Identification of Essential Lysines Involved in Substrate Binding of Vacular H\(^{+}\)-Pyrophosphatase*1

Chien-Hsien Lee, Yih-Ju Pan, Yun-Tzu Huang, Tseng-Huang Liu, Shen-Hsing Hu, Ching-Hung Lee, Yan-Wei Chen, Shih-Ming Lin, Lin-Kun Huang, and Rong-Long Pan

From the Department of Life Science and Institute of Bioinformatics and Structural Biology, College of Life Science, National Tsing Hua University, Hsin Chu 30043, Taiwan

Received for publication, September 30, 2010, and in revised form, January 26, 2011
Published, JBC Papers in Press, February 3, 2011, DOI 10.1074/jbc.M110.190215

H\(^{+}\)-translocating pyrophosphatase (H\(^{+}\)-PPase; EC 3.6.1.1) drives proton transport across an electrochemical potential gradient by hydrolyzing pyrophosphate (PP\(_i\)) and is found in various endomembranes of higher plants, bacteria, and some protists. H\(^{+}\)-PPase contains seven highly conserved lysines. We examined the functional roles of these lysines, which are, for the most part, found in the cytosolic regions of mung bean H\(^{+}\)-PPase by site-directed mutagenesis. Construction of mutants that each had a cytotoxic and highly conserved lysine substituted with an alanine resulted in dramatic drops in the PP\(_i\) hydrolytic activity. The effects caused by ions on the activities of WT and mutant H\(^{+}\)-PPases suggest that Lys-730 may be in close proximity to the Mg\(^{2+}\)-binding site, and the great resistance of the K694A and K695A mutants to fluoride inhibition suggests that these lysines are present in the active site. The modifier fluorescein 5’-isothiocyanate (FITC) labeled a lysine at the H\(^{+}\)-PPase active site but did not inhibit the hydrolytic activities of K250A, K250N, K250T, and K250S, which suggested that Lys-250 is essential for substrate binding and may be involved in proton translocation. Analysis of tryptic digests indicated that Lys-711 and Lys-717 help maintain the conformation of the active site. Proteolytic evidence also demonstrated that Lys-250 is the primary target of trypsin and confirmed its crucial role in H\(^{+}\)-PPase hydrolysis.

Native H\(^{+}\)-PPases consist of two identical polypeptides of molecular masses 64–91 kDa (6, 7), and each polypeptide contains 660–793 amino acid residues and 14–17 putative transmembrane segments (7–11). H\(^{+}\)-PPases are divided into K\(^{+}\)-dependent and K\(^{+}\)-independent subgroups, and many of the former can be stimulated more than 3-fold by a millimolar concentration of K\(^{+}\) (1, 11). Furthermore, Mg\(^2+\) is essential for the activity of H\(^{+}\)-PPase, whereas Ca\(^{2+}\) is inhibitory as it prevents the formation of the substrate complex. Fluoride is also an H\(^{+}\)-PPase inhibitor (12–17).

H\(^{+}\)-PPases from different species have ~30% sequence identity overall, and their conserved residues are clustered in three cytosolic loops, i.e. the third, the sixth, and the eighth (Fig. 1), which form an enzymatic domain (1, 18, 19). The conserved DX,KXE motif of the third cytosolic loop is also found in soluble PPase and has been implicated as a substrate catalysis-binding site (1, 11). Furthermore, chemical modification studies have identified several functional residues. Three mung bean H\(^{+}\)-PPase residues, Asp-283, Glu-301, and Asp-500, may be essential for PP\(_i\) hydrolysis and are located near the catalytic domain (20, 21). A sulfhydryl-modifying reagent, mersalyl, attacked the substrate-protectable cysteines of Rhodospirillum rubrum H\(^{+}\)-PPase (22). His-716 of mung bean H\(^{+}\)-PPase was the target of the noncompetitive inhibitor, diethyl pyrocarbonate (23, 24), and Arg-242 has been recently identified as a substrate-binding residue (14).

Mung bean H\(^{+}\)-PPase is inhibited by the lysine-specific chemical modifier FITC (25). Protection and stoichiometric analyses found a single FITC-attackable lysine within mung bean H\(^{+}\)-PPase that presumably participates in substrate binding (25). We set out to identify this lysine and to characterize the roles of the conserved lysines in mung bean H\(^{+}\)-PPase activity. We identified Lys-250 as the site for FITC modification. Furthermore, tryptic digests showed Lys-250 is the primary trypsin target. Lys-711 and Lys-717 were suggested to maintain the enzyme-substrate complex of H\(^{+}\)-PPase. Mutating at a highly conserved lysine may lead to misfolding of the active site. We propose a model illustrating the relationships among the conserved lysines, the Mg\(^{2+}\) cofactors, and the inhibitory fluorides all located at or near the putative catalytic site of H\(^{+}\)-PPase.

**EXPERIMENTAL PROCEDURES**

Manipulation and Expression of Lys → Ala-substituted H\(^{+}\)-PPase in Yeast Cells—Vacular H\(^{+}\)-PPase cDNA of Vigna radiata (GenBank\(^{TM}\) accession number AB009077) was cloned...
and inserted into pBlueScript II SK (+) between the HindIII and XbaI cutting sites for DNA preparation and manipulation as described previously (14). The site-directed mutagenesis was carried out by the PCR megaprimer method (26) with the primer containing mutated oligonucleotides (supplemental Table S1). Each mutation point was defined and confirmed by DNA sequencing. The Escherichia coli/Saccharomyces cerevisiae shuttle vectors, pYES2, containing WT or Lys→Ala-substituted H\(^+\)-PPase genes were transformed into protease-deficient haploid strain of \(S.\) cerevisiae, BJ2168 (14), by the LiAc/polyethylene glycol method (27). Heterologous expression of mung bean H\(^+\)-PPase in BJ2168 was carried out as described previously (24, 28).

Preparation of H\(^+\)-PPase-enriched Yeast Microsomes—Culturing and induction of the transformed yeast cells were performed according to a previous study (14). When \(A_{600}\) of medium reached 1.0, the cells were spun down by centrifugation at 4,000 \(\times\) 3 for 10 min. After washing with 100 mM Tris-HCl (pH 9.4) and 100 mM 2-mercaptoethanol at 37 °C for 20 min, the cells were treated with YP lysis medium, which contains 100 mM Tris-Mes (pH 8.0), 1% (w/v) yeast extract, 2% (w/v) peptone, 1% (w/v) glucose, 700 mM sorbitol, 5 mM 2-mercaptoethanol, and 1,200 units of lyticase per g wet weight of yeast cells, at 30 °C for 2 h with gentle shaking to generate spheroplasts. The spheroplasts were collected by centrifugation at 4,000 \(\times\) 3 for 10 min and resuspended in 50 mM Tris-ascorbate (pH 7.6), 5 mM EGTA-Tris, 10% (w/v) glycerol, 1.5% (w/v) polyvinylpyrrolidone (M\(_{r}\) 40,000), 1 mM phenylmethylene-sulfonyl fluoride (PMSF), and 10 \(\mu\)g/ml pepstatin A. The suspensions were homogenized with a Dounce glass homogenizer by 20 strokes with a tight fitting pestle. After centrifugation at 1,000 \(\times\) 3 for 10 min, the supernatant fractions were centrifuged at 84,000 \(\times\) 3 for 35 min to harvest the microsomal membrane fraction. The precipitates were resuspended in 5 mM Tris-Mes (pH 7.6), 1 mM EGTA-Tris, 1.1 mM glycerol, 2 mM DTT, 1 mM PMSF, and 10 \(\mu\)g/ml pepstatin A and carefully layered on the 10% (w/w) and 28% (w/w) of discontinuous sucrose density gradient, followed by centrifugation at 58,000 \(\times\) 3 for 2 h. The interface portion containing H\(^+\)-PPase-enriched membrane was collected and washed with 10-fold volumes of storage buffer, containing 5 mM Tris-Mes (pH 7.6) and 10% (w/v) glycerol, before centrifugation at 84,000 \(\times\) 3 for 1 h. Finally, the precipitate was resuspended in the storage buffer and stored at −80 °C for later use. Protein concentrations were measured by the Bradford method (29). For FITC inhibition, the 5 mM Tris-Mes was changed to 5 mM MOPS-KOH (pH 7.9) in the storage buffer.

Assays of H\(^+\)-PPase Activity—The PP\(_i\) hydrolysis activity was measured in the reaction medium and determined as described previously (14). PP\(_i\)-dependent H\(^+\) translocation was assayed as the fluorescence quenching of acridine orange in the assay medium (14) with 100 \(\mu\)g of microsomal proteins. The reaction was initiated by adding 1 mM Na\(_4\)PP\(_i\) (pH 7.6) and terminated by adding granamicidin D (5 \(\mu\)g/ml), which confirmed the integrity of the membrane. Fluorescence quenching was measured using excitation wavelength at 495 nm and emission wavelength at 530 nm. Coupling ratio (the ratio of initial proton pumping rate to that of PP\(_i\) hydrolysis) was defined as \((\Delta F/\%)/min)/(\mu\text{mol of PP}_i\text{ hydrolyzed per min}).

**SDS-PAGE and Western Blotting Analysis**—SDS-PAGE was performed according to Laemmli (30). Western blotting analysis was accomplished utilizing the polyclonal antibody against the keyhole limpet hemocyanin-conjugated synthetic polypeptide of a conserved hydrophilic loop sequence (KVERNIPEDPNPA275) of mung bean H\(^+\)-PPase and visualized by the Western Lightning™ kit (PerkinElmer Life Sciences).

**Chemical Modification of H\(^+\)-PPase by FITC**—Chemical modifications of H\(^+\)-PPase were carried out according to previous methods (14, 25) with minor modifications. The microsomes with H\(^+\)-PPase were incubated at 37 °C for 10 min in the medium containing 50 mM MOPS-KOH (pH 7.9), 10% (w/v) glycerol, and 10 mM FITC, the lysine modifier. The FITC labeling was stopped by 20-fold dilution using the PP\(_i\) hydrolysis reaction medium, and the enzymatic activities were measured after 10 min of incubation at 37 °C.
Roles of Cytosolic Lysine Residues in H⁺-PPase

Trypsin Proteolysis—The microsomal proteins were incubated with 1-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin at the ratio of 50:1 (w/w) at 37 °C for 15 min. The proteolysis was stopped by adding SDS sample buffer (30), containing 5 mM PMSF. The samples were subjected to 10% (w/v) SDS-PAGE and Western blotting analysis.

RESULTS

Heterologous Expression and Functional Characterization of the Mung Bean H⁺-PPase Lys → Ala Mutants—The mung bean H⁺-PPase-encoding cDNA was inserted into pYES2 for heterologous expression in the protease-deficient haploid yeast strain BJ2168 (Fig. 2A). The isolated H⁺-PPase-enriched membrane fraction was assayed for PP₁ hydrolytic and PP₁-dependent H⁺ translocation activities (Fig. 2, B and C). Proton pumping across the microsome membrane was initiated by adding Na₄PP₁. PP₁ hydrolysis was stimulated 6-fold by adding 50 mM K⁺ but was somewhat inhibited by adding 100 mM Na⁺, 100 μM Ca²⁺, or 5 mM F⁻ in addition to K⁺ (Fig. 2C). These observations agree with those found for the H⁺-PPase from a plant vacuole (1), indicating that the enzyme functioned properly in the yeast expression system and thus could be used for subsequent experiments.

We then investigated the role(s) of the cytosolic loop lysines (Fig. 1). These 18 lysines were divided into three groups. Group I includes six of the seven highly conserved lysines found in the H⁺-PPases of higher plants and bacteria, i.e. Lys-250, Lys-261, Lys-541, Lys-694, Lys-695, and Lys-730 (Fig. 1 and supplemental Fig. S1). Group II consists of the moderately conserved lysines, i.e. Lys-73, Lys-181, Lys-624, Lys-711, and Lys-717. Lys-711 and Lys-717 are highly conserved in higher plants but not in prokaryotes (Fig. 1 and supplemental Fig. S1). The lysines of group III are either less conserved or not conserved and are located sporadically in the nonconserved cytosolic loops. To determine the function(s) of the lysines, genes that each contained a single Lys → Ala codon substitution were first generated by PCR (26), and the substitutions were verified by DNA sequencing. The DNAs were then each transformed into yeast so that H⁺-PPase-enriched membranes could be prepared.

Microsomes containing WT or an H⁺-PPase mutant were isolated from the membrane fraction of yeast. Enzymatic activities and expression levels were characterized by activity assays and Western blotting, respectively. As shown in Fig. 3A, the expression levels of WT and all mutants, except that of K261A, were similar. K261A H⁺-PPase expression level as determined by silver staining was similar to the level of WT (data not shown), and thus the weak K261A immunoblotting signal may be attributed to the antigen-epitope KVERNIPEDDPRNPA275. To verify this possibility, a His₆ tag was added to the C terminus of K261A mutant as an expression reporter. The presence of the K261A mutant on membrane fractions but not in cytoplasmic fractions was subsequently detected, explicitly indicating the appropriate targeting of K261A mutant. Moreover, the expression level of K261A mutant is similar to that of WT (supplemental Fig. S2). H⁺-PPase expression was not observed in the control yeast that contained a virgin vector (Fig. 2A). Among the group I mutants, PP₁ hydrolytic activities were <20% that of WT H⁺-PPase. Among the group II mutants, K624A, K711A, and K717A hydrolyzed PP₁ at a level of 80, 130, and 50% of WT hydrolytic activity. The enzymatic activities of the group III mutants were similar to that of WT (Fig. 3B). PP₁-dependent H⁺ translocation was assessed by measuring the fluorescence quenching of acridine orange in microsomal preparations that contained WT or a mutant (Fig. 3B). The H⁺ translocation activities of the group I mutants were reduced almost to the background level, and the mutants of group II and III had no effect on proton pumping because no apparent difference in the coupling ratios (PP₁-dependent H⁺ translocation to the hydrolytic activity) of these mutants was observed as compared with that of WT (Fig. 3B). Possibly, the highly conserved lysines are...
Roles of Cytosolic Lysine Residues in H<sup>+</sup>-PPase

Effects of Ions on Lys→Ala Mutants—To examine the effects of ions, the enzymatic activities of the lysine mutants were determined in the presence and absence of K<sup>+</sup>, Ca<sup>2+</sup>, and F<sup>−</sup> (Table 1). The background concentrations in the reaction medium were as follows: for K<sup>+</sup> < 0.2 μM, Na<sup>+</sup> < 0.6 μM, and Ca<sup>2+</sup> < 1 μM, as determined by inductively coupled plasma mass spectroscopy. These levels were low enough that the medium could be used for the ion-effect assay. For WT, PP<sub>i</sub> hydrolysis was stimulated ~6-fold by 50 mM K<sup>+</sup>. The PP<sub>i</sub> hydrolytic activity of most mutants was stimulated by K<sup>+</sup> similarly to that of WT, with the exceptions of the mutants of K694A (11.8-fold stimulation) and K730A (2.2-fold stimulation). Furthermore, the PP<sub>i</sub> hydrolytic activities of WT and most mutants were suppressed between 40 and 60% by 100 μM Ca<sup>2+</sup>. However, K730A mutant, which retained ~80% of the WT activity, displayed relatively greater resistance to Ca<sup>2+</sup> inhibition. Moreover, the activities of WT and most mutants decreased ~40–50% in the presence of 5 mM F<sup>−</sup> and that of K711A decreased 60%. However, K694A and K695A mutants were almost completely resistant to 5 mM F<sup>−</sup> (Table 1), indicating that F<sup>−</sup> probably binds to these residues.

Identification of the FITC-targeted Lysine—Previous work demonstrated that H<sup>+</sup>-PPase could be competitively inhibited by the lysine modifier FITC (25). The stoichiometry of labeling suggested that only one lysine was labeled (25). For this study, we identified the FITC-targeted lysine. As shown in Fig. 4, the abilities of WT and most of the mutants to hydrolyze PP<sub>i</sub> were decreased 70–80% after FITC treatment. In contrast, K250A, K261A, K352A, K541A, K711A, and K717A decreased 60%. However, K694A and K695A mutants retained 90% of their hydrolytic activity, suggesting that Lys-250 is the primary FITC target involved in substrate binding. Moreover, more than 90% of the original hydrolytic activities of K694A and K730A mutants were inhibited by FITC, indicating that mutation at these positions increased the accessibility of FITC to Lys-250.

To further confirm that Lys-250 was the target of FITC, other mutants that contained Lys-250 substitutions were constructed. The levels of PP<sub>i</sub> hydrolysis by the mutants of K250T, K250S, K250V, and K250E were measured by the ability of these mutants to hydrolyze PP<sub>i</sub> (Table 1). The WT control enzymatic activity is 30.9 ± 0.2 μmol of PP<sub>i</sub> hydrolyzed per mg of protein.

| K<sup>+</sup> stimulation fold | Remaining activities + Ca<sup>2+</sup> fold | Remaining activities + F<sup>−</sup> fold |
|-----------------------------|-------------------------------------------|-----------------------------------------|
| Group I                     |                                           |                                         |
| WT                          | 6.4 ± 0.5                                 | 44.6 ± 0.1                               |
| K250A                       | 4.6 ± 0.1                                 | 54.7 ± 0.1                               |
| K261A                       | 8.0 ± 0.9                                 | 42.5 ± 0.8                               |
| K541A                       | *                                         | *                                       |
| K694A                       | 11.8 ± 0.5                                | 48.4 ± 0.6                               |
| K695A                       | 6.0 ± 0.1                                 | 44.3 ± 0.1                               |
| K730A                       | 2.2 ± 0.1                                 | 81.4 ± 0.2                               |
| Group II                    |                                           |                                         |
| K94A                        | 4.3 ± 1.0                                 | 63.6 ± 0.1                               |
| K181A                       | 5.2 ± 0.6                                 | 56.8 ± 0.1                               |
| K624A                       | 4.4 ± 1.5                                 | 44.2 ± 0.2                               |
| K711A                       | 6.2 ± 1.0                                 | 35.4 ± 0.2                               |
| K717A                       | 7.0 ± 0.1                                 | 58.0 ± 0.3                               |
| Group III                   |                                           |                                         |
| K73A                        | 5.0 ± 0.5                                 | 58.4 ± 0.1                               |
| K177A                       | 5.0 ± 0.9                                 | 53.3 ± 0.1                               |
| K352A                       | 4.6 ± 0.6                                 | 59.2 ± 0.1                               |
| K355A                       | 5.3 ± 0.2                                 | 37.5 ± 0.1                               |
| K603A                       | 5.4 ± 1.0                                 | 59.2 ± 0.2                               |
| K632A                       | 7.4 ± 1.2                                 | 32.5 ± 0.1                               |
| K640A                       | 6.4 ± 0.8                                 | 38.1 ± 0.1                               |

* Date were not detectable.
Roles of Cytosolic Lysine Residues in H\(^+\) -PPase

K250S, and K250N were 50, 40, and 15% that of WT, respectively, and K250E, K250M, K250Q, and K250R mutants completely lost the ability to hydrolyze PPi (Fig. 5A). In addition, K250T H\(^+\) -PPase retained ~30% of the proton translocation activity observed for WT and had a 40% decrease in the coupling ratio (Fig. 5, B and C). Furthermore, H\(^+\) translocation by K250N and K250S mutants was totally inhibited, indicating the possible involvement of Lys-250 in proton translocation. Even though the Lys-250 mutants retained PPi hydrolytic activity, they were also all very resistant to FITC inhibition (Fig. 6A), demonstrating that Lys-250 was the sole target of FITC.

**Proteolysis Analysis of H\(^+\) -PPase Lys \(\rightarrow\) Ala Mutants**—The Lys \(\rightarrow\) Ala mutants were then subjected to proteolysis to assess possible structural changes that resulted from the amino acid substitutions (Fig. 7). In the absence of Mg-PPi, WT is considerably digested upon treatment with trypsin. In contrast, Mg-PPi prevented tryptic digestion of WT (Fig. 7). Likewise, in the absence of Mg-PPi, the Lys \(\rightarrow\) Ala mutants, except K250A H\(^+\) -PPase, were easily digested with trypsin (Fig. 7), indicating that Lys-250 may be the primary tryptic cleavage site. This possibility was confirmed by examining the proteolytic profiles of other Lys-250 mutants. The Lys-250 mutants were resistant to trypsin to the same degree as K250A mutant (supplemental Fig. S3) with the exception of K250R mutant (Fig. 6B). K250R mutant was severely digested by trypsin in a manner similar to the other group I mutants (Fig. 7A), probably because arginines and lysines are the preferred targets for trypsin.

Most group I mutants and the group II mutants, K711A and K717A, were more severely digested by trypsin than was WT (Fig. 7), indicating that these mutations possibly induce structural changes. Moreover, the physiologic substrate Mg-PPi provided substantial protection for most Lys \(\rightarrow\) Ala mutants against trypsin (Fig. 7). However, the presence of Mg-PPi failed to substantially protect K711A, K717A, and K250R mutants against trypsin (Figs. 6B and 7B). We hypothesize that mutations at these sites may induce conformational changes, resulting in decreased substrate protection. The proteolytic properties of the group III mutants were similar to that of WT (data not shown).

**DISCUSSION**

Lysines located in the cytosolic side of H\(^+\) -PPase were classified into three groups according to sequence similarities among species (Fig. 1). Group I (highly conserved) lysines are essential for PPi hydrolysis. When mutated to alanines, the enzymatic and proton translocation functions of H\(^+\) -PPase were almost completely lost (Fig. 3B). Among the group II (moderately conserved) lysines, only the mutations of Lys-711 and Lys-717 increased or decreased PPi hydrolysis (Fig. 3B). Mutation of group III (less or not conserved) lysines had no significant effect on the enzymatic activity (Fig. 3A, B, and C). Among the group II lysines, the hydrolytic activity of the WT control was ~109.9 ± 0.8 μmol of PPi hydrolyzed per mg protein-h. ***, not detectable.

**Proteolysis Analysis of H\(^+\) -PPase Lys \(\rightarrow\) Ala Mutants**—The Lys \(\rightarrow\) Ala mutants were then subjected to proteolysis to assess possible structural changes that resulted from the amino acid substitutions (Fig. 7). In the absence of Mg-PPi, WT is considerably digested upon treatment with trypsin. In contrast, Mg-PPi prevented tryptic digestion of WT (Fig. 7). Likewise, in the absence of Mg-PPi, the Lys \(\rightarrow\) Ala mutants, except K250A H\(^+\) -PPase, were easily digested with trypsin (Fig. 7), indicating that Lys-250 may be the primary tryptic cleavage site. This possibility was confirmed by examining the proteolytic profiles of other Lys-250 mutants. The Lys-250 mutants were resistant to trypsin to the same degree as K250A mutant (supplemental Fig. S3) with the exception of K250R mutant (Fig. 6B). K250R mutant was severely digested by trypsin in a manner similar to the other group I mutants (Fig. 7A), probably because arginines and lysines are the preferred targets for trypsin.

Most group I mutants and the group II mutants, K711A and K717A, were more severely digested by trypsin than was WT (Fig. 7), indicating that these mutations possibly induce structural changes. Moreover, the physiologic substrate Mg-PPi, provided substantial protection for most Lys \(\rightarrow\) Ala mutants against trypsin (Fig. 7). However, the presence of Mg-PPi failed to substantially protect K711A, K717A, and K250R mutants against trypsin (Figs. 6B and 7B). We hypothesize that mutations at these sites may induce conformational changes, resulting in decreased substrate protection. The proteolytic properties of the group III mutants were similar to that of WT (data not shown).

**DISCUSSION**

Lysines located in the cytosolic side of H\(^+\) -PPase were classified into three groups according to sequence similarities among species (Fig. 1). Group I (highly conserved) lysines are essential for PPi hydrolysis. When mutated to alanines, the enzymatic and proton translocation functions of H\(^+\) -PPase were almost completely lost (Fig. 3B). Among the group II (moderately conserved) lysines, only the mutations of Lys-711 and Lys-717 increased or decreased PPi hydrolysis (Fig. 3B). Mutation of group III (less or not conserved) lysines had no significant effect on the enzymatic activity (Fig. 3B). Furthermore, K730A H\(^+\) -PPase was not stimulated by K\(^+\), indicating that Lys-730 probably is involved in K\(^+\) binding (Table 1). Moreover, H\(^+\) -PPase is inhibited by Ca\(^2+\) through competing with Mg\(^2+\) to form CaPPi (16). K730A H\(^+\) -PPase was highly...
Roles of Cytosolic Lysine Residues in H\(^{+}\)-PPase

resistant to Ca\(^{2+}\) inhibition, suggesting involvement of Lys-730 in Mg\(^{2+}\) binding. Although it is not common for a positively charged residue to coordinate Mg\(^{2+}\), it was suggested that the aspartate residues, such as Asp-731 and Asp-727, in the vicinity of Lys-730, along the acidic II motif, participated in Mg\(^{2+}\) bind-
ing (19). Consequently, the mutation at Lys-730 might bring about conformational changes, resulting in the Ca\(^{2+}\) resistance of the K730A mutant. In addition, K694A and K695A mutants were highly resistant to F\(^{-}\) inhibition (Table 1), indicating that this di-lysine motif participates in the binding of F\(^{-}\), which mimics the attacking nucleophile for hydrolyzing PPi, in soluble PPase, presumably in the vicinity of the active site (14, 32, 33). Nevertheless, the exact co-factor(s) binding and their positions in H\(^{+}\)-PPase still need to be examined.

The presence of the substrate, Mg-PPi, prevented H\(^{+}\)-PPase from labeling of FITC, a competitive inhibitor to H\(^{+}\)-PPase, demonstrating that the location of its target lysine is at the active site (25). In addition, all mutants, except K250A, were inhibited by FITC (Fig. 4), also suggesting that Lys-250 is the FITC target. Likewise, several other Lys-250 mutants, i.e. K250T, K250S, and K250N, retained hydrolytic activity and were very resistant to FITC, further confirming this hypothesis (Fig. 6A). When Lys-250 was mutated to the negatively charged glutamate, enzymatic activity was completely lost and was also lost when substituted with a positively charged arginine (Fig. 5A). Moreover, substitution of Lys-250 with a glutamine or a methionine also suppressed activity completely (Fig. 5A). These lines of evidence indicate that a bulky side chain or one without a positive charge cause the loss of enzymatic activity. Proton pumping by K250T, K250S, and K250N were somewhat inhibited, resulting in smaller values for the coupling ratio. We hypothesize that Lys-250 is crucial for energy transduction from substrate to the proton gradient (Fig. 5C).

WT and all group I–III mutants except K250A were digested to some extent by trypsin. Hypersensitivity of most group I mutants to proteolysis implies that mutation at these residues induced conformational changes making the enzyme more vulnerable to trypsin (Fig. 7A) and misfolding the active site for no activity. In contrast, Lys-250-substituted H\(^{+}\)-PPases were resistant to trypsin, suggesting that Lys-250 is the primary target of trypsin (Fig. 7A and supplemental Fig. S3). Furthermore, the trypsin sensitivity of K250R was similar to that of group I mutants (Fig. 6B). It is conceivable the mutation at group I residues (except Lys-250) promoted susceptibility of Lys-250 to trypsin. It was also well known that the binding of Mg-PPi decreases vulnerability of the H\(^{+}\)-PPase to trypsin (Fig. 7) (19). Nevertheless, the presence of Mg-PPi, did not effectively protect K250R mutant against trypsin, further suggesting that Lys-250 is essential for substrate binding (Fig. 6B).

It was demonstrated the substrate binding to H\(^{+}\)-PPase induces a conformational transition and stabilizes the enzyme to a higher order structure, preventing the trypsin digestion (Fig. 7) (16, 19). However, the mutation at Lys-711 and Lys-717 of H\(^{+}\)-PPase resulted in a greater susceptibility to trypsin, even in the presence of substrate, indicating more alterations for these mutants in the structure of the enzyme upon substrate binding (Fig. 7B). Previous study revealed that mutation at His-716, the amino acid next to Lys-717 but away from the active domain of H\(^{+}\)-PPase, displayed similar results in a substrate protection experiment (24, 34). Moreover, the attack of His-716 by the noncompetitive inhibitor, diethyl pyrocarbonate, could induce a conformational alteration at the active site of H\(^{+}\)-PPase (23, 24). Taken together, it is suggested the region
Roles of Cytosolic Lysine Residues in H\(^{+}\)-PPase

In phosphorous metabolism, there are several types of pyrophosphate-hydrolyzing enzymes, such as soluble and membrane-bound H\(^{+}\)-PPases (1, 32, 33). The structure of membrane-bound H\(^{+}\)-PPase is less understood, whereas the three-dimensional structure of soluble PPase has been resolved (32, 33). Although the homology in the amino acid sequence of soluble and membrane-bound H\(^{+}\)-PPases is low, they possess several similar motifs, such as the PP\(_{i}\)-binding motif (E/D)XXKXE and acidic motifs in their putative active domains (supplemental Fig. S4) (1, 11). In mung bean H\(^{+}\)-PPase, DXKXE and acidic motifs accommodate some group I lysines, Lys-261 and Lys-730, respectively. Furthermore, soluble and membrane-bound H\(^{+}\)-PPases share many common catalytic characteristics and contain many similar essential amino acid residues (14, 16, 18, 32, 33). Based on the structure of the soluble PPase active site, including the two motifs and a substrate-binding lysine (see Ref. 33 or see Protein Data Bank code 2AUU), and the analysis on the conserved lysines in this study, a working model is accordingly proposed to delineate the critical roles of the functional lysines for substrate binding of H\(^{+}\)-PPase (Fig. 8).

In summary, the conserved lysines in H\(^{+}\)-PPase are involved in substrate hydrolysis, cofactor binding, and structure maintenance. Furthermore, Lys-250 in mung bean H\(^{+}\)-PPase is primarily a trypsin target and may play a role in proton translocation. Nevertheless, the exact mechanisms for the enzymatic and proton translocation reactions still require further elucidations.

REFERENCES

1. Maeshima, M. (2000) Biochim. Biophys. Acta 1465, 37–51
2. Rea, P. A., and Poole, R. J. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 157–180
3. Li, J., Yang, H., Peer, W. A., Richter, G., Blakeslee, J., Bandyopadhyay, A., Titapivantakun, B., Undurraga, S., Khodakovsky, M., Richards, E. L., Kriek, B., Murphy, A. S., Gilroy, S., and Gaxiola, R. A. (2005) Science 310, 121–125
4. Guo, S., Yin, H., Zhang, X., Zhao, F., Li, P., Chen, S., Zhao, Y., and Zhang, H. (2006) Plant Mol. Biol. 60, 41–50
5. Park, S., Li, J., Pittman, J. K., Berkowitz, G. A., Yang, H., Undurraga, S., Morris, I., Hirschi, K. D., and Gaxiola, R. A. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 18830–18835
6. Wu, J. I., Ma, J. T., and Pan, R. L. (1991) FEBS Lett. 283, 57–60
7. Sato, M. H., Maeshima, M., Ohsumi, Y., and Yoshida, M. (1991) FEBS Lett. 290, 177–180
8. Serrano, A., Perez-Castanheira, J. R., Baltscheffsky, H., and Baltscheffsky, M. (2004) J. Bioenerg. Biomembr. 36, 127–133
9. Mimura, H., Nakanishi, Y., Hirono, M., and Maeshima, M. (2004) J. Biol. Chem. 279, 35106–35112
10. Baltscheffsky, M., Schultz, A., and Baltscheffsky, H. (1999) FEBS Lett. 457, 527–533
11. Drozdowicz, Y. M., and Rea, P. A. (2001) Trends Plant Sci. 6, 206–211
12. Fraichard, A., Trossat, C., Perotti, E., and Pugin, A. (1996) Biochimie 78, 259–266
13. Rea, P. A., Britten, C. J., Jennings, I. R., Calvert, C. M., Skiera, L. A., Leigh, R. A., and Sanders, D. (1992) Plant Physiol. 100, 1706–1715
14. Hsiao, Y. Y., Pan, Y. J., Hsueh, S. H., Huang, Y. J., Liu, T. H., Lee, C. H., Lee, C. H., Liu, P. F., Chang, W. C., Wang, Y. K., Chien, L. F., and Pan, R. L. (2007) Biochim. Biophys. Acta 1767, 965–973
15. Baykov, A. A., Dubnov, E. B., Bakuleva, N. P., Evtushenko, O. A., Zhao, R. G., and Rea, P. A. (1993) FEBS Lett. 327, 199–202
16. Maeshima, M. (1991) Eur. J. Biochem. 196, 11–17
17. Takeshige, K., and Hager, A. (1988) Plant Cell Physiol. 29, 649–657
18. Malinen, A. M., Belogurov, G. A., Salminen, M., Baykov, A. A., and Lahti, R. (2004) J. Biol. Chem. 279, 26811–26816
19. Nakanishi, Y., Saijo, T., Wada, Y., and Maeshima, M. (2001) J. Biol. Chem. 276, 7654–7660
20. Zhen, R. G., Kim, E. J., and Rea, P. A. (1997) J. Biol. Chem. 272, 22340–22348
21. Yang, S. J., Jiang, S. S., Kuo, S. Y., Hung, S. H., Tam, M. F., and Pan, R. L. (1999) Biochem. J. 342, 641–646
22. Belogurov, G. A., Turkina, M. V., Penttinen, A., Huopalahl, S., Baykov, A. A., and Lahti, R. (2002) J. Biol. Chem. 277, 22209–22214
23. Hsiao, Y. Y., Van, R. C., Hung, H. H., and Pan, R. L. (2002) J. Protein Chem. 21, 51–58
24. Hsiao, Y. Y., Van, R. C., Hung, S. H., Lin, H. H., and Pan, R. L. (2004) Biochim. Biophys. Acta 1608, 190–199
25. Yang, S. J., Jiang, S. S., Van, R. C., Hsiao, Y. Y., and Pan, R. L. (2000) Biochim. Biophys. Acta 1460, 375–383
26. Bank, S. (1995) Mol. Biotechnol. 3, 1–7
27. Gietz, R. D., Schiestl, R. H., Willems, A. R., and Woods, R. A. (1995) Yeast 11, 355–360
28. Kim, E. J., Zhen, R. G., and Rea, P. A. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 6128–6132
29. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
30. Laemmli, U. K. (1970) Nature 227, 680–685
31. Deleted in proof
32. Fabrichnyi, I. P., Lehtio, L., Tammenkoski, M., Zyrayan, A. B., Oksanen, E., Baykov, A. A., Lahti, R., and Goldman, A. (2007) J. Biol. Chem. 282, 1422–1431
33. Samygina, V. R., Moiseev, M. V., Rodina, E. V., Vorobyeva, N. N., Popov, A. N., Kurilova, S. A., Nazarova, T. I., Avaeva, S. M., and Bartunik, H. D. (2007) J. Mol. Biol. 366, 1305–1317
34. Huang, Y. T., Liu, T. H., Chen, Y. W., Lee, C. H., Chen, H. H., Huang, T. W., Hsu, S. H., Lin, S. M., Pan, Y. J., Lee, C. H., Hsu, I. C., Tseng, F. G., Fu, C. C., and Pan, R. L. (2010) J. Biol. Chem. 285, 23655–23664
35. Hsiao, Y. Y., Hsiao, Y. Y., Liu, P. F., Lin, S. M., Luo, Y. Y., and Pan, R. L. (2009) Bot. Stud. 50, 291–301
36. Dayhoff, M. O., Schwartz, R. M., and Orcutt, B. C. (1978) in Atlas of Protein Sequence and Structure (Dayhoff, M. O., ed) Vol. 5, supplement 3, pp. 345–351, National Biochemical Research Foundation, Washington, D.C.