Subunit interaction of vacuolar H⁺-pyrophosphatase as determined by high hydrostatic pressure

Su J. YANG, Shu J. KO, Yuan R. TSAI¹, Shih S. JIANG, Soong Y. KUO, Shu H. HUNG and Rong L. PAN²

Institute of Radiation Biology, College of Nuclear Science, National Tsing Hua University, Hsin Chu 30043, Taiwan, Republic of China

Vacuolar H⁺-pyrophosphatase (H⁺-PPase) from etiolated hypocotyls of mung bean (*Vigna radiata* L.) is a homodimer with a molecular mass of 145 kDa. The vacuolar H⁺-PPase was subjected to high hydrostatic pressure to investigate its structure and function. The inhibition of H⁺-PPase activity by high hydrostatic pressure has a pressure-, time- and protein-concentration-dependent manner. The $V_{\max}$ value of vacuolar H⁺-PPase was dramatically decreased by pressurization from 293.9 to 70.2 μmol of PP_i (pyrophosphate) consumed/h per mg of protein, while the K_m value decreased from 0.35 to 0.08 mM, implying that the pressure treatment increased the affinity of PP_i to vacuolar H⁺-PPase but decreased its hydrolysis. The physiological substrate

and its analogues enhance high pressure inhibition of vacuolar H⁺-PPase. The HPLC profile reveals high pressure treatment of H⁺-PPase provokes the subunit dissociation from an active into inactive form. High hydrostatic pressure also induces the conformational change of vacuolar H⁺-PPase as determined by spectroscopic techniques. Our results indicate the importance of protein–protein interaction for this novel proton-translocating enzyme. Working models are proposed to interpret the pressure inactivation of vacuolar H⁺-PPase. We also suggest that association of identical subunits of vacuolar H⁺-PPase is not random but proceeds in a specific manner.

INTRODUCTION

Plant vacuoles contain two types of proton-translocating pumps, H⁺-ATPase (EC 3.6.1.3) and H⁺-pyrophosphatase (H⁺-PPase, EC 3.6.1.1). Both enzymes catalyse electrogenic proton translocation to generate inside-acidic, inside-negative electrochemical potential for the energization of secondary transport [1,2]. Plant vacuolar H⁺-ATPase has been extensively studied and several of its properties were well characterized [1]. On the other hand, plant vacuolar H⁺-PPase was recently recognized as a new category of ion translocator using PP_i exclusively as an energy source and was subjected to identification, purification and characterization [2]. The vacuolar H⁺-PPase from mung bean consists of a single kind of polypeptide with a molecular mass of approx. 66000 Da [2–4]. The cDNA clones of tonoplast H⁺-PPase from *Arabidopsis thaliana* [5], *Hordeum vulgare* [6], *Beta vulgaris* [7], *Vigna radiata* [8], *Nicotiana tabacum* [9] and *Oryza sativa* [10] were obtained and the amino acid sequences deduced. Several amino acid residues conferring the inhibition by their respective modifiers were determined [11–14]. It was also demonstrated that tonoplast H⁺-PPase functions as a homodimer during PP_i hydrolysis and proton translocation as determined by gel filtration and radiation inactivation [5,15–19]. The target size analysis indicated that the functional mass and the subunit structure of vacuolar H⁺-PPase depend on the state of the enzyme [4]. However, the protein–protein interaction and the relationship between two identical subunits in this protein complex are still unclear.

Hydrostatic pressure is a potentially useful tool for probing the possible organization of many proteins [20–24]. Upon pressure treatment, a protein complex could be dissociated either irreversibly or reversibly into a monomer or smaller oligomer,

depending on the range of pressure employed and protein species of interest. High hydrostatic pressure below 3 kbar (1 kbar = 10⁵ kPa) seems to be the only tool that can provide information on protein–protein interaction with minimum direct effects on the tertiary structure of single-chain proteins [20,22,25]. In this study we used high hydrostatic pressure to investigate subunit interaction and relationship between structure and function of the vacuolar H⁺-PPase. The mechanism of pressure inactivation of vacuolar H⁺-PPase also provides insights to the assembly of the enzyme from two identical subunits.

MATERIALS AND METHODS

Materials

Seeds of *Vigna radiata* L. (mung bean) were soaked in tapwater for 6 h, and then germinated at room temperature in the dark in a commercial seedling incubator. Hypocotyls of 4-day-old etiolated seedlings were excised and used as starting materials. Fluorescein 5'-isothiocyanate (FITC) was obtained from Molecular Probes (Eugene, OR, U.S.A.) and dissolved in 50 mM Bicine-NaOH (pH 7.5) as the stock solution. All other chemicals were purchased from Sigma or Merck, and used without further purification.

Membrane preparation and purification of H⁺-PPase

Tonoplast vesicles were prepared from etiolated hypocotyls of mung bean seedlings as described elsewhere [4,26]. All steps were carried out at 4 °C except where otherwise indicated. Vacuolar H⁺-PPase was then purified from resealed tonoplast vesicles as

Abbreviation used: H⁺-PPase, H⁺-pyrophosphatase.

¹ Present address: Kao-Shiung Medical College, 100 Shih-Chuan 1st Road, Kao-Shiung, Taiwan, Republic of China.

² To whom correspondence should be addressed (e-mail rlpan@RB.nthu.edu.tw).

follows. Tonoplast vesicles were centrifuged at 120 000 *g* for 1 h and the pellet was resuspended in Buffer I (27 ml) [20 mM Tris-acetate (pH 7.5)/20 % (v/v) glycerol/1 mM dithiothreitol/1 mM Tris-EGTA/2 mM MgCl_2 /protein concentration of 1 mg/ml]. Sodium deoxycholate and KCl were added to final concentrations of 2 mg of protein/ml and 50 mM, respectively. Following 10 min incubation at 4 °C, deoxycholate-treated tonoplast vesicles were centrifuged at 150 000 *g* for 30 min. The membrane pellet was redissolved in Buffer I (14 ml) and solubilized by 0.4 % (w/v) L- α -lysophosphatidylcholine for 30 min at room temperature. After centrifugation at 150 000 *g* for 50 min, the supernatant (5–10 mg protein) was concentrated by Centricon-10 (Amicon, Inc., Danvers, MA, U.S.A.) to 200 μl and then applied to a FPLC column of Superose 12 HR 10/30 pre-equilibrated with a running buffer [20 mM Tris/HCl (pH 7.5)/20 % (v/v) glycerol/0.1 % (w/v) Triton X-100/1 mM Tris-EGTA/2 mM MgCl_2]. The flow rate was 0.4 ml/min at room temperature. Fractions (2.5 ml) with PPase activity were collected and then applied onto a Mono Q HR 5/5 FPLC column pre-equilibrated with the same buffer as mentioned above. The column was eluted at a flow rate of 0.5 ml/min with a salt gradient of 0–0.5 M NaCl. Fractions (1 ml) with highest activity were collected and stored immediately at –70 °C for further use.

Pressure treatment, enzyme assay, and protein determination

Tonoplast vesicles or purified vacuolar H^+ -PPase were placed in a bombardment cell of an SLM Aminco French Press (Model FA-030) at room temperature under different pressures for various time intervals. After the release of pressure, enzyme activities, proton translocation and spectrophotometric measurements were carried out accordingly. Enzyme activity was assayed in the reaction medium containing 25 mM Mops-KOH (pH 7.2), 1.0 mM PP_i , 1.0 mM MgSO_4 , 50 mM KCl, 0.02 % (w/v) Triton X-100, 80 $\mu\text{g/ml}$ phosphatidylcholine and approximately 10 $\mu\text{g/ml}$ purified enzyme [19]. For measurement of enzyme activity of vacuolar membrane, the protein concentration was 60 $\mu\text{g/ml}$ and inhibitors of possible contaminating enzyme activities (0.1 mM sodium orthovanadate, 0.5 mM NaN_3 and 50 mM KNO_3 for P-, F- and V-type ATPases, respectively, and 0.1 mM ammonium molybdate for acid phosphatases) were added. After a 15 min incubation at 33 °C, the reaction was terminated by adding a stop solution [1.7 % (w/v) ammonium molybdate/2 % (w/v) SDS/0.02 % (w/v) 1-amino-2-naphthol-4-sulphonic acid]. The released P_i was determined spectrophotometrically [27,28]. Protein concentration was determined by a modified Lowry method [29] with BSA as a standard.

Measurements of proton translocation

Proton translocation was measured as fluorescence quenching of Acridine Orange (excitation wavelength 495 nm, emission wavelength 530 nm). The reaction mixture contained 5 mM Mops-KOH (pH 7.2), 0.25 M sorbitol, 1 mM MgSO_4 , 50 mM KCl, 0.1 mM sodium orthovanadate, 0.1 mM ammonium molybdate, 50 mM KNO_3 , 0.5 mM NaN_3 , 5 μM Acridine Orange and 60 μg of membrane protein. The fluorescence quenching was initiated by adding 1 mM PP_i . The ionophore gramicidin (2 $\mu\text{g/ml}$) was added at the end of each assay.

Size-exclusion HPLC

Size-exclusion chromatography of purified vacuolar H^+ -PPase was performed on a HPLC Shodex WS-804F column pre-equilibrated with a buffer containing 50 mM Tris/HCl (pH 7.2), 100 mM K_2SO_4 and 0.05 % (w/v) NaN_3 . Elution was monitored

by the absorption at 254 nm. The flow rate was 0.75 ml/min. Carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), α -amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa) were used as molecular-size standards.

Labelling by FITC

Purified vacuolar H^+ -PPase (0.2 mg/ml) was incubated with 0.5 mM FITC in 50 mM Bicine-NaOH (pH 7.5) buffer at 33 °C for 20 min. Free modifier was removed by filter-centrifugation through a Sephadex G-25 (Superfine) column equilibrated in 50 mM Tris-acetate (pH 7.0) and 5 mM MgCl_2 .

Spectroscopic measurements

Absorption spectra of proteins were determined in a medium containing 50 mM Tris/HCl (pH 7.2) and 1 mM MgCl_2 at room temperature. Intrinsic and extrinsic fluorescence measurements of purified vacuolar H^+ -PPase and FITC-PPase were carried out with excitation wavelengths of 273 and 495 nm, respectively. Fluorescence polarization of purified vacuolar H^+ -PPase and FITC-PPase was measured conventionally using an SLM 4800 fluorescence spectrometer equipped with Glan-Thompson polarizers, at excitation wavelengths of 273 and 495 nm, respectively [30]. Secondary structure of purified vacuolar H^+ -PPase (1.2 μM) with/without pressure treatment was determined at 25 °C on a Lakewood N.J. Model 62A DS spectrometer using 0.1 cm pathlength quartz cells [31]. CD spectra shown were averages of three scans for each sample. The contents of secondary structure were calculated according to the method of Gans et al. [32].

RESULTS

Pressure effects on the PPase activity

Prior to the pressure study of vacuolar H^+ -PPase, we tested the purity and subunit structure of our preparation. The vacuolar H^+ -PPase purified from tonoplast of mung bean seedlings in this work contains only a single kind of polypeptide with a molecular mass of 73 kDa (Figure 1A). The contamination was negligible in our preparation as visualized on SDS/PAGE by silver staining, and also as examined on HPLC (results not shown). The native molecular mass of vacuolar H^+ -PPase was approximately 145 kDa as determined by size-exclusion gel filtration chromatography (Figure 1B). Our results thus concur with those of previous workers using chromatography [15,26], disulphide cross-linking on polyacrylamide gel [18] and radiation inactivation [4,16]. We were therefore convinced that the mung bean vacuolar H^+ -PPase is a dimeric enzyme and our preparation was pure enough for further studies.

Purified vacuolar H^+ -PPase was exposed to a range of high pressure. After 10 min treatment, the pressure was released and the enzymic activity measured. The PP_i hydrolysis reaction was markedly inhibited in a pressure-dependent manner up to 2.3 kbar, the upper limit of our system (Figure 2, ●). The P_{50} value, the hydrostatic pressure when 50 % activity of control is inhibited, occurs at approximately 0.88 kbar for purified vacuolar H^+ -PPase. At higher pressure, enzyme activity of purified vacuolar H^+ -PPase was substantially inhibited. In parallel experiments, the enzymic activity of the membrane-bound vacuolar H^+ -PPase was found to decline likewise as the pressure increased (Figure 2, ○). The half-maximal inhibition of the membrane-bound vacuolar H^+ -PPase was at $P_{50} = 1.25$ kbar. Furthermore, we investigated the time course of the pressure effect on both membrane-

Figure 1 SDS/PAGE pattern and size-exclusion gel filtration of the purified vacuolar H⁺-PPase

(A) SDS/PAGE pattern: lane 1, the standard markers with molecular masses (kDa) indicated on the left; lane 2, purified vacuolar H⁺-PPase. (B) Size-exclusive gel-filtration chromatography of the purified vacuolar H⁺-PPase. H⁺-PPase (10 μ g) was injected through a Shodex protein WS-804F column. The column was equilibrated and eluted by a buffer containing 50 mM Tris/HCl (pH 7.5), 100 mM K₂SO₄, 0.05% (w/v) NaN₃ and 0.1% (w/v) Triton X-100 with a flow rate of 0.75 ml/min at room temperature. Carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), α -amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa) were used as molecular-size (MW) standards. $\rightarrow$, The maximal peak (145 kDa) of vacuolar H⁺-PPase.

bound and purified vacuolar H⁺-PPases (Figure 3). Vacuoles and purified vacuolar H⁺-PPase were exposed to a high hydrostatic pressure of 1.1 kbar for various time intervals. PP_i hydrolysis activities of both membrane-bound and purified H⁺-PPases were progressively abolished with prolonged treatment (Figure 3). The $t_{1/2}$ value, the time required to bring the specific activity to 50% of control, was 2.1 min for the purified H⁺-PPase, whereas for vacuoles it was 7.0 min. Both pressure profile and time course indicate a slightly higher sensitivity of the purified vacuolar H⁺-PPase to high pressure, compared with membrane-bound PPase

Figure 2 Inactivation of enzymic activities and H⁺ translocation of vacuolar H⁺-PPase at various pressures

Vacuoles (60 μ g/ml) and purified vacuolar H⁺-PPase (10 μ g/ml) were placed in the pressure cell under different pressures for 10 min at room temperature in a medium containing 25 mM Mops-KOH (pH 7.2), 1 mM MgSO₄ and 50 mM KCl. After pressure treatment the PPase activity and proton translocation were assayed as described under Materials and methods. The control enzymic activities were approximately 16.9 and 198.4 μ mol of PP_i consumed/h per mg of protein for membrane-bound and purified vacuolar H⁺-PPases, respectively. $\circ$, Membrane-bound H⁺-PPase; $\bullet$, purified H⁺-PPase; $\blacktriangledown$, proton translocation. Each data point was the average of three experiments.

activity. We speculate that membrane may provide substantial protection to the vacuolar H⁺-PPase against high hydrostatic pressure. Alternatively, the structure of the vacuolar H⁺-PPase on membrane may differ from its solubilized form, resulting in distinct pressure sensitivities (see [4]).

Pressure response of PPase-associated proton translocation of vacuoles was also examined. The H⁺-pumping activity of vacuolar PPase was markedly suppressed, with a concomitant decrease in its capacity, as the pressure was raised. A P_{50} value of 1.09 kbar for PP_i-dependent proton translocation of vacuoles was observed. Possible leakage of the vacuolar membrane by pressure treatment is excluded since the coupling ratio (relative fluorescence change/specific activity) at each pressure dose remained constant (results not shown). Nevertheless, high pressure treatment inhibited both reactions of vacuolar H⁺-PPase and its associated proton translocation.

Semi-logarithmic plots of specific activities of purified vacuolar H⁺-PPase against pressure experienced yielded lines with characteristic break points (P_b) and slopes (results not shown). The biphasic responses demonstrate the complicated inhibition mechanism of enzymic activities of the vacuolar H⁺-PPase under the pressure treatment. The relative slopes within the pressure range lower (S_L) and higher (S_H) than P_b could be calculated for proton translocation and enzymic activities of H⁺-PPases. For instance, a S_L value of -1.12 and a S_H value of -1.40 (arbitrary units) were obtained for enzymic activity of purified H⁺-PPase. Similarly, S_L values of -0.38 and -0.87 and S_H values of -1.15 and -1.58 were determined for membrane-bound H⁺-PPase and its associated proton translocation, respectively. Steeper slopes (smaller S_H values) of all reactions suggest that major inactivation of the H⁺-PPases occurs within the high pressure region. Variation in S values for PP_i hydrolysis and proton translocation

Figure 3 Time courses of the inactivation of vacuolar H⁺-PPase by high hydrostatic pressure

Vacuole vesicles (60 µg/ml) and purified vacuolar H⁺-PPase (10 µg/ml) were incubated under 1.1 kbar pressure at room temperature for various time intervals in a medium containing 25 mM Mops-KOH buffer (pH 7.0) and 50 mM KCl. At time intervals, the pressure was released and the residual enzyme activity of the PPase was measured immediately as described under Materials and methods. The control enzymic activities were approximately 16.9 and 198.4 µmol of PP_i consumed/h per mg of protein for membrane-bound and purified vacuolar H⁺-PPase, respectively. ○, Membrane-bound H⁺-PPase; ●, purified vacuolar H⁺-PPase. Each data point was the average of three experiments.

Table 1 Ligand effects on the pressure-induced inactivation of vacuolar H⁺-PPase

Purified vacuolar H⁺-PPase (10 µg/ml) was pre-incubated with 3 mM of various ligands in the presence or absence of 3 mM MgSO₄ at room temperature for 10 min. After incubation, the reaction mixture was pressurized at 1.1 kbar for 10 min and enzymic activity was assayed as described in the Materials and methods section. Control specific activity was 114.3 µmol of PP_i consumed/h per mg of protein. Each value was the mean of three assays. Abbreviations used: pNPP, *p*-nitrophenyl phosphate; IDP, imidodiphosphate; PT, phosphothreonine; PS, phosphoserine; G-6-P, glucose-6-phosphate.

Ligand (3 mM)	Activity as substrate (%)	Relative activity (%)	
		—MgSO ₄	+MgSO ₄
I. Control		100.0	100.0
II. Pressure		40.9 ± 2.0	38.6 ± 5.5
PP _i	100	18.2 ± 2.7	10.3 ± 0.9
pNPP	4.2	28.9 ± 0.5	12.7 ± 1.1
IDP	5.7	35.3 ± 2.8	15.1 ± 4.3
PT	6.5	15.6 ± 5.5	3.2 ± 1.5
PS	< 1.0	14.2 ± 7.3	3.9 ± 2.4
P _i	—	< 1.0	< 1.0
G-6-P	8.5	11.3 ± 0.9	2.1 ± 0.7
ATP	3.7	46.0 ± 4.5	34.9 ± 3.1
ADP	4.6	42.1 ± 3.6	28.0 ± 6.7
AMP	< 1.0	43.5 ± 4.8	34.3 ± 2.7

provides evidence of different enzyme structure and/or mechanisms involved in pressure inactivation.

Effect of protein concentration on the pressure inhibition of the purified vacuolar H⁺-PPase was further scrutinized. Under 1.7 kbar of hydrostatic pressure, the specific activity of the

Figure 4 Size-exclusion HPLC of purified vacuolar H⁺-PPase after pressure treatment

Control and pressurized H⁺-PPase (20 µg) were subjected to size-exclusion gel-filtration chromatography on a HPLC Shodex WS-804F column. The flow rate was 0.75 ml/min, and the elution buffer was as described under Materials and methods. (A) Control; (B) 1.7 kbar, 10 min; (C) 2.3 kbar, 10 min; (D) 2.3 kbar, 30 min.

purified vacuolar H⁺-PPase was inhibited to 22% of control at concentrations below 10 µg/ml. However, the degree of pressure inactivation declined as the concentration of vacuolar H⁺-PPase in the pressure compartment increased. The concentration dependence of the pressure effect suggests that the vacuolar H⁺-PPase complex may experience dissociation–reassociation reactions [22].

Effects of high pressure (1.6 kbar for 10 min) on *K_m* and *V_{max}* of the purified vacuolar H⁺-PPase were also investigated. *K_m* and *V_{max}* of the control vacuolar H⁺-PPase activity were 0.35 mM and 293.9 µmol of PP_i consumed/h per mg of protein, respectively. After pressurization, *V_{max}* was decreased dramatically by four-fold to 70.2 µmol of PP_i consumed/h per mg of protein, and *K_m* to 0.08 mM. Pressurization on the vacuolar H⁺-PPase increased the accessibility of the physiological substrate to the active site, but decreased the turnover rate of the enzyme–substrate complex to the product.

To test whether pressure-induced inactivation was reversible, purified vacuolar H⁺-PPase was placed under atmospheric pressure at room temperature for various periods after moderate pressurization. Untreated enzyme was generally stable at room temperature under atmospheric pressure for more than 24 h (results not shown). However, upon releasing the pressure, the enzymic activity of the vacuolar H⁺-PPase decreased gradually as the time intervals increased. For instance, PP_i hydrolysis activity, measured immediately after 0.7 kbar pressure treatment, was 60% of control, whereas it declined slightly to approximately 35% when measured 60 min after pressure release. However, upon higher pressure treatment (2.3 kbar), the drift in the enzymic activity was not obvious, since vacuolar H⁺-PPase was almost completely inactivated. Nevertheless, we believe the inactivation continues after the withdrawal of high pressure.

Figure 5 Pressure effects on spectral properties of purified vacuolar H⁺-PPase

Purified vacuolar H⁺-PPase (25 μ g/ml) was treated with various pressures in a medium containing 50 mM Tris/HCl (pH 7.2) and 1 mM MgCl₂ for 10 min. After pressure treatment spectral properties of purified vacuolar H⁺-PPase were determined accordingly at room temperature. **(A)** Absorption spectra. Pressures from top are: atmospheric pressure, 0.23 and 0.35 kbar. **(B)** Intrinsic fluorescence spectra. The excitation wavelength was 275 nm and pressures from top are: atmospheric pressure, 0.35, 0.55, 1.1 and 2.3 kbar. **(C)** Extrinsic fluorescence spectra of FITC-PPase. Purified vacuolar H⁺-PPase (25 μ g/ml) was labelled with 0.5 mM FITC at 33 °C for 20 min as described under Materials and methods. The fluorescence of FITC-PPase was observed using an excitation wavelength of 495 nm in a medium containing 50 mM Tris/HCl (pH 7.2) and 1 mM MgCl₂. Pressures from top are: atmospheric pressure, 0.05 and 2.2 kbar. **(D)** CD. Purified vacuolar H⁺-PPase was treated under 2.3 kbar at room temperature for 10 min in the presence of 10 mM Tris-HCl (pH 7.2) and 1 mM MgCl₂. The secondary structure of control (●) and pressure-treated (○) vacuolar H⁺-PPase (1.2 μ M) was determined by CD. Each data point was the average of three experiments.

Ligand effect on pressure-induced inactivation

Purified vacuolar H⁺-PPase was preincubated for 10 min at 4 °C with different ligands in the presence or the absence of 3 mM MgSO₄, and then subjected to 1.1 kbar pressure treatment. Table 1 summarizes the effects of the physiological substrate PP_i, P_i, PP_i analogues and several nucleotides. In the absence of ligands and MgSO₄, the specific activity of PP_i hydrolysis decreased to 40.9% of control upon the pressure treatment. Addition of MgSO₄ during pressure treatment exerted no significant effect on the enzymic activity (38.6%). However, with PP_i under pressure, the specific activity declined markedly with concomitant increase

in PP_i concentration. Obviously, PP_i fails to protect the enzyme against pressure-induced inactivation; in contrast, it augments the inhibitory effect of hydrostatic pressure. Similarly, all the PP_i analogues also increased the sensitivity of the vacuolar H⁺-PPase to high hydrostatic pressure. Furthermore, in the presence of its product, P_i, the enzymic activity was completely inhibited by the high hydrostatic pressure. We believe the intercalation of the phosphate moiety in the active site may weaken the structure of the enzyme, resulting in an augmented pressure effect. On the other hand, ATP, ADP and AMP had negligible effects on the enzyme. The huge ribonucleotide moieties may hinder their accessibility to the active site of vacuolar H⁺-PPase.

Table 2 Effect of high pressure on fluorescence polarization

Purified vacuolar H⁺-PPase and FITC-PPase (15 µg/ml) were exposed to 2.3 kbar high hydrostatic pressure for the time intervals indicated. The excitation wavelengths were 275 and 495 nm for control and FITC-modified PPase, respectively.

	Exposure time (min)	Vacuolar H ⁺ -PPase			FITC-PPase		
		Polarization	% Of control	Anisotropy	Polarization	% Of control	Anisotropy
Control	0	0.121	(100)	0.084	0.172	(100)	0.122
2.3 kbar	10	0.100	(83)	0.069	0.056	(33)	0.038
	30	0.079	(65)	0.054	0.043	(25)	0.029

Size-exclusion HPLC of vacuolar H⁺-PPase after pressure treatment

Structural properties of purified vacuolar H⁺-PPase after pressure treatment were investigated by size-exclusion HPLC. Upon high hydrostatic pressure, a major portion of the enzyme appeared in fractions of larger molecular mass (original protein peak) with a minor portion of lower molecular mass on HPLC profile of the vacuolar H⁺-PPase (Figure 4). Moreover, prolonged treatment results in an increase in portions of lower molecular masses. For instance, after 2.3 kbar pressurization for 10 min, 25.9% of protein appeared in fractions of lower molecular mass, while 46.4% was observed for the same pressure but with longer treatment time, 30 min. After release of pressure, the subunits of the vacuolar H⁺-PPase may reorganize in various manners leading to distinct apparent molecular masses.

Effects of pressure on spectroscopic properties of PPase

Pressure effects on several spectroscopic properties of purified vacuolar H⁺-PPase were observed. Light scattering is a probe to investigate the surface roughness of macromolecules. After pressure treatment, light scattering by vacuolar H⁺-PPase was enhanced by several fold (results not shown), implying an obvious increase in surface roughness of protein. The enhancement in the surface roughness comes directly from a change in protein structure upon pressure. The purified vacuolar H⁺-PPase displays an intrinsic characteristic absorption spectrum in the region of 250–300 nm (Figure 5A). Upon the pressure treatment, absorption of the purified vacuolar H⁺-PPase decreased as the pressurization increased. The absorption drop illustrates again a possible conformational change in the vacuolar H⁺-PPase after the pressure treatment. This speculation was further verified by pressure effects on the intrinsic fluorescence of vacuolar H⁺-PPase. The intrinsic fluorescence spectra of the purified vacuolar H⁺-PPase were measured at an excitation wavelength of 273 nm (Figure 5B). Similarly, intrinsic fluorescence yields of vacuolar H⁺-PPase were decreased after the enzyme was subjected to the pressure treatment. Interestingly, the pressurization does not shift significantly the absorption and fluorescence maxima of vacuolar H⁺-PPase. These results indicate that the pressurization induced a conformational change, but did not expose the intrinsic chromophores to a medium of different polarity.

FITC was used as a spectroscopic probe to study the effects of high pressure on the structure of the modified domain of purified vacuolar H⁺-PPase. FITC could label the vacuolar H⁺-PPase with a concomitant loss of the enzyme activity in a concentration-dependent manner (data not shown). The half maximal inhibition (*I*₅₀ value) occurred at 0.5 mM for the purified vacuolar H⁺-PPase. The stoichiometry of the labelling was approximately 1 mol of FITC/mol of subunit. FITC-PPase displays an extrinsic

fluorescence at 530 nm upon excitation at 490 nm, an absorption peak of the FITC moiety. The effect of high pressure on extrinsic fluorescence of FITC-PPase was then determined to confirm the conformational change in the enzyme (Figure 5C). Fluorescence quenching of FITC-PPase was sensitive to the pressure applied. However, the maximal fluorescence peak of the FITC moiety was not found to shift upon pressure treatment. We suggest therefore that pressurization probably induces a conformational change of the enzyme without disturbing the polarity of the environment where the FITC-modified domain is located.

Table 2 shows the effects of high hydrostatic pressure on fluorescence polarization of control and FITC-modified vacuolar H⁺-PPases. The fluorescence polarization of the purified vacuolar H⁺-PPase and FITC-moiety of the labelled enzyme progressively declined with increase in pressure. For instance, the fluorescence polarization of the vacuolar H⁺-PPase changed from 0.121 to 0.1, whereas that of FITC-PPase changed from 0.172 to 0.056 when the hydrostatic pressure was increased to 2.2 kbar. Furthermore, prolonged incubation enhanced pressure effects on fluorescence polarization. The pressure effects on fluorescence polarization were more pronounced on the FITC moiety than on the enzyme itself, suggesting a higher sensitivity of the modified domain, presumably the active site, to pressurization. Nevertheless, changes in fluorescence polarization demonstrate an alteration in the mobility of several portions in the protein complex after pressurization. These results are explained as the consequence of the reorganization of protein subunits upon pressure treatment.

Secondary structure of the purified vacuolar H⁺-PPase was determined by CD. Figure 5(D) shows CD spectra of untreated and pressure-treated vacuolar H⁺-PPases. The untreated vacuolar H⁺-PPase contained approximately 61.4% α -helix. Upon pressure treatment, the CD spectrum of vacuolar H⁺-PPase was altered significantly and the content of α -helix obviously reduced to 50.2%. The CD spectra clearly indicate a pressure-induced change in secondary structure of vacuolar H⁺-PPase. Taken together, the spectroscopic analysis unequivocally demonstrates a conformational change of vacuolar H⁺-PPase upon the high hydrostatic pressure.

DISCUSSION

This work aims to examine the subunit interaction of plant vacuolar H⁺-PPase by high-pressure technique. The inhibition of vacuolar H⁺-PPase by high hydrostatic pressure was pressure-, time- and protein-concentration-dependent. The upper limit of the pressure employed in our system is much lower than 3.2 kbar, at which the change in tertiary conformation of single polypeptides is still negligible [20,22], but the dissociation of several dimers [23,33], tetramers [34–37] and larger aggregates [24,25,38]

was observed. The pressure effects observed in this work are primarily the consequence of the dissociation–reassociation of the identical subunits of vacuolar H⁺-PPase after pressurization.

Comparison of P_{50} values indicates that the membrane-bound H⁺-PPase is more resistant to hydrostatic pressure than its purified form. The distinct sensitivities to pressure treatment demonstrate that vacuolar membrane plays a crucial role in maintaining the structural integrity of H⁺-PPase. Alternatively, the subunit structure (interaction) of purified vacuolar H⁺-PPase might be different from its membrane-bound form. This token is also supported by radiation-inactivation analysis, which shows that the functional size of vacuolar H⁺-PPase differs as the consequence of various treatments, such as solubilization and the addition of ionophores [4].

Both K_m and V_{max} of vacuolar H⁺-PPase decreased upon high-pressure treatment, implying an increase in the affinity of the enzyme (E) for PP_i, but also a significant reduction in the turnover rate of E–PP_i to E + 2 × Pi. High pressure presumably provokes a conformational change in the vacuolar H⁺-PPase, allowing the enzyme more access to PP_i. However, the conformation imposed by high pressure is in turn inappropriate for the hydrolysis of PP_i to P_i in the active domain of vacuolar H⁺-PPase. Furthermore, ligand effects indicate that vacuolar H⁺-PPase is more sensitive to high pressure in the presence of physiological substrate and substrate analogues. This phenomenon is also found in vacuolar H⁺-ATPase [39]. We speculate that during hydrolysis of the substrates or on binding of substrate analogues, vacuolar proton-pumping enzymes are in transition states differing in subunit structure from their resting forms. The intercalation of these ligands in the active site of vacuolar H⁺-PPase consequently weakens the structural strength of the enzyme, thereby resulting in high sensitivity to stress environments.

Spectroscopic analysis further reveals in several ways changes in subunit interaction of vacuolar H⁺-PPase on high hydrostatic pressure. Light-scattering studies demonstrate an increase in the roughness of protein surface upon pressure treatment. In the native state, the surface of vacuolar H⁺-PPase is probably more smooth, but becomes irregular after being bombarded by high pressure. The surface change primarily reflects a modification of interior structure of vacuolar H⁺-PPase as also demonstrated by observations of secondary structure and other spectroscopic analysis. CD spectra show an alteration in the α -helix content of vacuolar H⁺-PPase in response to high hydrostatic pressure. Moreover, both intrinsic absorption and fluorescence intensities of vacuolar H⁺-PPase were markedly quenched after pressurization. However, the absorption and fluorescence maxima were not shifted, suggesting that the polarity of environments for the intrinsic chromophore(s) of vacuolar H⁺-PPase remains unchanged after pressure treatment. FITC labels a lysine residue, presumably at the active site of vacuolar H⁺-PPase, and is thus a good molecular probe to examine the conformation of catalytic domain. The quenching of extrinsic fluorescence of FITC–PPase upon high pressure implies a structural change in the labelled domain of the enzyme. Further investigation of the fluorescence polarization illustrate an increase in the degree of freedom of fluorophore moieties after pressure treatment. Thus, spectroscopic evidence confirms a conformational change in the interior of vacuolar H⁺-PPase upon pressure treatment.

The semi-logarithmic plots of specific activity versus pressure yield lines with characteristic break points and slopes, indicating the involvement of two or more steps during pressure treatment. The subunit interaction of vacuolar H⁺-PPase may be modified in different ways during each stage. The inactivation of the enzyme still proceeds irreversibly even after pressure release.

Size-exclusion HPLC profiles further suggest a reaggregation of protein complex into different inactive forms. Since the pressure applied herein is much lower than the threshold for direct tertiary conformational changes in single polypeptides [20,22], the results observed are the consequence of modified subunit interaction of vacuolar H⁺-PPase by pressurization. Two possibilities are thus proposed to interpret our findings. (i) An irreversible structural change of subunits occurs instantaneously upon dissociation by high pressure. Subsequent reassociation of the subunits results in altered dimeric conformation with a reduced enzyme activity. That is, correct contact (interaction) of subunits in the dimer is crucial and maintains in a specific way an appropriate conformation of the enzyme for its proper function. Release of the correct contact irreversibly alters the subunit structure with a concomitant loss in the enzymic activity. (ii) Another possibility is that no immediate conformational change, thereby no loss in enzyme activity, occurs when subunits of the dimer are separated from each other by high pressure. However, the majority of the subunits reassociate irreversibly into dimers with distinct contact points from the native one. Consequently, misplacement of proper subunit contact results in a conformational change followed by abolishment of the enzymic activity. We cannot distinguish between these two modes of pressure inactivation at present. Nevertheless, we believe protein–protein interaction of identical subunits determines the structure and the function of the vacuolar H⁺-PPase. Taken together, our results point to a notion that assembly *in vivo* of the two identical subunits of vacuolar H⁺-PPase is conceivably not random but proceeds in a novel manner that leads irreversibly to an appropriate function of the enzyme. The detailed mechanism of predetermined assembly of vacuolar H⁺-PPase subunits deserves further investigation.

This work was supported by the grant from National Science Council, Republic of China (NSC 0182-253K4) to RLP.

REFERENCES

- 1 Sze, H., Ward, J. M. and Lai, S. P. (1992) *J. Bioenerg. Biomemb.* **24**, 371–381
- 2 Rea, P. A. and Poole, R. J. (1993) *Annu. Rev. Plant Physiol. Mol. Biol.* **44**, 157–180
- 3 Rea, P. A., Britten, C. J. and Sarafian, V. (1992) *Plant Physiol.* **100**, 723–732
- 4 Tzeng, C. M., Yang, C. Y., Yang, S. J., Jiang, S. S., Kuo, S. Y., Hung, S. S., Ma, J. T. and Pan, R. L. (1996) *Biochem. J.* **316**, 143–147
- 5 Sarafian, V., Kim, Y., Poole, R. J. and Rea, P. A. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1775–1779
- 6 Tanaka, Y., Chiba, K., Maeda, K. and Maeshima, M. (1993) *Biochem. Biophys. Res. Commun.* **190**, 1110–1114
- 7 Kim, Y., Kim, E. J. and Rea, P. A. (1994) *Plant Physiol.* **106**, 375–382
- 8 Hung, S. S., Chiu, S. Y., Lin, L. Y. and Pan, R. L. (1995) *Plant Physiol.* **109**, 1125–1127
- 9 Lerhl, J., Konig, S., Zrenner, R. and Sonnewald, U. (1995) *Plant Mol. Biol.* **29**, 833–840
- 10 Sakakibara, Y., Kobayashi, H. and Kasamo, K. (1996) *Plant Mol. Biol.* **31**, 1029–1038
- 11 Kim, E. J., Zhen, R. G. and Rea, P. A. (1995) *J. Biol. Chem.* **270**, 2630–2635
- 12 Zhen, R. G., Kim, E. J. and Rea, P. A. (1994) *J. Biol. Chem.* **269**, 23342–23350
- 13 Kuo, S. Y. and Pan, R. L. (1990) *Plant Physiol.* **93**, 1128–1133
- 14 Yang, S. J., Jiang, S. S., Tzeng, C. M., Kuo, S. Y., Hung, S. S. and Pan, R. L. (1996) *Biochim. Biophys. Acta* **1294**, 89–97
- 15 Sarafian, V. and Poole, R. J. (1989) *Plant Physiol.* **91**, 34–38
- 16 Chanson, A. and Pilet, P. (1989) *Plant Physiol.* **90**, 934–938
- 17 Britten, C. J., Turner, J. C. and Rea, P. A. (1989) *FEBS Lett.* **256**, 200–206
- 18 Maeshima, M. (1990) *Biochem. Biophys. Res. Commun.* **168**, 1157–1162
- 19 Sato, M. H., Maeshima, M., Ohsumi, Y. and Yoshida, M. (1991) *FEBS Lett.* **290**, 177–180
- 20 Gross, M. and Jaenicke, R. (1994) *Eur. J. Biochem.* **221**, 617–630
- 21 Hawley, S. A. (1978) *Methods Enzymol.* **49**, 14–24
- 22 Weber, G. and Drickamer, H. G. (1983) *Q. Rev. Biophys.* **16**, 89–112

- 23 Silva, J. L., Mile, E. W. and Weber, G. (1986) *Biochemistry* **25**, 5780–5786
- 24 Gorovits, B., Raman, C. S. and Horowitz, P. M. (1995) *J. Biol. Chem.* **270**, 2061–2066
- 25 Silva, J. L. and Weber, G. (1986) *Fed. Proc.* **45**, 1919
- 26 Maeshima, M. and Yoshida, S. (1989) *J. Biol. Chem.* **264**, 20068–20073
- 27 Fiske, C. H. and Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 378–400
- 28 Wang, M. Y., Lin, Y. H., Chow, W. M., Chung, T. P. and Pan, R. L. (1989) *Plant Physiol.* **90**, 474–481
- 29 Larson, E., Howlett, B. and Jagendorf, A. T. (1986) *Anal. Biochem.* **155**, 243–248
- 30 Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, chap. 5, pp. 111–153, Plenum Press, New York
- 31 Chen, Y. H., Yang, J. T. and Martinez, H. M. (1972) *Biochemistry* **11**, 4120–4131
- 32 Gans, P. J., Lyu, P. C., Manning, M. C., Woody, R. W. and Kallenbach, N. R. (1991) *Biopolymers* **31**, 1605–1614
- 33 Paladini, A. A. and Weber, G. (1981) *Biochemistry* **20**, 2587–2593
- 34 King, L. and Weber, G. (1986) *Biochemistry* **25**, 3632–3637
- 35 King, L. and Weber, G. (1986) *Biochemistry* **25**, 3637–3640
- 36 Silva, J. L. and Weber, G. (1987) *J. Mol. Biol.* **199**, 149–159
- 37 Royer, C. A., Daly, T. T., Mathews, K. S. and Weber, G. (1986) *Biochemistry* **25**, 8308–8315
- 38 Dreyfus, G., Guimaraes-Motta, H. and Silva, J. L. (1988) *Biochemistry* **27**, 6704–6710
- 39 Tsai, Y. R., Yang, S. J., Jiang, S. S., Ko, S. J., Hung, S. H., Kuo, S. Y. and Pan, R. L. (1998) *J. Protein Chem.* **17**, 167–178

Received 30 June 1997/3 November 1997; accepted 15 January 1998