Journal of Plant Physiology **162** (2005) 129–138

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Proton pumping inorganic pyrophosphatase of endoplasmic reticulum-enriched vesicles from etiolated mung bean seedlings

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Received 2 June 2004; accepted 27 July 2004

KEYWORDS
Endoplasmic reticulum;
H^+-pyrophosphatase;
Proton translocation;
Tonoplast;
Vacuole

Summary

Endoplasmic reticulum (ER)-enriched vesicles from etiolated hypocotyls of mung bean seedlings (*Vigna radiata*) were successfully isolated using Ficoll gradient and two-phase (polyethylene glycol-dextran) partition. The ER-enriched vesicles contained inorganic pyrophosphate (PP_i) hydrolysis and its associated proton translocating activities. Antiserum prepared against vacuolar H^+-pyrophosphatase (V-PPase, EC 3.6.1.1) did not inhibit this novel pyrophosphatase-dependent proton translocation, excluding the possible contamination of tonoplast vesicles in the ER-enriched membrane preparation. The optimal ratios of Mg^{2+}/PP_i (inorganic pyrophosphate) for enzymatic activity and PP_i-dependent proton translocation of ER-enriched vesicles were higher than those of vacuolar membranes. The PP_i-dependent proton translocation of ER-enriched vesicles absolutely required the presence of monovalent cations with preference for K^+, but could be inhibited by a common PPase inhibitor, F^-. Furthermore, ER H^+-pyrophosphatase exhibited some similarities and differences to vacuolar H^+-PPases in cofactor/substrate ratios, pH profile, and concentration dependence of F^-, imidodiphosphate (a PP_i analogue), and various chemical modifiers.

Abbreviations: CCR, antimycin A-sensitive NADH-dependent cytochrome *c* reductase; DCCD, *N*, *N*′-dicyclohexylcarbodiimide; EDTA, *N*, *N*, *N*′*N*′-ethylenediamine tetraacetic acid; EGTA, ethyleneglycol-bis-(aminoethyl ether) *N*, *N*, *N*′, *N*′-tetraacetic acid; ER, endoplasmic reticulum; FITC, fluorescein 5′-isothiocyanate; NEM, *N*-ethylmaleimide; PGO, phenylglyoxal; Ppase, pyrophosphatase; TNM, tetranitromethane.

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doi:10.1016/j.jplph.2004.07.007

These results suggest that ER-enriched vesicles contain a novel type of proton-translocating PPase distinct from that of tonoplast from higher plants.
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Introduction

Acidification of subcellular compartments by proton pumping enzymes is essential to many physiological functions of higher plants. The major role of proton pumping enzymes is to generate a transmembrane H^+-electrochemical potential gradient which, in turn, provides the energy for secondary transport of solutes including sucrose, ions, and many metabolites (Maeshima, 2000, 2001; Palmgren, 2001; Capaldi and Aggeler, 2002; Nishi and Forgac, 2002; Weber and Senior, 2003). One of the most important and ubiquitous proton-pumping enzymes is proton-translocating ATPase (H^+-ATPases, EC 3.6.1.3). Higher plant cells possess at least three distinct types of H^+-ATPases embedded in the plasma membrane (P-ATPase), thylakoid and mitochondrial membranes (F-ATPases), and vacuolar membrane (V-ATPase), respectively (Palmgren, 2001; Capaldi and Aggeler, 2002; Nishi and Forgac, 2002; Weber and Senior, 2003). The structure and several features of these H^+-ATPases are obviously different. Moreover, a body of evidence indicates that plant vacuole contains another novel type of proton pumping enzyme, named vacuolar H^+-pyrophosphatase (H^+-PPase, EC 3.6.1.1) (Maeshima, 2000, 2001). H^+-pyrophosphatase has been found mainly in vacuoles, and was generally recognized as a distinct category of ion translocator using exclusively inorganic pyrophosphate (PP_i) as energy source (Maeshima, 2000, 2001). Both vacuolar H^+-ATPase and H^+-PPase are currently under extensive studies (Maeshima, 2000, 2001; Nishi and Forgac, 2002).

For years, investigators have been interested in whether any endomembrane other than vacuole contains also proton-pumping pyrophosphatase (Maeshima, 2000, 2001). Long et al. (1995) have shown the possible location of a PPase on phloem-specific plasma membrane from *Ricinus communis* seedlings. Using immunogold labeling, Robinson (1996) demonstrated the ubiquitous presence of vacuolar H^+-PPase in many organelles of the storage parenchyma cell from developing pea cotyledons. However, direct evidence showing PPase-dependent proton-pumping reaction on organellar membranes other than vacuoles has not been defined. Furthermore, after thermodynamic considerations Davies et al. (1997) ruled out the involvement of PPase in proton translocation across the plasma membrane of plant cells. Nevertheless, Vianello et al. (1991) detected a proton pumping activity on pea stem submitochondrial particles. The molecular mass of their H^+-PPase was markedly smaller than that from vacuole, indicating a possible new type of the H^+-PPase on mitochondrial membranes (Zancani et al., 1995). However, radiation inactivation analysis demonstrated that submitochondrial particles from etiolated mung bean seedlings contained a H^+-PPase with a functional size of 170 kDa (Jiang et al., 2000). Furthermore, it was revealed that the PPase purified from spinach thylakoid membrane lacked of any proton pumping activity (Jiang et al., 1997). Besides, using a GUS reporter system and a green fluorescent fusion protein (GFP), Mitsuda et al. (2001) investigated the tissue distribution and the subcellular localization of a novel H^+-PPase encoded by AVP2/AVPL1 in the *Arabidopsis thaliana* genome. This new H^+-PPase primarily resided in the Golgi apparatus rather than on the vacuolar membrane. The above works shed the light that other subcellular membrane may contain its own type of H^+-PPase. In addition, plant-like H^+-PPase were also recently discovered in acidocalcisomes of some protozoan (Scott et al., 1998) and prokaryotes (Drowzdowicz et al., 1999; Drowzdowicz and Rea, 2001; Perez-Castineira et al., 2001). It is thus believed that H^+-PPase is more widely distributed than previously believed in bioenergetic systems of higher plants.

In this study, we report a direct observation of PP_i-supported proton translocation in endoplasmic reticulum (ER)-enriched vesicles. Polyclonal antibody raised against vacuolar H^+-PPase could not inhibit this novel PP_i-dependent proton translocation, excluding possible contamination by tonoplast vesicles. Distinct characteristics of ER H^+-PPases from etiolated mung bean seedlings were also investigated.

Materials and methods

Plant materials

Seeds of mung beans (*Vigna radiata* L.), purchased from a local market, were soaked for 4 h in tap water and then germinated at room temperature in the dark using a commercial seedling incubator. Hypocotyls of 5-day-old etiolated seedlings were

excised, chilled on ice, and then used as starting materials.

Membrane preparations

ER-enriched vesicles were prepared according to a method of Yoshida (1994) with following modifications. Pre-chilled hypocotyls (300 g) were chopped into pieces by a knife. The tissue was then ground thoroughly with 300 mL homogenization medium [250 mM sorbitol, 50 mM Mops-KOH (pH 7.6), 5 mM *N,N,N′N′*-ethylenediamine tetraacetic acid (EDTA), 5 mM ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), 0.5% (w/v) defatted BSA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL tertiary-butylated hydroxytoluene, and 2.5 mM dithiothreitol]. The crude microsomal fraction (10,000–156,000 g pellet) was collected and resuspended in 34 mL of sorbitol suspension medium [250 mM sorbitol, 5 mM Mops-KOH (pH 7.3), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM KCl, 10 μg/mL tertiary-butylated hydroxytoluene, and 2 mM dithiothreitol]. The solution was then divided into two parts (17 mL each) and loaded on 8 mL of 5.5% (w/w) Ficoll made up in 15% (w/w) sucrose in the same sorbitol suspension medium above. After centrifugation at 200,000 g for 2.5 h, plasma membranes were spun down as "Ficoll-pellet". Tonoplast and ER-enriched vesicles were co-banded at the interface between the sample load and the Ficoll gradient. The Ficoll interface was collected, diluted with 2 volumes of sorbitol suspension medium and centrifuged at centrifugation at 156,000 g for 20 min. The membrane pellet was resuspended in 16 mL of sucrose-phosphate buffer [250 mM sucrose and 10 mM potassium phosphate (pH 7.8)], and subjected to further separation by two-polymer phase partition.

The two-phase partition system consisted of 5.6% (w/w) each of polyethylene glycol (PEG 3350; Sigma, St. Louis, MO, USA) and Dextran T500 (Pharmacia, Uppsala, Sweden) freshly made in sucrose-phosphate buffer and 10 mM NaCl. The interfacial fraction (8 mL) above was added into 32 mL pre-cooled (4 °C) two-phase solution, mixed by several inversions, and then centrifuged at 4500 g for 5 min at 4 °C. The endoplasmic reticulum preferentially partitioned into lower phase. The lower phase was collected and mixed with a second upper phase of PEG freshly made in sucrose-phosphate buffer alone for second partition. The consequent lower phase containing ER-enriched vesicles was washed with 4 volumes of sorbitol suspension medium above, centrifuged at 156,000 g for 30 min, and finally resuspended in sorbitol suspension buffer at a final protein concentration of 3 mg/mL. The ER-enriched preparation was ready for immediate use or stored at −70 °C.

Tonoplast vesicles of high purity were prepared by a floating centrifugation method of Maeshima and Yoshida (1989) with minor modifications. The crude microsomal fraction in grinding buffer was prepared as described above and then suspended in SPED buffer [300 mM sucrose, 10 mM potassium phosphate (pH 7.8), 1 mM EGTA, and 2 mM dithiothreitol]. The membrane solution (15 mL) was overlaid with 10 mL of SMED medium [0.25 M sorbitol, 5 mM Mops-KOH (pH 7.3), 1 mM EGTA, and 2 mM dithiothreitol] and centrifuged at 123,000 g for 30 min. After centrifugation, tonoplast vesicles banded at the interface of SMED and SPED solutions were collected, diluted in a half-volume of SMED solution, and centrifuged at 130,000 g for 20 min. The resulting pellets were resuspended in a buffer containing 20 mM Tris-acetate (pH 7.5), 20% (w/v) glycerol, 2 mM dithiothreitol, 1 mM EGTA, and 2 mM $MgCl_2$ at a final protein concentration of 3 mg/mL.

Enzyme assay and protein determination

ATPase was assayed in a reaction medium [3 mM ATP, 3 mM $MgSO_4$, 30 mM Tris/Mes (pH 7.0), 50 mM KCl, 1 mM ammonium molybdate, 5 mM sodium azide, approximately 20 μg/mL membrane protein, and 0.03% (w/v) Triton X-100 where indicated]. For NO_3^- and vanadate-sensitive ATPase activities, 100 mM KNO_3 and 100 μM sodium vanadate were added, respectively. PPase activity was assayed in a reaction medium containing 25 mM Mops-KOH (pH 7.9), 0.2 mM PP_i, 3.0 mM $MgSO_4$, 50 mM KCl, 0.1 mM sodium vanadate, 1.0 mM sodium molybdate, 5 mM sodium azide, 100 mM KNO_3, and approximately 20 μg/mL membrane protein. After incubation at 37 °C for 15 min, the reaction was terminated by a solution containing 1.7% (w/v) ammonium molybdate, 2% (w/v) sodium dodecyl sulfate, and 0.02% (w/v) 1-amino-2-naphthol-4-sulfonic acid. The released P_i was determined spectrophotometrically (Fiske and Subbarow, 1925; Wang et al., 1989). Antimycin A-sensitive NADH-dependent cytochrome *c* reductase (CCR; EC 1.6.99.3) activity was assayed as previously reported (Hodges and Leonard, 1974; Yoshida et al., 1986).

Protein concentration was determined by the method of Bradford (1976) using BSA as a standard.

Measurement of proton translocation

Proton translocation was measured as fluorescence quenching of acridine orange (excitation

wavelength 495 nm, emission wavelength 530 nm) with a Hitachi F-4000 fluorescence spectrophotometer. The reaction mixture (2 mL) for ER-enriched vesicles contained 5 mM Mops-KOH (pH 7.9), 250 mM sorbitol, 3 mM $MgSO_4$, 50 mM KCl, 0.2 mM PP_i, 5 μM acridine orange, and 30 μg/mL membrane protein. The H^+-pumping medium (2 mL) for tonoplast vesicles consisted of 5 mM Mops-KOH (pH 7.0), 250 mM sorbitol, 1 mM $MgSO_4$, 50 mM KCl, 1 mM PP_i, 5 μM acridine orange, and 30 μg/mL membrane protein. The fluorescence quenching of ER-enriched vesicles and tonoplast were initiated by adding PP_i. The ionophore gramicidin (2 μg/mL) was added at the end of each assay (Maeshima and Yoshida, 1989; Kuo and Pan, 1990).

Preincubation with inhibitors

Prior to incubation with inhibitors, vesicles were centrifuged at 80,000 g for 35 min and resuspended in 250 mM glycerol and 20 mM Tris-HCl (pH 7.9) buffer. The standard preincubation mixture contained inhibitor at concentration as indicated, 20 mM Tris-HCl (pH 7.9), and 1 mg/mL membrane proteins. The preincubation was carried out at 25 °C for each inhibitor except NEM, which was used at 4 °C. The inhibition was started by adding inhibitors for various periods and terminated either by addition of 2.5 mM dithiothreitol (for Cys-reactive inhibitor) or by 37.5-fold dilution directly into the H^+-PPase reaction mixture (for other inhibitors). After incubation, subsequent assay of H^+-PPase activity was performed as mentioned above.

Preparation of antibody

Antibodies against mung bean vacuolar H^+-PPase were raised in mice by injection of the purified vacuolar H^+-PPase as described previously (Mao et al., 1989; Yang et al., 1996). For immunization, the purified vacuolar H^+-PPase (200 μg) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis was sliced and homogenized in 2 mL complete Freud adjuvant and inoculated subcutaneously to mice at multiple sites. After 6–10 weeks, anti-vacuolar H^+-PPase serum was collected without further purification.

Chemicals

ATP and antimycin A-sensitive NADH-dependent cytochrome *c* reductase were purchased from Sigma (St. Louis, MO, USA), and PP_i from E. Merck (Damstadt, Germany). Dextran T500 was provided by Pharmacia (Uppsala, Sweden). All other chemicals were of analytic grade and used without further purification.

Results and discussion

Isolation of endomembranes

Firstly, the purity of ER-enriched vesicles isolated accordingly was examined using ER marker enzymes, such as antimycin A-insensitive NADH-dependent cytochrome *c* reductase (CCR) and Triton-/ VO_4^{3-}-sensitive H^+-ATPase (Kawata and Yoshida, 1988; Sze et al., 1992). Table 1 shows that

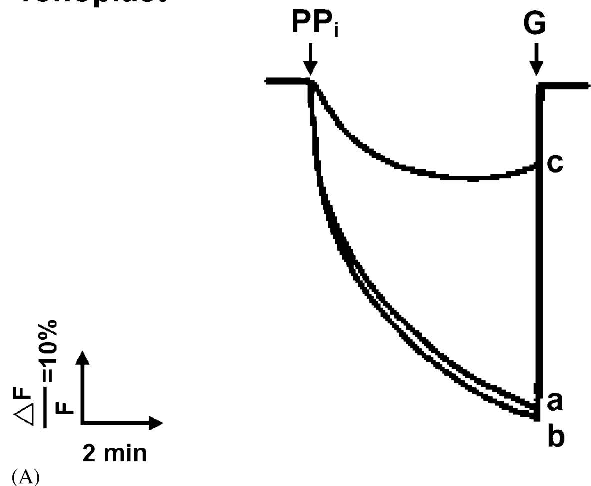
Table 1. Specific activities of maker enzymes in various fractions

Marker enzymes + inhibitors	Ficoll-pellet (plasma membrane)	Upper phase (Tonoplast)	Lower phase (ER)
NADH-cyt *c* Reductase (μmol/mg h)	0.9±0.1	0.5±0.1	5.8±0.3
ATPase ($\mu mol\ P_i$/mg h)			
Control	9.7±0.8 (100)	13.7±0.2 (100)	15.8±0.7 (100)
Bafilomycin (0.1 μM)	8.5±0.4 (88)	6.3±0.2 (46)	12.9±0.6 (82)
KNO_3 (100 mM)	6.6±0.2 (68)	6.0±0.3 (44)	12.3±0.4 (78)
Na_3VO_4 (100 μM)	5.4±0.2 (56)	9.6±0.4 (70)	5.8±0.3 (37)
Triton X-100 (0.03%)	13.7±0.5 (142)	24.4±0.7 (178)	2.4±0.3 (15)

Antimycin A-insensitive NADH-dependent cytochrome *c* reductase activity was measured as described under "Materials and methods". ATPase was assayed in a 1 mL-reaction medium containing 3 mM ATP, 3 mM $MgSO_4$, 30 mM Tris-Mes (pH 7.0), 50 mM KCl, 1 mM ammonium molybdate, 5 mM sodium azide, approximately 20 μg/mL membrane proteins. Membrane proteins were preincubated with bafilomycin for 10 min at 25 °C and diluted 25-fold into the assay mixture. Other inhibitors were added at the time of assay. Each value is the mean of three independent measurements.

the ER-enriched vesicles (lower phase) possess significantly higher enzymatic activities of CCR than tonoplast-enriched fraction (upper phase), suggesting high purity of the ER preparation (cf., Yoshida, 1994). Moreover, ATP hydrolysis of ER-enriched vesicles was substantially inhibited by Triton X-100 (Kawata and Yoshida, 1988; Yoshida, 1994), at concentration higher than 2.0 μg detergent/μg protein. In contrast, Triton X-100 enhanced activity of H^+-ATPase in the plasma membrane-enriched fraction (Ficoll pellet). Furthermore, H^+-ATPase activity of ER-enriched vesicles was partially inhibited in the presence of VO_4^{3-}, since ER contains a P-type H^+-ATPase (Hwang et al., 1997). However H^+-ATPase of ER-enriched vesicle was less sensitive to bafilomycin and NO_3^-, the characteristic inhibitors of tonoplast H^+-ATPase (Sze et al., 1992). In addition, the ATPase activity of ER-enriched vesicles was independent on Cl^-, whereas vacuolar H^+-ATPase was preferentially stimulated by Cl^- (Kawata and Yoshida, 1988; Yoshida, 1994). Besides, sensitivity of the H^+-ATPase activity of ER-enriched vesicles to F^- was relatively higher than tonoplast (see below). These results revealed that the ER-enriched vesicles obtained by our protocol were certainly distinct from tonoplast membrane preparations (Kawata and Yoshida, 1988). It was also demonstrated that ER-enriched vesicles were highly purified and the contamination by tonoplast was substantially negligible.

Figure 1 shows that both ER-enriched vesicles and tonoplasts were capable of translocating proton across their respective membranes. Upon preincubation with anti-mung bean vacuolar H^+-PPase antibody, proton translocation of tonoplast membrane was markedly inhibited. However, under similar conditions, the anti-mung bean vacuolar H^+-PPase antibody did not inhibit proton translocation of ER-enriched vesicles. Conceivably, the H^+-PPase on ER-enriched vesicles was not the target for anti-vacuolar H^+-PPase antibody. This negative response further excluded the possible contamination of vacuolar type H^+-PPase in our ER-enriched preparations. In other words, proton translocation of the ER-enriched vesicles came directly from its own H^+-PPase, suggesting certainly another distinct type of the proton-translocating enzyme. Taken together, these ER-enriched preparations were free from contamination of vacuole and suitable for studying enzymatic reaction and proton translocation as shown below.



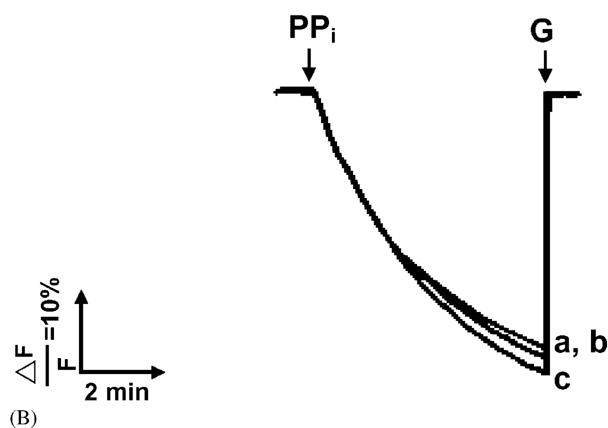


Figure 1. Effects of anti-vacuolar H^+-PPase serum on PP_i-dependent H^+-pumping of tonoplast and ER-enriched vesicles. Aliquot (20 μL) of anti-mung bean vacuolar H^+-PPase serum was incubated with 20 μL of tonoplast (Fig. 1A, curve c) and ER-enriched vesicles (Fig. 1B, curve c) at 25 °C for 30 min, respectively. Both protein concentrations of tonoplast and ER are 3 mg protein/mL, respectively. After incubation, PP_i-dependent H^+-translocation was measured at 25 °C by fluorescence quenching of acridine orange. Curves a and b represent membranes incubated with sorbitol suspension buffer and pre-immune serum, respectively. G, gramicidin.

Some characteristics of ER H^+-PPase

The substrate concentration curves of PP_i hydrolysis and the proton translocation of ER-enriched vesicles were determined (Fig. 2). At 3 mM $MgSO_4$, both reaction rates increased concomitantly with the increase of substrate concentrations and reached maxima when the Mg^{2+}/PP_i ratio was approximately 30–60:1 (Fig. 2). As the concentration of PP_i was higher than that at this ratio, both reactions were gradually inhibited by the substrate. This inhibitory effect is not surprising, since substrate inhibition is very common to most types of PPases (Leigh et al.,

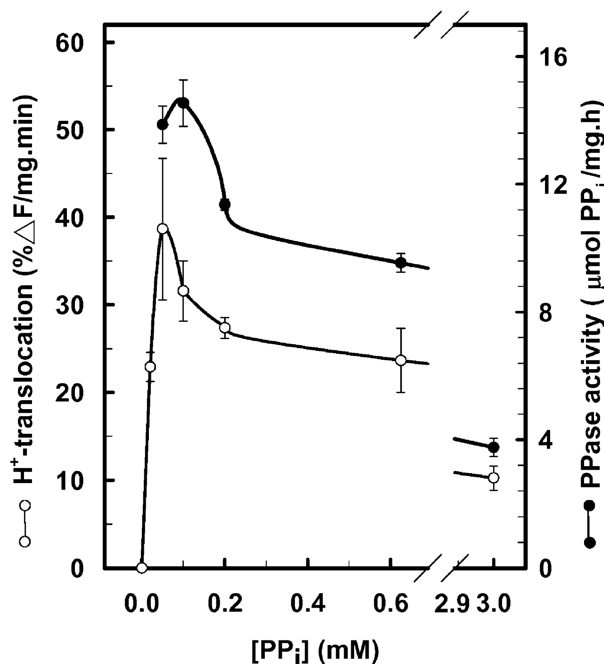


Figure 2. Effects of PP_i concentrations on H^+-pumping and PP_i hydrolysis of ER-enriched vesicles. PP_i-dependent H^+-pumping (o) and PP_i hydrolysis (•) activities were determined as described under "Materials and methods". $MgSO_4$ was kept constant at 3 mM. Each value is the mean of three independent measurements.

Table 2. Effect of monovalent ions on ER H^+-PPase

Addition (50 mM)	H^+-translocation (% ΔF/mg protein min)	Specific activity (μmol PP_i/mg protein h)
None	0	10.2±0.9
LiCl	11.6±0.6	10.5±0.6
NaCl	14.1±0.2	10.4±0.5
KCl	33.6±3.7	13.1±0.1
RbCl	26.1±1.0	14.0±1.0
CsCl	12.8±0.5	10.4±1.2
KF	0	1.1±0.1
KCl	33.6±3.7	13.1±0.1
KI	10.7±2.4	14.0±0.7
KNO_3	19.6±0.7	14.6±0.5
K_2SO_4*	23.0±0.1	13.7±0.3

Proton translocation of ER-PPase was measured as changes of fluorescence quenching (ΔF) in a medium containing 5 mM Mops-KOH (pH 7.9), 0.25 M sorbitol, 3 mM $MgSO_4$, 50 mM KCl, 5 μM acridine orange, and 30 μg/mL membrane proteins. The fluorescence quenching was initiated by adding 0.2 mM PP_i at 25 °C. The initial rate of proton translocation and the specific activity of enzyme were determined as described under "Materials and methods". Each value is the mean of three independent measurements.

*The concentration of K_2SO_4 was 25 mM.

1992). However, the optimal Mg^{2+}/PP_i ratio for mung bean vacuolar H^+-PPase is well known as approximately 1:1 under the same conditions (Maeshima and Yoshida, 1989). The relatively high optimal Mg^{2+}/PP_i ratio suggests that ER-enriched vesicles contain a distinct type of its own PP_i hydrolase.

It has been long known that tonoplast H^+-PPase could be stimulated by K^+ but inhibited by F^- (Maeshima, 2000). We therefore scrutinized effects of several monovalent ions on ER H^+-PPase (Table 2). In the absence of monovalent ions, the enzymatic activity of ER H^+-PPase is 10.2 μmol PP_i hydrolyzed/mg protein h. However, no trace of PP_i-supported proton translocation of ER-enriched vesicles was detected under similar conditions. Nonetheless, in the presence of 50 mM KCl, PP_i hydrolysis of ER-enriched vesicles increased by approximately 30% and ER H^+-PPase supported proton translocation was then observed. Other monovalent cations could replace K^+ for PP_i-dependent proton translocation of ER-enriched vesicles, albeit, with lesser effect. The absolute requirement of monovalent cation for PP_i-associated proton translocation but not PP_i hydrolysis of ER-enriched vesicles indicates that monovalent cations play presumably a role in coupling these reactions. This property was obviously different from that of tonoplast H^+-PPase.

Moreover, using K^+ as counterion, I^-, NO_3^-, and SO_4^{2-} did not change the enzymatic activity of ER H^+-PPase, but showed some degree of effects on PP_i-dependent proton translocation of ER-enriched vesicles. The extent of the coupling between enzymatic activity and proton pumping of ER H^+-PPase depended on the species of monovalent ions in the medium. Furthermore, both enzymatic activity and proton translocation of ER H^+-PPase were sensitive to F^-, a common inhibitor of most PPases. The requirement of divalent cations for ER H^+-PPase was also examined (Table 3). ER H^+-PPase absolutely requires Mg^{2+} for its enzymatic activity and proton translocation. In contrast, other divalent cations scrutinized were ineffective for ER H^+-PPase. Nevertheless, the presence of Ca^{2+} in the reaction medium could inhibit enzymatic activity of ER H^+-PPase, a phenomenon also observed in tonoplast (data not shown; cf., Maeshima, 1991, 2000).

The optimal pH for ER H^+-PPase was in the weak alkaline range (Fig. 3), similar to tonoplast H^+-PPase. However, the enzymatic reaction of tonoplast H^+-PPase was almost abolished at pH below 6.0, while that of ER H^+-PPase still retained approximately one-third of the maximal enzymatic activity. ER H^+-PPase was therefore more resistant to the acid medium than vacuoles.

Table 3. Effects of divalent cations on ER H^+-PPase

Addition (3 mM)	H^+-translocation (% ΔF/mg protein min)	Specific activity (μmol PP_i/mg protein h)
Mg^{2+}	28.6±3.7	10.1±0.1
Mn^{2+}	1.7±0.6	0
Ni^{2+}	0	0.3±0.1
Ca^{2+}	0	0.2±0.1
Cd^{2+}	0	0.3±0.2
Co^{2+}	0	0
Cu^{2+}	0	0
Zn^{2+}	0	0

Proton translocation of ER H^+-PPase was measured in a medium containing 5 mM Mops-KOH (pH 7.9), 0.25 M sorbitol, 3 mM divalent cations, 50 mM KCl, 5 μM acridine orange, and 60 μg/mL membrane proteins. The fluorescence quenching was initiated by adding 0.2 mM PP_i at 25 °C. The initial rate of proton translocation and the specific activity of enzyme were determined as described under "Materials and methods". Each value is the mean of three independent measurements.

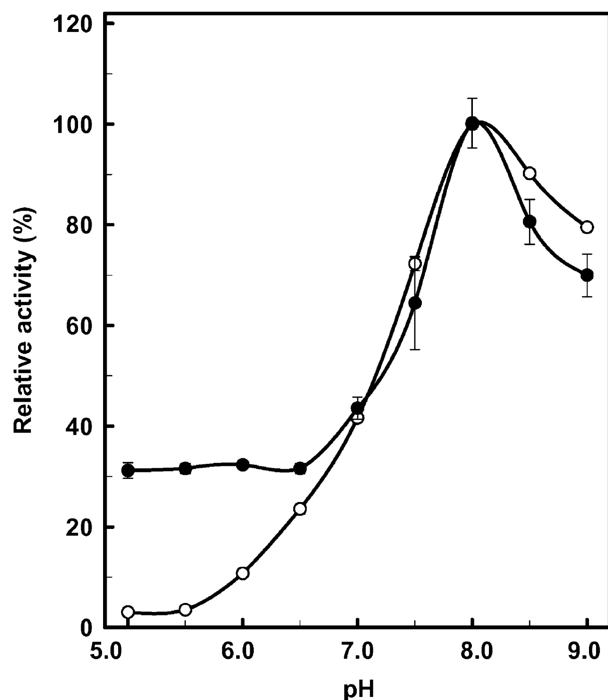


Figure 3. The pH profiles on pyrophosphatase activities of tonoplast and ER-enriched vesicles. PP_i hydrolysis activities of both ER (●) and tonoplast (o) were measured as described under "Materials and methods" in 30 mM Tris-Mes solution of various pH values. Both $MgSO_4$ and PP_i were kept constant at 1 mM. Each value is the mean of three independent measurements.

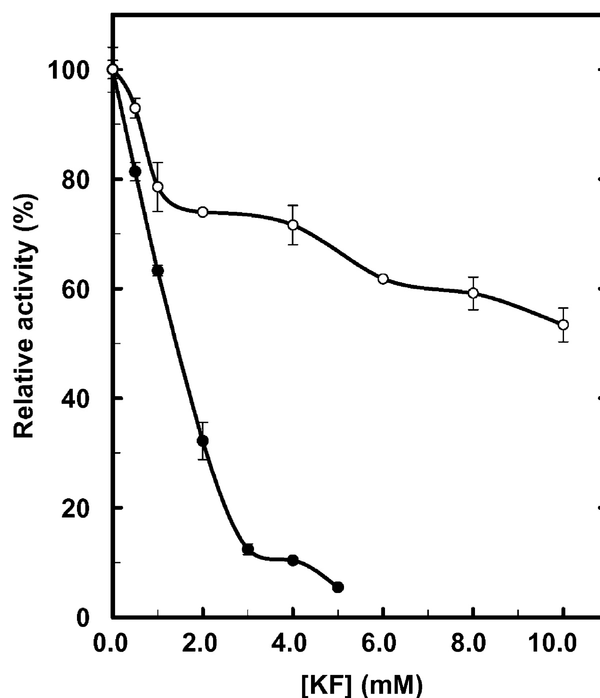


Figure 4. Effects of KF on vacuolar and ER H^+-PPases. PP_i hydrolysis activities of vacuolar (o) and ER (●) H^+-PPases were determined as described in Fig. 3 except 25 mM Mops/KOH (pH 7.9) was added in the reaction medium. KF at indicated concentrations was added for inhibition. Control specific activities for vacuolar and ER H^+-PPases were approximately 44.6±1.3 and 9.4±0.3 μmol PP_i hydrolyzed/mg protein h, respectively. Each value is the mean of three independent measurements.

Inhibitor sensitivities of ER H^+-PPase

A series of experiments were carried out to compare inhibitor sensitivities of vacuolar and ER H^+-PPases. Concentration dependence of ER H^+-PPase on F^- is shown in Fig. 4. At 5 mM of KF, PP_i hydrolysis of ER-enriched vesicles was almost completely inhibited. However, vacuolar H^+-PPase still possessed 60% of the control enzymatic activity under the same conditions. Furthermore, we demonstrated that imidodiphosphate, a pyrophosphate analogue, exerted more severe inhibition on H^+-PPase of ER than vacuolar membrane (Fig. 5; cf., Zhen et al., 1994). The different sensitivities of ER H^+-PPase to various inhibitors from vacuolar H^+-PPase again reveal they belong to distinct types of the PP_i-hydrolase.

Finally, the sensitivities of ER H^+-PPase to group-specific modifiers were also determined (Table 4). TNM completely diminished the enzymatic activity of ER H^+-PPase, indicating tyrosine residues may play a crucial role in the catalytic domain (cf., Yang et al., 1996). PGO and NEM gave partially inhibitory effects on ER H^+-PPase. In contrast, DCCD and FITC (fluorescein 5′-isothiocyanate) exerted lesser degree of inhibition on the ER H^+-PPase. Nevertheless, the sensitivities of ER H^+-PPase to modifiers

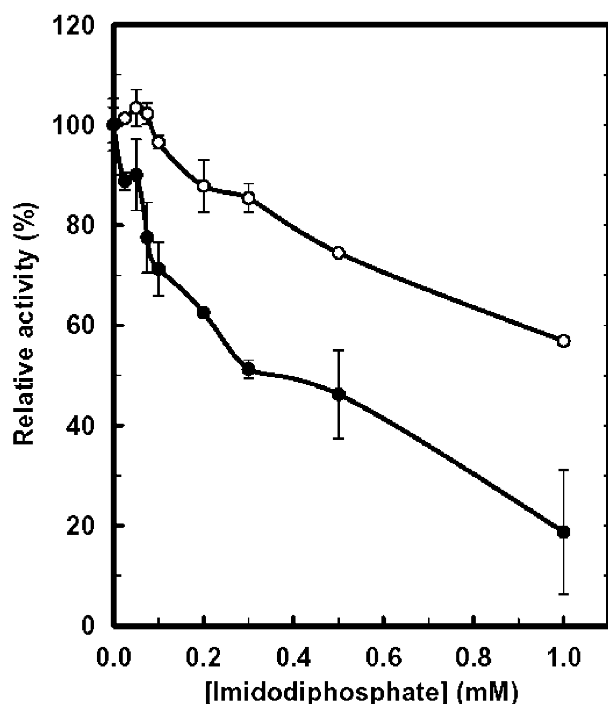


Figure 5. Effect of imidodiphosphate on vacuolar and ER H^+-PPases. PP_i hydrolysis activities of vacuolar (o) and ER (●) H^+-PPase were analyzed as described in Fig. 4. Imidodiphosphate of indicated concentrations was added for inhibition. Control specific activities for vacuolar and ER H^+-PPases were approximately 26.6±1.3 and 6.2±0.3 μmol PP_i hydrolyzed/mg protein h, respectively. Each value is the mean of three independent measurements.

Table 4. Inhibitor sensitivities of H^+-PPase of ER-enriched and tonoplast vesicles

Inhibitor	Incubation conditions	Residual activity (%)	
		ER	TP
–	–	100	100
DCCD	20 mM, 60 min	75.4±0.9	58.6±1.3
FITC	2 mM, 30 min	87.0±5.3	64.3±3.0
NEM	40 μM, 10 min	65.1±0.8	58.4±3.9
PGO	50 mM, 20 min	26.9±3.8	46.3±1.9
TNM	4 mM, 20 mM	0	0

The preincubation with inhibitors and measurements of specific activities of both enzymes were carried out as described under "Materials and methods". Control PP_i hydrolysis activities for ER and tonoplast vesicles were approximately 10.2±0.3 and 49.0±1.5 μmol PPi consumed/mg protein h, respectively. Each value is the mean of three independent measurements. TP, tonoplast vesicles.

are slightly different from those of tonoplast (approximately 6.7–22.7%). We speculate that both types of H^+-PPases may share common catalytic mechanism but with different structure in the active domain.

Many studies have attempted to determine whether all kinds of organellar membranes contain proton-pumping pyrophosphatase (cf., Maeshima, 2000; Mitsuda et al., 2001). Robinson (1996) showed the ubiquitous distribution of vacuolar H^+-PPase within the parenchyma cell from pea cotyledons. Possible existence of a pyrophosphatase on plasma membranes from *Ricinus communis* seedlings (Long et al., 1995), cauliflower inflorescence cells (Ratajczak et al., 1999), and tobacco culture cells (Sikora et al., 1998) was suggested. In addition, several groups successfully observed a proton pumping activity of submitochondrial particles (Vianello et al., 1991; Jiang et al., 2000). A pyrophosphatase was also purified from thylakoid membrane of spinach (Jiang et al., 1997). Recently, it was revealed that Golgi body contains a new type of H^+-pyrophosphatase (Mitsuda et al., 2001). Here, our results further provide direct evidence on the presence of PP_i-supported proton translocation of ER-enriched vesicles. Distinct characteristics of ER H^+-PPases from vacuolar H^+-PPases exclude possible contamination by latter species and more importantly indicate that they belong to different types of PP_i-dependent proton translocase, an analog to all kinds of H^+-ATPases on various organellar membranes. Taken together, we believe that most subcellular membranes of higher plants may contain their own types of proton pumping PPases. Ubiquitous coexistence of proton translocating ATPases and PPases on the same subcellular membranes, for similar functions but at the expense of different energy sources, may provide advantages in the bioenergetic systems of higher plants. Further studies such as purification and detailed characterization of each unique type of H^+-PPase from these endomembranes are recommended.

Acknowledgements

This work was supported by a grant from National Science Council, Republic of China (NSC92-2311-B-007-005) to RLP.

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