Roles of histidine residues in plant vacuolar H\textsuperscript{+}-pyrophosphatase

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Abstract

Vacuolar proton pumping pyrophosphatase (H\textsuperscript{+}-PPase; EC 3.6.1.1) plays a pivotal role in electrogenic translocation of protons from cytosol to the vacuolar lumen at the expense of PP\textsubscript{i} hydrolysis. Alignment analysis on amino acid sequence demonstrates that vacuolar H\textsuperscript{+}-PPase of mung bean contains six highly conserved histidine residues. Previous evidence indicated possible involvement of histidine residue(s) in enzymatic activity and H\textsuperscript{+}-translocation of vacuolar H\textsuperscript{+}-PPase as determined by using histidine specific modifier, diethylpyrocarbonate [J. Protein Chem. 21 (2002) 51]. In this study, we further attempted to identify the roles of histidine residues in mung bean vacuolar H\textsuperscript{+}-PPase by site-directed mutagenesis. A line of mutants with histidine residues singly replaced by alanine was constructed, over-expressed in \textit{Saccharomyces cerevisiae}, and then used to determine their enzymatic activities and proton translocations. Among the mutants scrutinized, only the mutation of H716 significantly decreased the enzymatic activity, the proton transport, and the coupling ratio of vacuolar H\textsuperscript{+}-PPase. The enzymatic activity of H716A is relatively resistant to inhibition by diethylpyrocarbonate as compared to wild-type and other mutants, indicating that H716 is probably the target residue for the attack by this modifier. The mutation at H716 of V-PPase shifted the optimum pH value but not the \textit{T}_{1/2} (pretreatment temperature at which half enzymatic activity is observed) for PP\textsubscript{i} hydrolytic activity. Mutation of histidine residues obviously induced conformational changes of vacuolar H\textsuperscript{+}-PPase as determined by immunoblotting analysis after limited trypsin digestion. Furthermore, mutation of these histidine residues modified the inhibitory effects of F\textsuperscript{−}/C\textsubscript{0} and Na\textsuperscript{+}, but not that of Ca\textsuperscript{2+}. Single substitution of H704, H716 and H758 by alanine partially released the effect of K\textsuperscript{+} stimulation, indicating possible location of K\textsuperscript{+} binding in the vicinity of domains surrounding these residues.

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1. Introduction

Vacuolar types of H\textsuperscript{+}-pyrophosphatase (V-PPase; EC 3.6.1.1), found mainly in higher plants, some protozoa, and several species of eubacteria and archebacteria, catalyze electrogenic H\textsuperscript{+}-translocation from the cytosol to the vacuolar lumen to generate an inside-acidic and inside-positive membrane potential for the secondary transport of ions, metabolites, and toxic substances [1–4]. Vacuolar H\textsuperscript{+}-PPase has been successfully purified from various sources and was identified as a homodimer with a molecular mass of approximately 145 kDa [1,2]. The cDNA and its deduced amino acid sequences of vacuolar H\textsuperscript{+}-PPases from several higher plants and bacteria possess high degree of homology and identity (86–91%) [1,3,4]. However, phylogenetic analysis indicates that vacuolar H\textsuperscript{+}-PPases are distinct from three other types of proton pumping enzymes, i.e., P\textsuperscript{−}, F\textsuperscript{−}, and vacuolar H\textsuperscript{+}-ATPases, belonging to a novel proton translocase family [4]. Vacuolar H\textsuperscript{+}-PPase requires Mg\textsuperscript{2+} as a cofactor and the binding of Mg\textsuperscript{2+} can stabilize and activate the enzyme [1,5]. Relatively high concentration of K\textsuperscript{+} can stimulate vacuolar H\textsuperscript{+}-PPase, while excess PP\textsubscript{i}, Ca\textsuperscript{2+}, Na\textsuperscript{+}, and F\textsuperscript{−} inhibit its enzymatic activity [6–8]. It is conceivable that vacuolar H\textsuperscript{+}-PPase accommodates binding domains for the substrate and above ligands, as well as proton translocation.

Amino acid sequence alignment of vacuolar H\textsuperscript{+}-PPases from various organisms revealed several highly conserved regions involved in substrate binding [1,4,9]. For instance, a putative substrate binding motif of DX\textsubscript{3}KXE on loop e

Abbreviations: DEPC, diethylpyrocarbonate; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(\textit{\textalpha}-aminoethylether)\textsubscript{N,N,N,N\textsuperscript{\textalpha}}-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; V-PPase, vacuolar H\textsuperscript{+}-pyrophosphatase

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and two acidic DX3DX3D motifs on both loop e and m of cytosolic side have been proposed, respectively [4,9,10]. Nevertheless, essential regions and residues in the V-PPase for the enzymatic function, proton translocation, and ligand binding still require further elucidation. By using chemical modification, previous studies have found some important residues involved in the enzymatic activities [9,11–18]. Inhibition of vacuolar H⁻-PPase by arginine-specific reagents, phenylglyoxal and 2,3-butanedione, showed that at least one arginyl residue locates at its active site [11].

Results from tetranitromethane modification of vacuolar H⁻-PPase suggested that the enzyme contains a substrate-protectable tyrosine residue in a domain sensitive to treatment of Cys-634 by NEM (N-ethylmaleimide) [12,14]. The PP₇ hydrolytic activity of vacuolar H⁻-PPase was markedly decreased in a concentration-dependent manner by fluorescein S⁻-isothiocyanate, indicating the involvement of a lysine residue in enzymatic reactions [15]. Radioactively labeling technique demonstrated Asp-283 of vacuolar H⁻-PPase is essential to the enzymatic reaction [14,16]. Furthermore, parallel application of mutational and conventional biochemical methods revealed that the Glu-305 and Asp-504 directly participate in DCCD (N,N'-dicyclohexylcarbodiimide) binding and are also critical for catalysis [17]. Moreover, mutagenic analysis demonstrated that Lys-261 and Glu-263 might be related to the energy conversion from PP₇ hydrolysis to proton translocation and several Asp residues might confer the binding of Mg²⁺-PP₇ [9]. Notwithstanding, the exact structure and catalytic properties of vacuolar H⁻-PPase still remain to be determined.

It has been recently shown that a histidine residue is possibly involved in the inhibition of enzymatic and H⁺ translocating activities of vacuolar H⁻-PPase by diethylpyrocarbonate (DEPC), a histidine-specific modifier [18]. Furthermore, analysis of the amino acid sequence demonstrates that vacuolar H⁺-PPase of mung bean contains six histidine residues (Fig. 1). Most of these histidine residues are relatively well conserved in higher plants. Since several lines of evidence also indicate that histidine residues are involved in proton transport [19–21], H⁺/peptide co-transport [22], ion-coupled amino acid transport [23], activation and pH sensing of ion channel [24–26], and structure or assembly of the enzymes [27,28], it is thus particularly interesting for us to investigate the importance of these histidine residues in vacuolar H⁺-PPase. In this study, we attempted to identify the roles of histidine residues in vacuolar H⁺-PPase by site-directed mutagenesis [13]. A series of mutants with histidine residues singly replaced by alanine was constructed, over-expressed in Saccharomyces cerevisiae, and then used to determine their enzymatic activities and proton translocations [29]. Our results indicate that only did the mutation of histidine residue H716 induce a significant decline in enzymatic activity, proton translocation, and coupling ratio of vacuolar H⁺-PPase. Chemical modification of mutants revealed that H716 is probably the target residue for the attack by this modifier. Mutation of histidine residues obviously induced a conformational change of vacuolar H⁺-PPase as determined by limited proteolysis. Furthermore, single substitution of histidine residues by alanine provoked different modification in ion effects on PP₇, hydrolytic activity of V-PPase. Accordingly, we suggest K⁺ binding site of vacuolar H⁺-PPase possibly resides at the vicinity of domains embedding C-terminus.

2. Materials and methods

2.1. Microorganisms

The vacuolar protease-deficient haploid strain of S. cerevisiae, BJ2168 (MATα, pre1Δ407, prblΔ1122, pep4−3, leu2, trpl, ura3−52), was used for heterologous expression of mung bean V-PPase [29]. BJ2168 was grown in YEPD medium [1% (w/v) yeast extract (DIFCO, Detroit, MI, USA), 2% (w/v) bactopeptone (DIFCO), and 2% (w/v) dextrose]. The mutated V-PPase cDNA was constructed into an E. coli/S. cerevisiae shuttle vector, pYES2 (Invitrogen, Carlsbad, CA, USA) after GAL1 promoter between HindIII and XbaI, for heterologous expression in S. cerevisiae. XL1-Blue (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1,

Fig. 1. Amino acid alignment of fragments containing histidine residues from various vacuolar H⁺-PPases. Amino acid sequence of fragments containing histidine residues from various sources were aligned using Clustal W program [40]. Amino acids located between each fragment have been omitted for clarity. Residue numbers on the top of alignments indicate the location of histidine residues of mung bean vacuolar H⁺-PPase.
lac [F’ proAB, lacF9ΔM15, Tn10 (Tet’)] was used for plasmid manipulation. The transformation of yeast was performed by the method of Gietz et al. [30]. All the plasmid DNA’s were extracted using Gene-Spin™ DNA extraction kit (Protech, Taiwan) as recommended by the manufacturer.

2.2. Site-directed mutagenesis

All mutations were generated by PCR megaprimer method using Vent® as DNA polymerase [31]. Mutagenic oligonucleotides were designed to singly substitute each His codon on cDNA sequence of V-PPase. The sequences of the mutated oligonucleotides (positions of His codons presented in brackets and positions of mutation underlined) were:

H68A 5'-CGACGCAGTT[GGC]GTCGTTGATGC-3'
H319A 5'-GTTGAAAT[GCC]GAGTTGACTGC-3'
H520A 5'-TCACGATAATC[GGC]ACTCATGCGACAG-3'
H740A 5'-CTCTCTGAG[GC]GCCAGAAGCC-3'
H716A 5'-CAGATTCG[GCC]AAGGCAAGCAG-3'
H758A 5'-TTGCTACC[GGC]GTGTGGTTTAC-3'

The final PCR products were cut with appropriated restriction enzymes and subsequently introduced to pYES2 following the confirmation of the sequences at the mutated sites by DNA sequencing.

2.3. Preparation of V-PPase-enriched microsomes from yeast cells

Crude membrane fractions were prepared from the heterologously expressing yeast as described by Kim et al. [29] with minor modifications. Yeast cells were cultured by diluting 400 ml of stationary phase cells into 1 l in CM medium [0.5% (w/v) ammonium sulfate, 2% (w/v) galactose, 0.2% (w/v) yeast nitrogen base without amino acid and ammonium sulfate, 60 μg/ml leucine, and 40 μg/ml tryptophan] at 30 °C. The cells were grown for 3 days and collected by centrifugation at 4000 × g for 10 min. The cells were resuspended in 100 mM Tris/HCl (pH 9.4) and 10 mM dithiothreitol (DTT). The suspension was incubated at 30 °C for 20 min with gentle shaking. After centrifugation at 4000 × g for 10 min, the cells were treated with theYP medium at 30 °C for 2 h. The YP medium contained 100 mM Tris/Mes (pH 7.6), 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, 1% (w/v) galactose or glucose, 0.7 M sorbitol, 5 mM DTT, and lyticase (Sigma, St. Louis, MO, USA) 1200 U/g wet weight of cell pellet. Spheroplasts were collected by centrifugation at 4000 × g for 10 min followed by resuspension in an冰冷 homogenization buffer containing 10% (w/v) glycerol, 5 mM EGTA/Tris, 50 mM Tris/ascorbate (pH 7.6), 2 mg/ml BSA, 1.5% (w/v) polyvinylpyrrolidone (M, 40000), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml pepstatin A. The spheroplast cells were homogenized using a motor-driven Dounce homogenizer for 10 cycles, followed by centrifugation at 4000 × g for 15 min to remove cell debris. The supernatant was then aspirated and subjected to centrifugation at 84 000 × g for 1 h. The pellet was resuspended in suspension buffer [1.1 M glycerol, 1 mM Tris/EGTA, 5 mM Tris/Mes (pH 7.6), 2 mg/ml BSA, 2 mM DTT, 1 mM PMSF, and 10 μg/ml pepstatin A] and then layered on to a discontinuous sucrose density gradient consisting of 10% (w/w) and 28% (w/w) sucrose in suspension buffer. Following centrifugation at 58 000 × g for 2 h, the V-PPase-enriched microsomes were withdrawn from the 10%/28% interface and diluted to 10-fold of the storage buffer [5 mM Tris/Mes (pH 7.6), and 10% (w/v) glycerol]. After centrifugation at 84 000 × g for 55 min, the precipitate was resuspended in the storage buffer and stored at −80 °C for later use.

2.4. Enzyme assay and protein determination

PPi, hydrolytic activity was measured as the liberation of P_i from PP_i in a reaction medium [30 mM Tris/Mes (pH 8.0), 1 mM MgSO_4, 0.5 mM NaF, 50 mM KCl, 1 mM PP_i, 1.5 μg/ml gramicidin D, and 20–30 μg of microsome protein] at 37 °C for 15–20 min. Under these conditions, the hydrolysis of pyrophosphate is linear with concentration of vacuolar H^+-Pase and time. After incubation the reaction was terminated by a stop solution [1.7% (w/v) ammonium molybdate, 2% (w/v) SDS, and 0.02% (w/v) 1-amino-2-naphthol-4-sulfonic acid]. The released P_i was determined spectrophotometrically as described elsewhere [32,33]. For determination of optimal pH, different pH values of reaction media were maintained by adjusting pH value of Tris/Mes buffer. Lineweaver–Burk plot was obtained conventionally and values of K_M and V_max were thus determined using a SigmaPlot 5.0 software (SPSS, Chicago, IL, USA).

Protein concentration was measured by a modified Lowry method [34] with BSA as the standard.

2.5. Measurement of proton translocation

Proton translocation was measured as fluorescence quenching of acridine orange (excitation wavelength 495 nm, emission wavelength 530 nm). Reaction mixture for proton translocation contained 5 mM Tris/HCl (pH 7.6), 1 mM EGTA/Tris, 400 mM glycerol, 100 mM KCl, 1.3 mM MgSO_4, 0.5 mM NaF, and 5 μM acridine orange, and 200 μg microsome proteins. The fluorescence quenching was initiated by adding 1 mM sodium pyrophosphate (pH 7.6). The ionophore, gramicidin D (5 μg/ml), was included at the end of each assay to confirm the integrity of the membrane. The initial rate of fluorescence quenching was calculated as the proton transport activity. Coupling ratio (the ratio of proton pumping to the rate of PP_i
hydrolysis) was measured as \((\Delta F\% / \text{min}) / (\mu \text{mol PPi hydrolyzed/min})\) [17].

2.6. Treatment of V-PPase with DEPC

DEPC was dissolved as stock with absolute alcohol, in which it was stable for several weeks (data not shown). The alcohol concentration in the incubation mixture was kept below 5% (v/v) at which no alcohol effect was observed. DEPC modification was conducted in a medium containing 0.4 mg/ml of microsome protein with 20 mM Tris/Mes (pH 7.0) and 1.0 mM DEPC at 37 °C for 10 min. After incubation, the reaction mixture was diluted 25-fold with enzyme assay solution (as mentioned above) and the enzymatic activity was assayed immediately.

2.7. SDS-PAGE and Western analysis

SDS-PAGE was performed according to Laemmli [35]. The samples were delipidated in 50 volumes of 1:1 (v/v) mixture of chloroform/methanol at −20 °C for 3–4 h. The solution was centrifuged at 10000 x g for 10 min. The supernatants were aspirated, air-dried, and resuspended in 50 μl denaturation buffer [5% (w/v) SDS, 5% (w/v) 2-mercaptoethanol, 10 mM Tris/HCl (pH 6.8), 10% (w/v) glycerol, and 0.002% (w/v) bromophenol blue]. The solution was heated at 70 °C for 20 min before 10% (w/v) SDS-PAGE. The gels were stained with Coomassie Blue or electrotransferred to PVDF membrane using the semi-dry electrotransblotting apparatus (Nova Blot, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The blots were incubated with the rabbit polyclonal antibody raised against the MAP-conjugated synthetic peptide of the sequence KVERNIPEDDPRNPA, which corresponds to positions 261–275 of the substrate-binding domain of mung bean MAP-conjugated synthetic peptide of the sequence KVERNIPEDDPRNPA, which corresponds to positions 261–275 of the substrate-binding domain of mung bean V-PPase. Bands of immunoblots were visualized using the Western Lightning™ kit (New England Nuclear, Boston, MA, USA) as recommended by the manufacturer.

2.8. Trypsin proteolysis assay

The TPCK-treated trypsin (Promega, Madison, WI, USA) was dissolved in 1 mM HCl to make 0.1 mg/ml of stock solution. The microsomal proteins (15 μg) were incubated with trypsin solution at ratio of 100:1 (w/w) on 37 °C bath for 20 min. The proteolysis was stopped by adding the denaturation buffer followed by heating at 95 °C for 15 min. The samples were subjected immediately to 10% (w/v) SDS-PAGE. Western analysis was performed as described above.

2.9. Control over K\(^{+}\), Na\(^{+}\), and Ca\(^{2+}\) contamination

The background concentrations of K\(^{+}\), Na\(^{+}\), and Ca\(^{2+}\) in our assay media were below 4.0, 5.0 and 20 nM, respectively, as determined by inductively coupled plasma-atomic emission spectrometer (ICP-AES) at NTHU Instrument Center, National Tsing Hua University, Hsin Chu, Taiwan.

2.10. Chemicals

Restriction endonucleases and T\(_{4}\) DNA ligase were provided from New England Biolabs (Beverly, MA, USA). Immunoblotting reagents were obtained from Bio-Rad (Hercules, CA, USA). DEPC was purchased from Sigma, and PP\(_{i}\) from E. Merck (Damstadt, Germany). All other chemicals were of analytic grade and used without further purification.

3. Results and discussion

3.1. Expression and H\(^{+}\)-PPase activities

The mung bean vacuolar H\(^{+}\)-PPase encoding cDNA (VVP) was incorporated into yeast expression vector pYES2 and transcribed efficiently in yeast under the control of the promoter of GAL1 (cf. [13,17,29]). The V-PPase-enriched membranes from transformants were successfully prepared by sucrose gradient centrifugation and then subjected to SDS-PAGE and immunoblotting with polyclonal antibodies specific to the deduced substrate-binding site of V-PPase. These results demonstrated that the expressed protein is in a good agreement with mung bean vacuolar H\(^{−}\)-PPase and our expression system is feasible for further studies.

To investigate the roles of histidine residues in vacuolar H\(^{−}\)-PPase, a series of mutant DNA with histidine residues singly replaced by alanine were routinely generated and heterologously expressed in yeast. The microsomal membranes of transformants contained over-expressed V-PPase as visualized by immunoblotting at positions in the vicinity of molecular mass of 73 kDa (Fig. 3A). The efficiencies of the expression were similar, regardless the mutation at different histidine residues. PP\(_{i}\) hydrolysis and its associated proton translocation of mutants were measured as shown in Fig. 3B and C. Most transformants except H716A retained...
relatively similar hydrolytic activities (ranging from 75% to 95%) to the wild-type. The PPi hydrolysis of H716A was considerably inhibited by 30–50% of the wild-type, suggesting that this histidine residue probably plays a crucial role in sustaining the enzymatic reaction of vacuolar H⁺-PPase. Furthermore, the proton pumping activities of mutants were decreased variously to 80–25% of the wild-type. Among variants, proton transport reactions of H319A and H716A were remarkably declined to 49% and 26% of the wild-type, respectively, implying that these two histidine residues might be involved in the H⁺ translocation of V-PPase. Moreover, the coupling ratios were calculated and results showed those of both mutants were approximately decreased to 50% of the wild-type (Fig. 3D), confirming possible contribution of these two residues in proton transport. However, the involvement of these residues may be indirect, since there exists partially residual activities in both enzymatic and proton translocating reactions. Nevertheless, these alterations came directly from the effect of single amino acid substitution, since all mutants displayed similar efficiency of expression in yeast microsomes.

3.2. Kinetic properties of mutants

Effects of histidine mutation on kinetic properties of V-PPase were scrutinized. A conventional Lineweaver–Burk plot of vacuolar H⁺-PPase yielded an apparent $K_M$ value of $142.8 \pm 1.3$ μM and an apparent $V_{max}$ value of $45.5 \pm 0.5$ μmol PPi consumed/mg protein h for substrate Mg²⁺-PPi, respectively (data not shown). The apparent $K_M$ and $V_{max}$ values of each mutant were then determined and are also summarized in Table 1. The apparent $K_M$ values of H716A was similar to wild-type; albeit, its apparent $V_{max}$ value was decreased to approximately 40%. We speculate that the mutation at this residue brought the conformation into a state hindering only the hydrolytic reaction but not affinity of the substrate. Alternatively, we could not exclude other possibilities rendering the decrease in $V_{max}$ but not $K_M$ values of H716A. Furthermore, apparent $V_{max}$ values of H319A, H704A, and H758A were slightly altered (76–85% of the wild-type), whereas those of H68A and H520A remained relatively unchanged. Besides, apparent $K_M$ values of most mutants were not modified, thus indicating the mutation at these histidine residues exert no significant effect on the accessibility of substrate to the active site of vacuolar H⁺-PPase. These results concur with those using DEPC as inhibitor [18], implicating possible involvement of the essential histidine residue in catalytic reaction of V-PPase.

The $pK_a$ value of imidazole moiety of histidine is presumably at pH 6.0. We are thus interested in determining
the optimum pH of the vacuolar H+-PPase after its histidine residues are singly replaced. Fig. 4 depicts the pH profile of several histidine mutants of vacuolar H+-PPase. For the wild-type, the optimum pH of vacuolar H+-PPase is approximately at pH 7.6. Upon substitution of histidine residue, optimum pH values of most mutants remained unchanged, except H704A at pH 7.1 and H716A at 6.8. The shift of optimum pH reveals that the enzyme conformation of these two mutants might be different from the wild-type, resulting in the exposure of other acidic residues to the environments. The conformational change of H716A is likely more drastic than other mutants, since it showed a broader drift in optimum pH.

3.3. Sensitivities of mutants to DEPC

Previous studies demonstrated that histidine-specific modifier, DEPC, could inhibit the enzymatic activity and
proton translocation of V-PPase, suggesting the involvement of an essential histidine residue in these reactions [18]. It is particularly interesting to identify histidine residue(s) conferring DEPC inhibition. At 1.0 mM DEPC, the enzymatic activity of the wild-type V-PPase was reduced to approximately 38% of the untreated (Fig. 5). At the same concentration of DEPC, most mutants, except H716A, could be inhibited to a similar extent as that of the wild-type. In the presence of DEPC, H716A retained approximately 80% of the enzymatic activity as compared to in its absence. The extent of DEPC inhibition on enzymatic activities of mutant vacuolar H+-PPases depends on concentrations of inhibitor and incubation time (data not shown). The relative resistance of H716A against DEPC inhibition leads to a suggestion that this residue is the major target for the attack of the modifier in wild-type as well as other mutant H+-PPases. However, we cannot exclude the possibility that mutation at H716 may induce a conformational change, preventing the access of DEPC to target residue(s).

### 3.4. Ion effects on enzymatic activities

Vacuolar H+-PPase requires relatively high concentration of K+ for optimum activities, while Ca²⁺, Na⁺ and F⁻ could substantially inhibit its enzymatic reaction (cf. Fig. 2; [6–8]). The ion effects on the wild-type and the mutant V-PPases were examined and summarized in Table 2. K⁺ (50 mM) could stimulate PPi hydrolysis of vacuolar H+-PPase by approximately 7.2-fold. Mutation of histidine residues brought about different degree of modification in the stimulation of vacuolar H+-PPase activities by K⁺. For instance, the stimulation of H704A, H716A and H758A by K⁺ was decreased to 6.1-, 2.8- and 5.6-fold, respectively. It is generally accepted that there are mainly two subcategories of V-PPases regarding the sensitivities to K⁺-stimulation [3,36–38]. Type I V-PPases are sensitive to K⁺, but type II

### Table 1

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Vₘₐₓ (μmol/mg protein h)</th>
<th>Kₘₐₚ (μM)</th>
<th>pH optimum</th>
<th>T₁/₂ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>45.5±0.5 (1.00)</td>
<td>142.8±1.3 (1.00)</td>
<td>7.6</td>
<td>62.8</td>
</tr>
<tr>
<td>H68A</td>
<td>43.5±0.4 (0.96)</td>
<td>129.8±1.1 (0.91)</td>
<td>7.6</td>
<td>64.3</td>
</tr>
<tr>
<td>H319A</td>
<td>38.7±0.2 (0.85)</td>
<td>140.8±0.6 (0.99)</td>
<td>7.6</td>
<td>60.8</td>
</tr>
<tr>
<td>H520A</td>
<td>48.7±1.8 (1.07)</td>
<td>136.9±4.2 (0.96)</td>
<td>7.6</td>
<td>63.8</td>
</tr>
<tr>
<td>H704A</td>
<td>34.5±0.7 (0.76)</td>
<td>123.4±1.8 (0.86)</td>
<td>7.1</td>
<td>62.5</td>
</tr>
<tr>
<td>H716A</td>
<td>18.8±0.2 (0.41)</td>
<td>135.3±0.8 (0.95)</td>
<td>6.8</td>
<td>63.0</td>
</tr>
<tr>
<td>H758A</td>
<td>35.7±0.6 (0.78)</td>
<td>128.2±1.7 (0.90)</td>
<td>7.6</td>
<td>61.5</td>
</tr>
</tbody>
</table>

Microsomes containing heterologously expressed V-PPase were prepared from _S. cerevisiae_ as described under Materials and methods. Histidine residue singly substituted by alanine is indicated as the names of each mutant shown. Values are means±S.D. from at least three separate experiments.

Fig. 4. The pH profile of enzymatic activities of each mutant V-PPase. PPi hydrolysis of each mutant V-PPases was measured at different pHs of reaction medium as described under Materials and methods. The pH values were maintained using 30 mM Mes/Tris. (●), WT; (▲), H319A; (▽), H704A; (◇), H716A. Values are means±S.D. from at least three separate experiments.

Fig. 5. Inhibition of vacuolar H⁺-PPase by DEPC. The DEPC pretreatment of each mutant and enzyme assay were as described under Materials and methods. The concentration of DEPC was 1.0 mM. SA₁(DECPC), specific activity in the presence (+) and absence (−) of DEPC. Values are means±S.D. from at least three separate experiments.
enzymes are not [3,36,37]. Moreover, a recent report demonstrated that replacement of A460 of type I H+-PPase from *C. hydrogenoformans* by lysine residue successfully turned the enzyme from K+-sensitive (type I) into K+-insensitive (type II) forms, indicating possible involvement of K+ binding at this site [36]. In the present study, K+ stimulation of vacuolar H+-PPase activities was significantly alleviated upon mutation at H716. We speculate that the loop accommodating H716 may fold into the vicinity of A537, the putative K+ binding site of vacuolar H+-PPase equivalent to A460 of that from *C. hydrogenoformans*, (cf., Ref. [36] and Fig. 2 of Ref. [9]). Furthermore, it has been shown that H716 locates in the vicinity of the proposed “Acidic 2” motif, DX(D/X)D, in an active domain on cytosolic side of microsomal membrane (loop *m*; cf., Ref. [9]). It is likely that the fragment containing H716 and “Acidic 2” motif on C-terminus probably plays an indispensable role in sustaining enzymatic reaction and K+ stimulation of V-PPase. Alternatively, we cannot exclude the possibility that the mutation of H716 may induce a long-distance conformational change, resulting in the decrease in enzymatic activity and the K+-stimulation of V-PPase.

Furthermore, the PPi hydrolytic activity of the wild-type could be 66% inhibited in the presence of 5 mM F-. However, most mutants displayed lower sensitivity to F- inhibition under similar conditions. For example, H716A was only 27% inhibited, whereas H68A and H319A were 39% and 37% suppressed by F-, respectively. It is possible that the mutation of histidine residues changes the structure of vacuolar H+-PPase, preventing further inhibitory effects of F- on V-PPase. The binding site of F- is not identified yet; albeit, it is suggested that F- may interfere the chelating of Mg2+ as a cofactor, consequently reducing V-PPase activity [39]. The environment of F- binding site is obviously more sensitive to structural variations induced by the mutation at histidine residues.

Na+ is also known to inhibit the enzymatic activity of vacuolar H+-PPase. At 100 mM of Na+, the enzymatic activity of vacuolar H+-PPase declined to about 40% of that in its absence. Upon mutation at histidine residues, the Na+ inhibition of PPi hydrolytic activities of most mutants, except H520A, were relieved by approximately 2-fold. Obviously, these mutants provide a better environment to avoid the binding of Na+, consequently attenuating its inhibitory effect. In contrast, the mutation of histidine residues of vacuolar H+-PPase did not provoke any change in Ca2+-inhibition of its enzymatic activities. The binding cage of Ca2+ is presumably more resistant to the conformational modification induced by mutation at histidine residues. The various effects of mutation at histidine residues on ion sensitivities indicate that the binding sites and interaction mechanisms of these ligands in vacuolar H+-PPase are distinct, subject to different degrees of changes in responsiveness.

### 3.5. Thermal stability and proteolytic analysis

Analysis of thermal stability of enzymes provides structural information of proteins interested. We pretreated the vacuolar H+-PPase at different temperature, cooled it on ice bath, and then measured its enzymatic activity. The thermal stability of vacuolar H+-PPase was scrutinized and *T*1/2 values (pretreatment temperature at which half enzymatic activity is then observed) were determined (Table 1). The PPi hydrolytic activity of wild-type V-PPase was relatively constant at pretreatment temperature below 50 °C, beyond which the activity declined instead (data not shown). The temperature profile displays a *T*1/2 value of 62.8 °C for wild-type V-PPase. Similarly, *T*1/2 values of mutants were accordingly determined. Our results indicate, however, *T*1/2 of H68A was slightly increased, while that of H319A mildly decreased (Table 1). The rest of the mutants failed to show any significant difference in *T*1/2 values from the wild-type. It is obvious that possible conformational changes following substitution of most histidine residues by alanine did not trigger drastic variation in their heat stability.

Protease vulnerability of enzymes is a good marker for examining the structural changes of proteins. Microsomes

<table>
<thead>
<tr>
<th>Mutants</th>
<th>K+ -stimulation (fold)</th>
<th>F- -inhibition (%)</th>
<th>Na+ -inhibition (%)</th>
<th>Ca2+ -inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7.2 ± 0.2</td>
<td>66 ± 6</td>
<td>40 ± 1</td>
<td>64 ± 2</td>
</tr>
<tr>
<td>H68A</td>
<td>7.6 ± 0.4</td>
<td>39 ± 3</td>
<td>22 ± 3</td>
<td>68 ± 1</td>
</tr>
<tr>
<td>H319A</td>
<td>8.2 ± 0.2</td>
<td>37 ± 5</td>
<td>20 ± 3</td>
<td>67 ± 3</td>
</tr>
<tr>
<td>H520A</td>
<td>7.1 ± 0.3</td>
<td>54 ± 2</td>
<td>38 ± 2</td>
<td>70 ± 4</td>
</tr>
<tr>
<td>H704A</td>
<td>6.1 ± 0.2</td>
<td>43 ± 1</td>
<td>29 ± 2</td>
<td>68 ± 1</td>
</tr>
<tr>
<td>H716A</td>
<td>2.8 ± 0.5</td>
<td>27 ± 2</td>
<td>21 ± 2</td>
<td>57 ± 1</td>
</tr>
<tr>
<td>H758A</td>
<td>5.6 ± 0.3</td>
<td>67 ± 1</td>
<td>24 ± 1</td>
<td>70 ± 3</td>
</tr>
</tbody>
</table>

Table 2

Ion effects on mutant V-PPases

Microsomes containing heterologously expressed V-PPase were prepared from *S. cerevisiae* as described under Materials and methods. Histidine residue singly replaced by alanine is indicated as the names of each mutant shown. The concentration of ions: 50 mM K+, 5 mM F-, 100 mM Na+ and 0.1 mM Ca2+. Values are means ± S.D. from at least three separate experiments.
containing V-PPase were subjected to limited trypsin digestion and then visualized by immunoblotting (Fig. 6). Upon incubation with TPKC-treated trypsin, at protein/protease ratio of 100:1 (w/w) at 37 °C for 20 min, V-PPase was partially digested. Under similar conditions, however, most mutant proteins were almost completely digested as compared to wild-type protein. The mutation at histidine residues presumably exposed more cleavage sites of V-PPase to trypsin attack. Furthermore, in the presence of physiological substrate, Mg\(^{2+}\) PP\(_i\), different degree of partial protection of mutant H\(^{-}\)-PPases against trypsin digestion was observed. Under the protection of physiological substrate, H68A, H704A, H758A were relatively resistant, while H319A, H520A, and H716A were still seriously vulnerable to trypsinolysis. Conceivably, these histidine residues may participate in the maintenance of the architecture of V-PPase and their replacements by alanine may result in a structure more susceptible to the attack by trypsin.

In summary, this work shows H716A, probably a major target conferring DEPC inhibition, plays an essential role in the enzymatic and translocating reactions of vacuolar H\(^{-}\)-PPase. Substitution of H716 by alanine residue might change the structure of V-PPase and consequently alter its characteristics, such as susceptibility to proteolysis, ion effects, and optimal pH. The exact mechanism of the involvement of H716 in structure–function relationship of vacuolar H\(^{-}\)-PPase remains to be determined.

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References


