0.27 mL of 0.175 M TDAB was added and another 2 min of vacuum treatment was applied. Then, 1.5 mL of freshly prepared 0.03% (v/v) H_2O_2 was added and the mixture was stirred under vacuum for 30 s. The solution was then poured into a gel matrix (120 × 140 × 1 mm). The 4% stacking gel solution was made by sequential addition of 0.67 mL of 30% (w/v) acrylamide and 0.8% (w/v) *N,N'*-methylenebisacrylamide, 2.17 mL of distilled water, 1.26 mL of 0.5 M KH_2PO_4 adjusted to pH 4 with H_3PO_4, 0.38 mL of 1% (w/v) freshly prepared ascorbic acid, 0.038 mL of 0.03% (w/v) freshly dissolved $FeSO_4 \cdot 7H_2O$, 0.1 mL of 0.175 M TDAB, 0.38 mL of 0.15% (v/v) freshly prepared H_2O_2. After a vacuum treatment similar to that applied to the running gel, the solution was loaded on top of the running gel. The buffer solution was composed of 75 mM Gly and 0.125% (w/v) TDAB adjusted to pH 3.0 with H_3PO_4. The gel was run at a constant current of 14 mA for 15 h at 4°C. After electrophoresis the gel was incubated for 5 min in 1% (v/v) glycerol at 20°C, dried under vacuum, and exposed to Hyperfilm-MP (Amersham). Polypeptides were colored with Coomassie blue or silver reagent.

SDS-PAGE

SDS-PAGE was conducted using a 12% acrylamide gel by the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue or silver reagent.

Immunoblotting

Western blotting and immunodetection were performed as described by Parry et al. (1989) with monoclonal antibodies raised to the 70-kD polypeptide of the vacuolar ATPase of oat roots (1:200 dilution) or with polyclonal antibodies raised to the plasmalemma ATPase of *Arabidopsis thaliana* (1:5000 dilution).

Enzyme Activities

G6PDH activity was measured by monitoring the reduction of $NADP^+$ at 340 nm. The reaction started with the addition of 10 μL of sample to 3 mL of 84 mM triethanolamine (pH 7.6), 6.5 mM $MgCl_2$, 2.25 mM Glc-6-P, and 0.35 mM $NADP^+$.

ATPase activity was determined by measuring the release of Pi. Twenty microliters of solubilized membrane or 150 μL of collected fractions (10 μg of protein) were incubated for 30 min at 37°C in 1 mL containing 25 mM Tris-Mes (pH 7.3), 50 mM KCl, 3 mM ATP (disodium salt), 4 mM $MgSO_4$, 1 mM EDTA, and 0.2 mM ammonium molybdate. The reaction was stopped by the addition of Ames reagent containing 1.8% (w/v) SDS when Triton X-100 was present in assays (Ames, 1966). The A_{830} values were measured 1 h later. The effect of polyclonal antibodies raised to the 66-kD polypeptide was measured after 30 min of incubation at 4°C of tonoplast vesicles with the antiserum (1:200 dilution).

Protein Determination

Protein concentration was determined as previously described (Peterson, 1977) with BSA as the standard.

Phosphorylation Procedure

The 100-μL reaction mixture contained 50 mM Tris-Mes (pH 6.5), 1 mM $MgSO_4$, approximately 50 μM [γ-^{32}P]ATP (specific activity 0.5–3 Ci $mmol^{-1}$), and 100 to 150 μg of protein or 2 μg of purified ATPase. The reaction was started (4°C) by the addition of [γ-^{32}P]ATP and terminated 30 s later by the addition of 1 mL of an acid solution containing 12% (w/v) TCA, 40 mM NaH_2PO_4, and 10 mM cold ATP. After 60 min at 4°C, precipitated proteins were sedimented at 13,000*g* for 15 min. The pellet was resuspended twice in 1 mL of acid solution, then washed with 1 mL of cold acetone before solubilization with 60 μL of a pH 4 solution containing 250 mM Suc, 50 mM DTT, 35 mM TDAB, 100 mM KH_2PO_4 (1 h, 25°C, stirring). Treatment of the pellet with 0.25 M hydroxylamine (pH 5.5, 30 min, 25°C) and alkaline pH buffers (30 min, 25°C, pH 4.0, 7.5, 9.0, and 10.8) occurred before acetone extraction.

For the pulse-chase experiment, tonoplasts or the purified ATPase were phosphorylated (4°C) with [γ-^{32}P]ATP, and 30 s later 2 mM cold ATP was added for 1 min (4°C). The chase was terminated by the addition of 1 mL of the acid solution. Then, precipitated proteins were washed as above. Control for nonwashed radioactivity was run by employing boiled tonoplast.

RESULTS

Estimation of the Functional Molecular Mass of the ATPase by Radiation Inactivation

Plots of the residual activities of ATPase and G6PDH as percentages of their original activities versus time of irradiation showed that the activity decay obeyed a single-exponential function of the absorbed dose (Fig. 1). Thus, the target theory could be applied for the determination of the functional molecular mass. The apparent molecular mass of the vacuolar ATPase was calculated by multiplying the molecular mass of G6PDH (104 kD) by D37 (G6PDH)/D37 (ATPase), where D37 is the radiation time required to

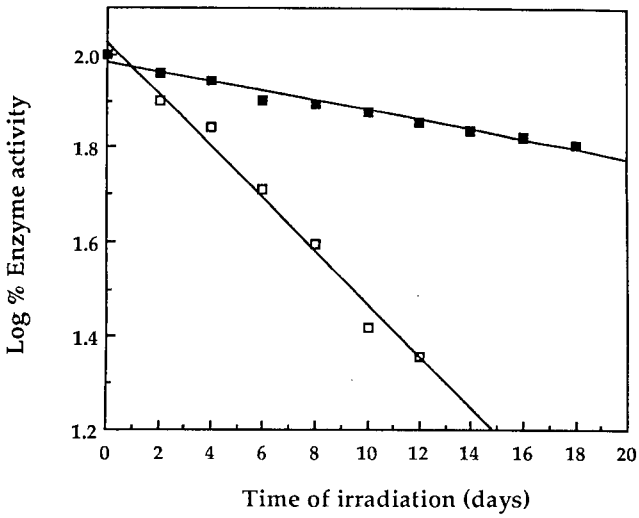

Figure 1. Functional molecular mass of the tonoplast H^+-ATPase estimated by radiation inactivation as described in "Materials and Methods." G6PDH was used as an internal standard. Points are means for three assays of a typical experiment. Data were analyzed by linear regression. The equations for ATPase (□) and G6PDH (■) inactivation profiles were $y = 2.024 - 0.056x$ ($R = 0.99$) and $y = 1.988 - 0.013x$ ($R = 1.00$), respectively. The ratio D37 (G6PDH)/D37 (ATPase) was 3.97.

reduce the measured activity to 37% of its initial value (Chanson and Pilet, 1989). From three tonoplast preparations, the experiment was repeated three times using triplicates, and mean values were calculated. Molecular masses of 366, 381, and 412 kD were obtained for the tonoplast ATPase (mean value ± SE: 386 ± 24 kD).

Solubilization and Purification of the H^+-ATPase

Triton X-100 was used to solubilize the ATPase. The optimal experimental procedure consisted of a 30-min period of incubation at 4°C with a Triton X-100 to protein ratio of 3 (w/w). This ratio corresponded to a final concentration of Triton X-100 of 0.09% (w/w). Under these conditions, about 75% of the ATPase activity was found in the supernatant. The 18% remaining activity was in the pellet, which indicated that the ATPase was not inactivated during the solubilization process.

Chromatography of the solubilized tonoplast on Sephacryl S-400 HR yielded only one peak of ATPase activity (Fig. 2). The addition of glycerol and phospholipids to the running buffer was required for maximal ATPase activity.

The purification was continued by anion-exchange chromatography. At pH 6.0, the tonoplast ATPase was retained on the resin. Only one peak of ATPase activity was eluted with a 0 to 1 M KCl gradient (Fig. 3). The tonoplast ATPase was eluted when the KCl concentration reached about 0.25 M. The pooled ATPase-containing fractions had a specific activity of 22 μmol Pi min^{-1} mg^{-1} protein.

The identity of the purified ATPase with the tonoplast-vesicle ATPase was confirmed by their sensitivities to Cl^-, NO_3^-, DCCD, thiol reagents, and vanadate (Table I). Both

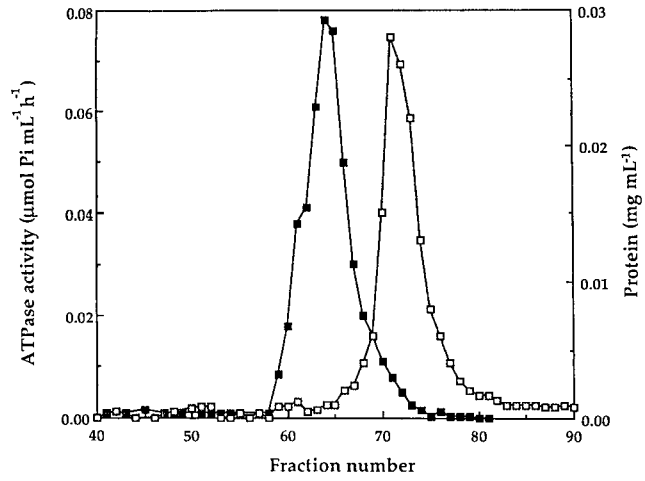

Figure 2. Purification of the solubilized tonoplast ATPase by gel filtration on a Sephacryl S-400 HR column as described in "Materials and Methods." Fractions (4 mL) were assayed for ATPase activity (■) and protein (□).

activities were about 60 to 70% stimulated by 50 mM Cl^- and 70 to 80% inhibited by 100 μM vanadate. However, increasing the amount of Triton X-100 in the reaction mixture decreased the sensitivity of the purified ATPase to vanadate (results not shown). Inhibition by nitrate in the presence of 50 mM KCl was slightly higher with the purified enzyme. Both activities were equally inhibited by 100 μM DCCD, 1 mM *N*-ethylmaleimide, or 100 μM *p*-chloromercuribenzoic acid. The purified enzyme was not inhibited by oligomycin and ammonium molybdate, which confirmed the absence of mitochondrial ATPase and nonspecific phosphatase.

IEF of the purified ATPase gave only one protein band at pH 5.2 (data not shown).

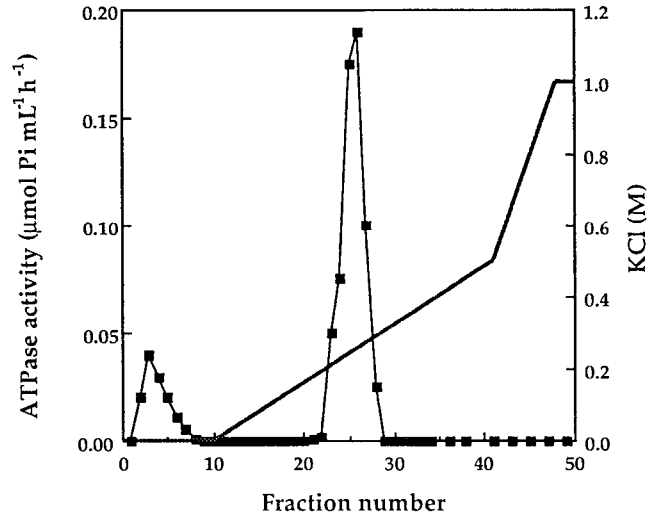

Figure 3. Purification by ion-exchange chromatography on a Mono Q column (0.5 × 5 cm) of the tonoplast ATPase previously purified by gel filtration. Fractions (1 mL) were assayed for ATPase activity (■) after elution with a 0 to 1 M KCl linear gradient (—) as described in "Materials and Methods."

Table I. *Sensitivity of tonoplast ATPase and purified ATPase*

ATPase activity was measured with tonoplast and pooled fractions from Sephacryl S-400 HR as described in "Materials and Methods." The specific ATPase activities of tonoplast and purified ATPase were, respectively, 0.35 and 4 μmol Pi min^{-1} mg^{-1} protein. Shown are means ± SD (*n*) of the activity as a percent of the control.

Assay	Activity (Percent of Control)	
	Tonoplast-bound ATPase	Purified ATPase
Control	100	100
+ Cl^- (50 mM)	155 ± 11 (15)	172 ± 14 (4)
Control (50 mM KCl)	100	100
KNO_3 (50 mM)	68 ± 11 (25)	49 ± 12 (3)
Vanadate (0.1 mM)	21 ± 8 (25)	29 ± 3 (7)
DCCD (0.1 mM)	6 ± 3 (6)	10 ± 4 (3)
N-Ethylmaleimide (1 mM)	22 ± 7 (5)	16 ± 4 (3)
p-Chloromercuribenzoic acid (0.1 mM)	17 ± 5 (4)	15 ± 5 (3)
Molybdate (0.2 mM)	98 ± 2 (11)	98 ± 6 (4)
Oligomycin (1 μg mL^{-1})	99 ± 4 (7)	98 ± 4 (3)

Multisubunit Composition of the Tonoplast H^+-ATPase

SDS-PAGE analysis of the purified tonoplast ATPase showed eight polypeptides of 95, 66, 56, 54, 40, 38, 31, and 16 kD (Fig. 4).

Immunological Cross-Reactivity

A polyclonal antibody raised to the plasmalemma H^+-ATPase of *A. thaliana* did not cross-react (western blot) with tonoplast polypeptides or with purified ATPase (Fig. 5A, lanes 1, 3, and 5). This antibody cross-reacted with two polypeptides of about 100 and 66 kD from the microsomal fraction of *A. pseudoplatanus* (Fig. 5A, lanes 2, 4, and 6). The 100-kD polypeptide may correspond to the plasma membrane H^+-ATPase. The 66-kD polypeptide could be a proteolysis product of the plasma membrane H^+-ATPase.

Monoclonal antibodies raised to the 70-kD subunit of the vacuolar ATPase of oat roots reacted with the 66-kD subunit of the purified vacuolar ATPase of *A. pseudoplatanus* (Fig. 5B).

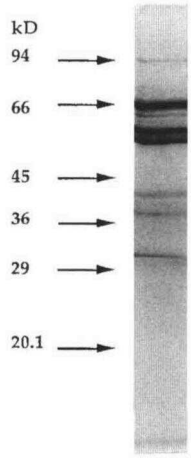

Figure 4. Subunit composition of the tonoplast H^+-ATPase. After purification by ion-exchange chromatography, an aliquot of fractions 20 to 30 was subjected to SDS-PAGE. The gel was stained with silver reagent. The figures on the ordinate indicate the positions of the size markers.

Phosphorylation of the Tonoplast H^+-ATPase

A very short incubation period (30 s at 4°C) of tonoplast proteins (Fig. 6A, lane 1) or purified ATPase (Fig. 6B, lanes 7 and 8) with [γ-^{32}P]ATP resulted in the phosphorylation of the 66-kD polypeptide. Evidence for the rapid turnover of the intermediate was provided by a pulse-chase experiment with 2 mM nonradioactive ATP, which resulted in the total loss of radioactivity in the 66-kD polypeptide (Fig. 6A,

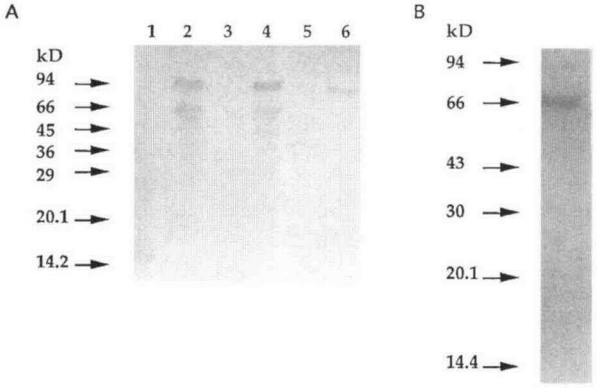

Figure 5. A, Immunoblot of the tonoplast ATPase and microsomal fraction of *A. pseudoplatanus* cells. Lanes 1 and 3, Tonoplast with 50 and 30 μg of protein, respectively; lane 5, 10 μg of purified ATPase; lanes 2, 4, and 6, microsomal fraction with 50, 30, and 10 μg of protein, respectively, probed with antibodies (dilution 1:5000) raised to the plasmalemma ATPase of *A. thaliana*. The microsomal fraction was obtained by resuspending protoplasts of *A. pseudoplatanus* in a 30-mM Tris-Mes osmotic shock buffer (pH 7.8) containing 10 mM EDTA, 1 mM PMSF, 10 μg mL^{-1} chymostatin. The homogenate was centrifuged at 6,000*g* for 20 min, then at 100,000*g* for 1 h. The microsomal pellet was homogenized in the osmotic shock buffer. The blots were prepared and probed as described in "Materials and Methods." B, Immunoblot of the purified tonoplast ATPase (5 μg) probed with a monoclonal antibody (dilution 1:200) raised to the 70-kD polypeptide of the vacuolar ATPase of oat roots.

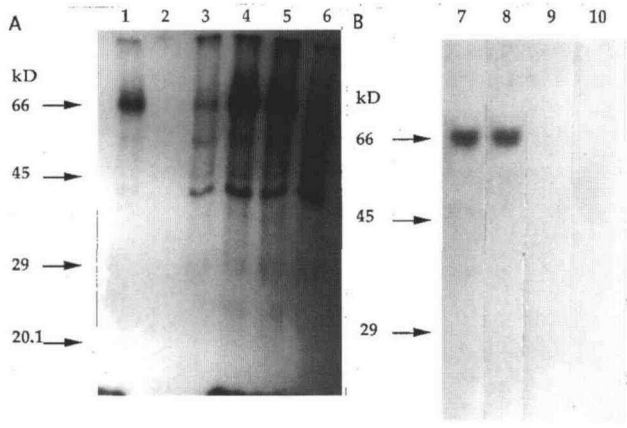

Figure 6. Autoradiogram of the tonoplast (A) and the purified tonoplast ATPase (B) after phosphorylation with [γ-^{32}P]ATP and PAGE at acidic pH in the presence of a cationic detergent. Lane 1, Control tonoplast; lane 2, boiled tonoplast before phosphorylation; lanes 7 and 8, purified tonoplast ATPase; lane 3, hydroxylamine treatment after phosphorylation; lanes 4, 5, and 9, incubation of tonoplast or purified ATPase with 0.1 mM (lanes 4 and 9) and 1 mM (lane 5) vanadate during phosphorylation; lanes 6 and 10, pulse-chase experiment with unlabeled ATP as described in "Materials and Methods." The numbers on the left are the molecular masses of the marker proteins.

lane 6, and Fig. 6B, lane 10). Treatment with 0.5 M hydroxylamine (pH 5.0) after TCA precipitation of the phosphorylated samples removed the label from the 66-kD polypeptide (Fig. 6A, lane 3).

The chemical nature of the phosphoryl ATPase linkage was further investigated by determining the stability of the intermediate at different pH values. When tonoplast proteins were phosphorylated, TCA-precipitated, and suspended in buffers of increasing pH it could be seen that the amount of radioactivity associated with the 66-kD polypeptide decreased as the pH became more alkaline (Fig. 7). When phosphorylation assays were performed with tonoplast preparations, 0.1 to 1 mM vanadate increased the phosphorylation of the 66-kD polypeptide (Fig. 6A, lanes 4 and 5). Nevertheless, when phosphorylation assays were performed with the purified vacuolar ATPase, 0.1 mM vanadate totally inhibited the 66-kD phosphorylation (Fig. 6B, lane 9).

DISCUSSION

The molecular mass of the vanadate-sensitive H^+-ATPase of the tonoplast of *A. pseudoplatanus* has been estimated at about 400 kD by radiation inactivation analysis. This value is probably an underestimate because if one adds up the identified subunits and takes into account that the A and B subunits are present in triplicate, the molecular mass should be above 600 kD. The 400-kD value could correspond to the catalytic and regulatory subunits. This value fit with that of a V-type ATPase, i.e. 400 to 750 kD (Nelson and Taiz, 1989; Forgac, 1992). The functional molecular mass of the tonoplast ATPase from corn coleoptiles (Mandala and Taiz, 1985) and mung bean seedlings (Wang et al., 1989) was about 400 kD, whereas those of maize roots (Chanson and Pilet, 1989) and *Neurospora crassa* (Bowman et al., 1986) were 542 and 520 kD, respectively, based on radiation inactivation analysis. Fractionation of the solubilized tonoplast by gel filtration and ion-exchange chromatography yielded only one peak of ATPase activity, the pI of which was close to 5.2. A similar value characterized the tonoplast H^+-ATPase of *Hevea* (Marin et al., 1985). The identity of the purified H^+-ATPase with the H^+-ATPase in native tonoplast vesicles was confirmed by their sensitivity to various effectors. The purified ATPase was more sensitive to nitrate than the ATPase in the native tonoplast and was inhibited by low concentrations of vanadate as much as the ATPase in native tonoplast. Nevertheless, we observed that increasing the amount of Triton X-100 in the reaction mixture decreased the ATPase sensitivity to vanadate.

Analysis of the purified ATPase on SDS-PAGE showed at least eight polypeptides of 95, 66, 56, 54, 40, 38, 31, and 16 kD. Thus, the subunit composition of the vanadate-sensitive tonoplast H^+-ATPase of *A. pseudoplatanus* was consistent with those described for V-type ATPases present on the endomembrane system of eukaryotic cells, including vacuoles (Mandala and Taiz, 1985, 1986; Bremberger et al., 1988; Kaestner et al., 1988; Bowman et al., 1989; Kane et al., 1989; Moriyama and Nelson, 1989a), lysosomes (Ohkuma et al., 1982), endosomes (Galloway et al., 1983), Golgi (Moriyama and Nelson, 1989b), chromaffin granules (Moriyama and Nelson, 1988), and coated vesicles (Arai et al., 1988). In particular, the subunit composition of the vacuolar ATPase of *A. pseudoplatanus* was similar to that of beet root vacuolar ATPase (Parry et al., 1989). Moreover, the cross-reaction of this 66-kD subunit with antibodies raised against the 70-kD subunit of the vacuolar H^+-ATPase of oat roots indicates that this polypeptide corresponds to the 66-kD subunit of other vacuolar H^+-ATPases. Taken together, our results clearly indicate that the ATPase under study was a V-type ATPase and not a P-type ATPase or a phosphatase.

Vanadate seems to inhibit only those ATPases in which a phosphorylated intermediate is formed during turnover.

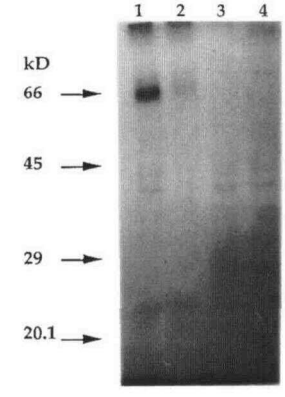

Figure 7. Effect of pH on the stability of the phosphorylated tonoplast bound-ATPase as described in "Materials and Methods." The following buffers were used: lane 1, untreated control; lane 2, 100 mM Tris (pH 7.5 with HCl); lane 3, 100 mM sodium borate (pH 9 with HCl); lane 4, 100 mM sodium borate (pH 10.8).

As previously reported (Lindquist et al., 1973; Lopez et al., 1976), vanadate adopts a stable trigonal bipyramidal structure that resembles the transition state of the phosphate and competes with phosphate for binding. These observations led us to determine if the tonoplast H^+-ATPase of *A. pseudoplatanus* could operate via a phosphoenzyme intermediate as the P-ATPase. The use of an acid gel electrophoresis system in the presence of a cationic detergent established that the 66-kD subunit of the tonoplast H^+-ATPase was phosphorylated when the purified enzyme or tonoplast was incubated with [γ-^{32}P]ATP. The short period of incubation at 4°C indicated that the phosphorylation did not depend on protein kinases. The short chase period with unlabeled ATP resulted in the complete loss of radioactivity in the 66-kD subunit and reflected a rapid turnover, which characterizes the phosphorylated intermediate (Goffeau and Slayman, 1981). The chemical nature of the phosphoryl ATPase linkage was further investigated by determining the stability of the intermediate in the presence of hydroxylamine and at different pH values. The results rule out phosphoserine or phosphothreonine intermediates, which are resistant to hydrolysis by hydroxylamine and phosphohistidine, or phospholysine intermediates, which are stable at alkaline pH (Weller, 1979). Thus, the acyl phosphate of the tonoplast H^+-ATPase of *A. pseudoplatanus* may be an aspartyl or a glutamyl derivative. It will be necessary to carry out a digestion of the phosphorylated protein to confirm the identity of the phosphorylated amino acid.

Vanadate had opposite effects on the phosphorylated intermediate when the enzyme was tonoplast-bound or purified. This discrepancy probably results from the different enzyme surroundings, as previously reported (Medda and Hasselbach, 1984). The higher phosphorylation of the tonoplast-bound 66-kD polypeptide in the presence of vanadate could be explained by an inhibition of dephosphorylation of the phosphorylated intermediate or, since vanadate is a permeant ion, it may increase the rate of phosphorylation by dissipating the membrane potential in the absence of chloride in the reaction medium (R. Poole, personal communication). On the other hand, we cannot exclude that when tonoplast-bound ATPase was used, vanadate affected the activity of protein kinases or protein phosphatases associated with the ATPase. Further experiments are necessary to resolve these contradictory findings.

The subunit composition of the tonoplast H^+-ATPase of *A. pseudoplatanus* and the results of phosphorylation assays lead us to the intriguing possible conclusion that this enzyme is a V-type ATPase that operates with a P-type catalytic mechanism. Our results are nevertheless consistent with several recent findings: (a) A residual sequence from the phosphorylation domain of P-type ATPases is present in the α and β chain of F0F1-ATPases (Pedersen and Carafoli, 1987b), and this region exhibits homology to the 69-kD sequence of the carrot vacuolar ATPase (Zimniak et al., 1988; Taiz et al., 1989). (b) The Asp residue of P-type ATPases, which is phosphorylated during the catalytic process, is replaced with a Glu residue in the 69-kD subunit of carrot V-type ATPase (Zimniak et al., 1988). One cannot rule out the eventual phosphorylation of this Glu residue. (c) This critical aspartate residue is conserved in the V-type ATPase of *M. thermolithotrophicus*, which may have a P-type catalytic mechanism (Taiz et al., 1989). Because other vacuolar ATPases from chromaffin granules and yeast were recently reported to be inhibited by vanadate (Beltran and Nelson, 1992), there are now indications that some vacuolar H^+-ATPases may have a phosphorylated intermediate.

ACKNOWLEDGMENTS

We are indebted to A. Pelletier (Institut National de la Recherche Agronomique, Dijon) and Dr. B. Lamy (Centre Régional de Transfusion Sanguine, Besançon) for radiation assays, to J.P. Rémi-Martin and Dr. Nicollier (Institut National de la Santé et de la Recherche Médicale, Besançon) for IEF and fast protein liquid chromatography experiments, and to Dr. R. Serrano (University of Valencia, Spain) and Dr. H. Sze (University of Maryland, College Park) for generous gifts of antibodies to the plasmalemma ATPase of *A. thaliana* and antibodies to the 70-kD polypeptide of the vacuolar ATPase of oat roots, respectively. We thank A. La Roche for technical assistance.

Received April 10, 1995; accepted June 8, 1995.

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