Mutagenic Analysis of Functional Residues in Putative Substrate-binding Site and Acidic Domains of Vacuolar H\textsuperscript{+}-Pyrophosphatase* 

Received for publication, October 25, 2000, and in revised form, December 11, 2000
Published, JBC Papers in Press, December 11, 2000, DOI 10.1074/jbc.M009743200

Yoichi Nakanishi‡§, Takanori Saijo‡, Yoh Wada¶, and Masayoshi Maeshima||

From the ‡Laboratory of Biochemistry, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan and the ¶Division of Biological Science, Institute of Science and Industrial Research, Osaka University, Osaka 567-0047, Japan

Vacuolar H\textsuperscript{+}-translocating inorganic pyrophosphatase (V-PPase) uses PP\textsubscript{i} as an energy donor and requires free Mg\textsuperscript{2+} for enzyme activity and stability. To determine the catalytic domain, we analyzed charged residues (Asp\textsuperscript{253}, Lys\textsuperscript{261}, Glu\textsuperscript{263}, Asp\textsuperscript{279}, Asp\textsuperscript{283}, Asp\textsuperscript{287}, Asp\textsuperscript{720}, Asp\textsuperscript{722}, and Asp\textsuperscript{731}) in the putative PP\textsubscript{i}-binding site and two conserved acidic regions of mung bean V-PPase by site-directed mutagenesis and heterologous expression in yeast. Amino acid substitution of the residues with alanine and conservative residues resulted in a marked decrease in PP\textsubscript{i} hydrolysis activity and a complete loss of H\textsuperscript{+} transport activity. The conformational change of V-PPase induced by the binding of the substrate was reflected in the susceptibility to trypsin. Wild-type V-PPase was completely digested by trypsin but not in the presence of Mg\textsuperscript{2+}, while two V-PPase mutants, K261A and E263A, became sensitive to trypsin even in the presence of the substrate. These results suggest that the second acidic region is also implicated in the substrate hydrolysis and that at least two residues, Lys\textsuperscript{261} and Glu\textsuperscript{263}, are essential for the substrate-binding function. From the observation that the conservative mutants K261R and E263D showed partial activity of PP\textsubscript{i} hydrolysis but no proton pump activity, we estimated that two residues, Lys\textsuperscript{261} and Glu\textsuperscript{263}, might be related to the energy conversion from PP\textsubscript{i} hydrolysis to H\textsuperscript{+} transport. The importance of two residues, Asp\textsuperscript{253} and Glu\textsuperscript{263}, in the Mg\textsuperscript{2+}-binding function was also suggested from the trypsin susceptibility in the presence of Mg\textsuperscript{2+}. Furthermore, it was found that the two acidic regions include essential common motifs shared among the P-type ATPases.

Vacuolar H\textsuperscript{+}-pyrophosphatase (V-PPase)\textsuperscript{1} belongs to the fourth class of electrogenic proton pump in addition to the P-, F-, and V-type ATPases. The proton pumping reaction couples with the hydrolysis of PP\textsubscript{i}, V-PPase acidifies vacuoles together with vacuolar H\textsuperscript{+}-ATPase in the plant cell and actively exports protons from the cytosol in the bacterial plasma membrane (1–3). V-PPase has the simplest structure among the proton pumps except for bacteriorhodopsin, a light-driven proton pump. The molecular mass calculated from the cDNA sequences range from 80 to 81 kDa for V-PPases of land plants and algae (for a review, see Refs. 3 and 4), while V-PPases in photosynthetic bacteria *Rhodospirillum rubrum* (5) and archaebacteria *Pyrobaculum aerophilum* (6) are relatively small. The simplicity of the enzyme structure and its substrate is an advantage to analyze the structure-function relationship. The enzyme activity is stimulated by K\textsuperscript{+} at relatively high concentrations. Also, Mg\textsuperscript{2+} is essential to form a Mg-PP\textsubscript{i} complex and to keep the active conformation of V-PPase (1, 7, 8). Ca\textsuperscript{2+} prevents formation of a Mg-PP\textsubscript{i} complex (8) and directly inhibits V-PPase (9). Thus, V-PPase should have a specific binding site for its substrate (Mg-PP\textsubscript{i}), Mg\textsuperscript{2+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} in addition to a H\textsuperscript{+} transport channel.

Multiple amino acid sequence alignment of V-PPases of various organisms revealed highly conserved regions (2, 3, 10). There is a putative substrate-binding motif of DXXXXXXXKEE in the cytoplasmic loop (1, 11). This was supported by immunological study with an antibody specific to this sequence (DVGADLVGKEVE) (12). This sequence is common among V-PPases not only from land plants but also from *Chara corallina* (10), *Acetabularia acetabulum* (13), *R. rubrum* (5), *Thermotoga marititia* (GenBank™ accession number AE001702), and *P. aerophilum* (6). Studies using substrate analogs, such as amidomethylenebisphosphonate, have also provided information on the catalytic domain (13–14). Furthermore, the N-ethylmaleimide-binding cysteine residue (Cyse\textsuperscript{634}) (17) and the N,N’-dicyclohexylcarbodiimide-binding residues (Glu\textsuperscript{605} and Asp\textsuperscript{624}) (18) have been identified by a combination of site-directed mutagenesis of *Arabidopsis* V-PPase and heterologous expression in yeast.

The aim of this study is to clarify the substrate-binding site of V-PPase by the method of site-directed and random mutagenesis. We prepared a line of constructs, in which charged residues in a putative substrate-binding site were replaced, expressed in *Saccharomyces cerevisiae*, and then examined for enzymatic properties. V-PPase has been proposed to have three conserved regions (3, 10). In addition to a putative PP\textsubscript{i}-binding site in the first conserved region, we investigated the two acidic motifs in the first and third conserved regions. Each aspartic acid residue in the two acidic regions was substituted and conservative residues resulted in a marked decrease in PP\textsubscript{i} hydrolysis activity and a complete loss of H\textsuperscript{+} transport activity. The conformational change of V-PPase induced by the binding of the substrate was reflected in the susceptibility to trypsin. Wild-type V-PPase was completely digested by trypsin but not in the presence of Mg\textsuperscript{2+}, while two V-PPase mutants, K261A and E263A, became sensitive to trypsin even in the presence of the substrate. These results suggest that the second acidic region is also implicated in the substrate hydrolysis and that at least two residues, Lys\textsuperscript{261} and Glu\textsuperscript{263}, are essential for the substrate-binding function. From the observation that the conservative mutants K261R and E263D showed partial activity of PP\textsubscript{i} hydrolysis but no proton pump activity, we estimated that two residues, Lys\textsuperscript{261} and Glu\textsuperscript{263}, might be related to the energy conversion from PP\textsubscript{i} hydrolysis to H\textsuperscript{+} transport. The importance of two residues, Asp\textsuperscript{253} and Glu\textsuperscript{263}, in the Mg\textsuperscript{2+}-binding function was also suggested from the trypsin susceptibility in the presence of Mg\textsuperscript{2+}. Furthermore, it was found that the two acidic regions include essential common motifs shared among the P-type ATPases.

* This work was supported by Grants-in-Aid for Scientific Research 10219203 and 11163212 (to M. M.) from the Ministry of Education, Science, and Sports of Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of Japan Society for the Promotion of Science for Young Scientists Research Fellowship 11000510.
¶ To whom correspondence should be addressed. Tel.: 81-52-789-4096; Fax: 81-52-789-4094; E-mail: maeshima@agr.nagoya-u.ac.jp.
|| The abbreviations used are: V-PPase, vacuolar H\textsuperscript{+}-translocating inorganic pyrophosphatase; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; Mes, 4-morpholineethanesulfonic acid.

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
ScGAP2

F668

TABLE I

| Name          | Primer sequence          | Name          |
|---------------|--------------------------|---------------|
| D253A         | 5′-CCAGCTGCTGTCAGGGTTTCGCTGACT-3′ | R1251         |
| D253E         | 5′-AAGGCTCCTGAGATGGGTGCTGACT-3′ | R1251         |
| K261A         | 5′-CTTTCCTCACAAGCCGAAGCTGACT-3′ | F668          |
| K261R         | 5′-ACAGTTGTGGAGAGATGGAAGAC-3′ | R1251         |
| E283A         | 5′-CTCGTTGGAAGATGGATGAAAC-3′ | R1251         |
| E283D         | 5′-TGGCAAGGTTGAAGAACATCCCG-3′ | R1251         |
| E283F         | 5′-CTGATTGGGTAAGATGGATGAAAC-3′ | R1251         |
| D279C         | 5′-GAGATTTGGGTAAGATGGATGAAAC-3′ | ScGAP2        |
| D279E         | 5′-GAGATTTGGGTAAGATGGATGAAAC-3′ | ScGAP2        |
| D283A         | 5′-GAGATTTGGGTAAGATGGATGAAAC-3′ | ScGAP2        |
| D283E         | 5′-GAGATTTGGGTAAGATGGATGAAAC-3′ | ScGAP2        |
| D287A         | 5′-GAGATTTGGGTAAGATGGATGAAAC-3′ | ScGAP2        |
| D287E         | 5′-GAGATTTGGGTAAGATGGATGAAAC-3′ | ScGAP2        |
| D253A         | 5′-GAATGATTTGGAAGATGGATGAAAC-3′ | R1251         |
| D253E         | 5′-GAATGATTTGGAAGATGGATGAAAC-3′ | R1251         |
| D279C         | 5′-GAATGATTTGGAAGATGGATGAAAC-3′ | ScGAP2        |
| D279E         | 5′-GAATGATTTGGAAGATGGATGAAAC-3′ | ScGAP2        |

EXPERIMENTAL PROCEDURES

Heterologous Expression of Mung Bean V-PPase in Yeast Cell—A EcoRI-SalI fragment of VVP2 cDNA encoding mung bean V-PPase (Ref. 19; DDBJ accession number AB009077) was inserted into a URA3-marked, high copy (2 µm) yeast expression vector pKT10 (20, 21). The obtained pKVVP2 plasmid was introduced into a S. cerevisiae strain BJ5458, which was deficient in major vacuolar proteinases (22), by the lithium acetate/single-stranded DNA/polyethylene glycol transformation method (23). Positive Ura + colonies were selected, and the expression of V-PPase was confirmed by immunoblotting with the anti-V-PPase antibodies previously prepared (24).

Plasmid Preparation for V-PPase Mutants—Site-directed mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene) by the method of Kirsch and Joly (25). Mutagenic and antisense standard primers used in this study are listed in Table I. The DNA sequences of at least two independent plasmids for each mutant were determined to confirm the mutation points.

Preparation of a Random Mutation Library and Screening of V-PPase Mutant—For convenience of genetic manipulation, the AatII site of pKVVP2 plasmid in pKT10 vector was removed, and a SacI site at position 715 in the plasmid was introduced by substitution with a synonymous codon. The resulting pKVVP2/S plasmid was used as a seed for hypermutagenic polymerase chain reaction (26). The reaction mixture contained 40 pg/ml pKVVP2/S plasmid, 250 mM primers (Table I, F668 and R1251), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin, 2.5 mM MgCl₂, 0.5 mM MnCl₂, 0.1 mM dATP, 0.1 mM dCTP, 1 mM dGTP, 1 mM dTTP, and 0.08 units/ml Taq DNA polymerase. Amplification was done using 20 cycles of a set of 30 s at 95°C, 45 s at 55°C, and 5 min at 72°C. The obtained polymerase chain reaction fragments were digested and inserted into the SacI–AatII site of the pKVVP2/S plasmid. This library was amplified in E. coli and introduced into yeast BJ5458. A region that was mutagenized in each mutant was amplified by polymerase chain reaction using F668 and R1251 primers. Mutation points were confirmed by DNA sequencing. In most mutants, plural sites were mutated.

Preparation of V-PPase Enriched Membrane Fraction—Crude membrane fractions were prepared from yeast cells by the method of Kim et al. (27) with a few modifications. Yeast cells were precultured at 30°C for 2 days in AHCW/Glc medium that contained 50 mM potassium phosphate buffer, pH 5.5, 0.002% (w/v) adenine sulfate, 0.002% tryptophan, 2% glucose, 1% casamino acids (Nihon Pharmaceutical Co.), and 0.67% yeast nitrogen base without amino acids and adenine sulfate (Difco). The cell culture was diluted 64-fold and then grown for 12 h to reach an exponential phase. After being washed with 0.1 M Tris-HCl, pH 9.4, 50 mM 2-mercaptoethanol, and 0.1 M glucose at 30°C for 10 min, cells were treated with a zymolase medium at 30°C for 1 h with gentle agitation. The medium contained 0.05% zymolase 20T (Seikagaku Kogyo Co.), 0.9% sorbitol, 0.1 M glucose, 50 mM Tris-Mes, pH 7.6, 5 mM DTT, 0.043% yeast nitrogen base without amino acids and ammonium sulfate, and 0.25× dropout solution composed of all amino acids and adenines (28). Spheroplasts were collected from the suspension by centrifugation at 3,000 × g for 10 min and washed with 1× sorbitol.

The spheroplasts were resuspended in 50 mM Tris-acetate, pH 7.6, 1.1 mM glyceral, 1.5% polyvinylpyrrolidone (Mr, 40,000), 5 mM EDTA-Tris, 1 mM PMSF, 0.2% bovine serum albumin, 1 mM EDTA, and 1 µg/liter leupeptin and then homogenized with a motor-driven Teflon homogenizer. After centrifugation at 2,000 × g for 10 min, the precipitate was suspended in the same buffer and centrifuged again. All of the supernatant fractions were pooled and centrifuged at 120,000 × g for 30 min. The precipitate (membranes) was suspended in 15% (w/w) sucrose and layered on a 35% (w/w) sucrose solution. Both sucrose solutions contained 1 M NaCl, 50 mM sodium molybdate, 0.02% Triton X-100, and 30 mM Tris-Mes, pH 7.2 in the absence (right) or presence (left) of 0.5 mM KF. In an experiment (NaCl at 50 mM was used instead of KCl). Released Pi was measured colorimetrically.

The membrane vesicles were assayed for the PP₇-dependent H⁺ transport activity with 1 µM acridine orange. Reaction was started by the addition of PP₇ (1 mM). The initial rate of fluorescence quenching was defined as the H⁺ transport activity.
RESULTS

Expression of Mung Bean V-PPase in Yeast as a Functional Enzyme—A yeast expression vector pKT10 was used to express a cDNA (VVP2) encoding mung bean V-PPase in yeast (Fig. 1A). For DNA manipulation, an EcoRI site in VVP2 was eliminated by substitution with synonymous codons. VVP2 was efficiently transcribed under the control of the promoter of glyceraldehyde-3-phosphate dehydrogenase in a medium supplemented with glucose (30). The V-PPase-enriched membrane fraction was prepared by a stepwise sucrose gradient centrifugation and then subjected to immunoblotting with the polyclonal antibodies specific to V-PPase. Transformants produced a 73-kDa protein that was reacted with the antibodies (Fig. 1B). The recombinant V-PPase accounted for 2 to 3% of the total membrane protein. The membranes showed the PP_i hydrolysis activity that was insensitive to KF, an inhibitor of acid phosphatase (Fig. 1C). The activity was 3.5 times enhanced by 50 mM KCl but not by 50 mM NaCl. The membrane vesicles prepared from VVP2 transformant gave the PP_i-dependent H^+ transport activity (Fig. 1D). The pH gradient was collapsed by the addition of membrane-permeable ammonium ion, indicating the electrogenic H^+ transport in the vesicles. The elimination of an EcoRI site from the original VPP2 sequence did not affect the expression level and the enzymatic activity. The results indicate that V-PPase expressed in yeast functions normally in the heterologous system.

A Series of V-PPase Mutants in Respect to Putative Functional Motifs—The sequence DVGADLVGKVE is a putative substrate-binding site of V-PPase (2, 11, 31). This motif has been demonstrated to be exposed to the cytosol (12). To evaluate the functional significance of the charged residues in this motif, we generated a series of V-PPase mutants, in which the residues were replaced with alanine (D253A, K261A, E263A) or conservative residues (D253E, K261R, E263D).

A comparison of the primary structures of V-PPases of various organisms revealed that there are two consensus acidic regions, DNVGDNVGD (acidic region 1) and DTGADLVGKVE (acidic region 2). The former is near the DVGADLVGKVE motif but not by 50 mM NaCl. The membrane vesicles prepared from VVP2 transformant gave the PP_i-dependent H^+ transport activity (Fig. 1D). The pH gradient was collapsed by the addition of membrane-permeable ammonium ion, indicating the electrogenic H^+ transport in the vesicles. The elimination of an EcoRI site from the original VPP2 sequence did not affect the expression level and the enzymatic activity. The results indicate that V-PPase expressed in yeast functions normally in the heterologous system.
interaction with the substrate and Mg2+, we generated a series of mutants substituted with alanine (D279A, D283A, D287A, D723A, D727A, and D731A) and glutamic acid (D279E, D283E, D287E, D723E, D727E, and D731E). These V-PPase mutants were assayed for the enzymatic activities.

**Enzymatic Activities of V-PPase Mutants**—The V-PPase protein in each mutant was clearly detected as a 73-kDa protein in the absence or low expression of V-PPase. As a control, we obtained a V262A mutant from a random mutation library. This V262A mutant had the same V-PPase activity and protein level as the wild type. These results proved that aspartate residues in the putative PPi-binding site and the acidic motifs are involved in the binding and hydrolysis of the substrate. There was no difference in the protein level of V-PPase in the membranes among the mutants (Fig. 3A). Thus, the loss of activity was not due to the absence or low expression of V-PPase.

Interestingly, the K261R and E263D mutants retained 25 and 50% of the original PPi hydrolytic activity, respectively, at 1 mM MgCl2, but they had no H+ transport activity. Thus, Lys261 and Glu263 may be involved in the energy transduction from PPi hydrolysis to H+ translocation. Furthermore, three other mutants (E263G, V259A, and C304R) with a single substitution of an amino acid were obtained by a random mutation technique. The E263G and C304R mutants gave no activity of PPi hydrolysis or H+ transport (Fig. 4). The V259A mutant had 60% of the original activity of PPi hydrolysis but no H+ transport activity as well as K261R and E263D.

**Mg2+ and Substrate Binding Properties in V-PPase Mutants**—V-PPase requires Mg2+ for activation, structural stabilization, and protection from protease digestion (1, 7, 8). In this study, yeast vacuolar membranes containing V-PPase were treated with trypsin in the presence or absence of Mg2+, PPi, and Mg-PPi. Trypsin preferentially cleaves at the carboxyl sides of arginine and lysine residues. As shown in Fig. 5A, Mg2+ and Mg-PPi, but not PPi, at relatively high concentrations partially prevented digestion of V-PPase by trypsin. Thus, the trypsin susceptibility is a good marker for the structural change caused by the binding of Mg2+ and Mg-PPi.

All V-PPase mutants were thoroughly digested by trypsin in the absence of Mg2+ (Fig. 5B, panels none and 1 mM PPi). Therefore, the amino acid substitution did not affect the trypsin cleavage site. It should be noted that Mg2+ protected the enzyme from trypsin digestion in mutants of K261A, D279A, D283A, D287A, D723A, D727A, and D731A as compared with the wild type V-PPase (Fig. 5B, 1 mM Mg2+). Neither Mg2+ nor PPi has a direct inhibitory effect on trypsin, since V-PPase in the E263A mutant was completely digested even in the presence of 1 mM Mg2+ and 1 mM PPi (Fig. 5B). This observation indicates that these amino acid substitutions in-
crease the affinity of the enzyme for Mg$^{2+}$. In other words, the charged residues of Lys 261, Asp 279, Asp 283, Asp 287, Asp 723, Asp 727, and Asp 731 may have a negative effect on the Mg$^{2+}$-binding property of V-PPase.

In the presence of both Mg$^{2+}$ and PPi, certain mutants were resistant to trypsin as was the wild type V-PPase. However, the K261A, E263A, and K261R mutants were sensitive to trypsin, and the other alanine mutants (D279A, D723A) and the glutamate mutants (D253E, D279E, D283E, D287E, and D723E) were partially sensitive under the assay conditions. These results suggest that Lys261 and Glu263 are essential for the substrate-binding function. The other aspartate residues at 253, 279, 283, 287, and 723 may also be implicated in the substrate-binding function or located near the substrate-binding site of V-PPase.

**DISCUSSION**

V-PPase has been estimated to possess the binding sites for its substrate (Mg-PPi), Mg$^{2+}$, K$^+$, and Ca$^{2+}$, independently, and a H$^+$ transport pathway. In the present study, we examined the functional role of charged residues in the putative substrate-binding site (DVGADLVGKVE) and two conservative acidic regions of V-PPase by site-directed and random mutagenesis in combination with a heterologous expression system in *S. cerevisiae*. The V-PPase expressed in yeast exhibited the PPi hydrolysis and proton pump activities, and the enzyme protein was detected by immunoblotting. V-PPase translated in yeast was considered to be localized mainly in vacuolar membranes judging from the following three observations: vacuolar type H$^+$-ATPase was detected in the same membrane fraction; a PPi-dependent H$^+$ current was detected in intact vacuoles prepared from the VVP2-transformed yeast cells by the patch clamp technique; and the amount of the V-PPase protein in a yeast strain that lacked the major vacuolar proteases was higher than that in the normal strain.

**Role of a DVGADLVGKVE Motif and Acidic Regions in Enzyme Activity**—The substitution of acidic residues in the putative substrate-binding motif and two acidic regions had a negative effect on the V-PPase activity (Fig. 3). This is a direct effect of amino acid substitution, since all V-PPase mutants expressed in yeast were accumulated in the membrane at equal levels. The PPi hydrolysis activity was markedly decreased even in the case of conservative substitution (D253E, K261R, and E263D), although the V262A mutant retained the original activity. By comparison, between wild type and mutant, the role of each original residue is evaluated to be positive (P) or negative (N) for activity to protect the enzyme from trypsin.

![FIG. 5. Trypsin digestion assay of V-PPase mutants in the presence of Mg$^{2+}$ and PPi. A, yeast membrane preparation (25 μg) containing wild type V-PPase was mixed with 0.5% detergent and then incubated with 0.1 μg of trypsin in the presence of MgCl$_2$ and PPi at the indicated concentrations. Samples (5 μg) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-V-PPase antibody. NT, nontreatment with trypsin. B, membrane preparations of V-PPase mutants were treated with trypsin in the presence of 1 mM PPi, 1 mM MgCl$_2$, or 1 mM MgCl$_2$ plus 1 mM PPi. The remaining V-PPase protein was determined by immunoblotting.](image)

![FIG. 6. Schematic model for substrate- and Mg$^{2+}$-binding sites of V-PPase. A, nine amino acid residues of V-PPase were substituted to the indicated residues. The trypsin susceptibility of the mutants in the presence of Mg$^{2+}$ (Mg) or Mg$^{2+}$ plus PPi (Mg + PPi) were examined as shown in Fig. 5. The intensity of immunostained band of V-PPase was determined densitometrically and compared with the wild type V-PPase. The original residues are circled. The mutant with the indicated residue on the left of each column is more resistant to trypsin. By comparison, between wild type and mutant, the role of each original residue is evaluated to be positive (P) or negative (N) for activity to protect the enzyme from trypsin. B, the amino acid residues that are involved in binding of Mg$^{2+}$ (left) and a Mg$^{2+}$-PPi complex (right) are shown with solid lines. Some charged residues that have a negative effect on Mg$^{2+}$ binding are shown with broken lines.](image)
susceptibility even at 1 mM, but PPi plus Mg$^{2+}$ makes V-PPase resistant to trypsin (Fig. 5A). Furthermore, the trypsin susceptibility assay revealed that Lys$^{261}$ and Glu$^{263}$ are essential for the binding of the substrate (Fig. 5). Fig. 6B shows a scheme of our model.

This work demonstrated the functional significance of acidic residues in the acidic regions (DNSVGDNVDG$^{287}$ and DTXGD-PXK$^{731}$) (Fig. 2). The sequences are conserved among V-PPases of various organisms including R. rubrum (5) and T. maritima (GenBank$^{TM}$ accession number AE001702). Substitution of the aspartate residue at 279, 283, 287, 723, 727, and 731 with alanine or glutamate residue resulted in the complete loss of the activity (Fig. 3). The digestion assay with trypsin also supported the implication of aspartate residues at 279, 283, 287, and 731 in the binding of Mg-PP (Fig. 5). These residues may be adjacent to the DVGADLVGKVE motif in the tertiary structure of V-PPase as discussed later.

The present study also suggested the residues involved in energy coupling of V-PPase. The K261R and E263D mutants partially retained the PP hydrolysis activity, 25 and 50% of that of the wild-type enzyme, respectively, but the mutants did not exhibit the H$^+$ transport activity (Fig. 3). The V259A mutant also retained partial activity (60%) of PP hydrolysis but no proton pump activity (Fig. 4). These three residues are located in the putative PP-binding site. Thus, the conserved motif including Val$^{255}$, Lys$^{261}$, and Glu$^{263}$ may be implicated in the initial step of the energy transfer from PP, hydrolysis to the H$^+$ translocation. In Arabidopsis V-PPase, Glu$^{427}$ in the transmembrane domain has been demonstrated to be involved in the energy coupling by the site-directed mutagenesis (18). The role of several charged residues in the transmembrane domains remains to be investigated for their role in H$^+$ translocation.

**Residues Involved in Binding of Free Mg$^{2+}$.** The presence of Mg$^{2+}$ decreased the susceptibility of V-PPase to trypsin (Fig. 5A), which is consistent with a previous report (8). Interestingly, V-PPase became resistant to trypsin in several V-PPase mutants (K261A, D279A, D283A, D287A, D723A, D727A, D731A, E263D, and D727E) compared with the wild-type V-PPase, while the D253A and E263A mutants were completely digested by trypsin (Fig. 5). This suggests that the two residues Asp$^{253}$ and Glu$^{263}$ are essential for Mg$^{2+}$ binding (Fig. 6). On the other hand, Lys$^{261}$ and other aspartate residues in the acidic regions seem to interfere with V-PPase in the binding of Mg$^{2+}$ (Fig. 6B, broken lines). Probably, these aspartate residues weaken the interaction between Mg$^{2+}$ and a binding site that includes Asp$^{263}$ and Glu$^{263}$ (Fig. 6B). Since Asp$^{253}$ and Glu$^{263}$ may be involved in the binding of a Mg-PP complex as mentioned above, the two acidic residues may be located in the substrate-binding pocket and also have an ability to interact with free Mg$^{2+}$. Probably, there is another, high affinity binding site for free Mg$^{2+}$ independent of the substrate-binding site. However, those residues could not be detected in the present assay system of trypsin susceptibility in the presence of 1 mM Mg$^{2+}$.

**Common Motifs in Acidic Regions among V-PPase and P-type ATPase.** With respect to the conserved acidic regions, we found that V-PPases share common motifs with the P-type ATPases such as Ca$^{2+}$-ATPase. In the P-type ATPase, the ATP-binding and ATP-hydrolyzing domain has been proposed to consist of several conserved motifs of DKTG7, DPPR (or DKVR), (T/S)GD(N/K), and GDGXND (32, 33). Among these motifs, the TGDN and GDGXND motifs are conserved in V-PPases as a GDN motif including Asp$^{263}$ in the first acidic region and a GDTIGD motif including Asp$^{723}$ and Asp$^{727}$ in the second acidic region (Fig. 7). It has been proposed that the TGDN motif is involved in ATP hydrolysis together with a DPPR motif in the P-ATPases and that the adenosine moiety of ATP interacts with the other motifs such as a KGAP motif of the P-ATPase (32, 33). It has been recently reported that the amino acid residues that are involved in the binding of adenosine moieties and the hydrolysis of phosphoanhydride bond of ATP can be topologically distinguished in the crystal structure of sarcoplasmic reticulum Ca$^{2+}$-ATPase (34). The DPPR (or DKVR) motif, which is located in the hydrolysis pocket, is present as DRRR$^{723}$ in loop e and DNAR$^{693}$ in loop m of V-PPases except for A. acetabulum V-PPase (3, 13), but an adenosine-binding KGAP motif is not conserved in the V-PPase. Aravind et al. (35) have proposed that two aspartate residues in the GDGXND motif of the P-ATPases have a role in hydrolysis of the phosphoanhydride bond of ATP. The present study revealed not only the importance of the two acidic regions in addition to the PP-binding motif proposed previously but also the functional and sequence similarity of V-PPases to the P-type ATPases. However, it cannot be concluded that V-PPase is a member of the P-type ATPase family, since V-PPase lacks a phosphorylation domain (DKTGTLT) that is common to the P-ATPases including plasma membrane H$^+$-ATPase, Ca$^{2+}$-
ATPase and other heavy metal-transporting ATPase (32). At present, it is unclear whether V-PPase forms an intermediate phosphorylation form during PPi hydrolysis.

In summary, Lys261 and Glu263 of mung bean V-PPase are essential for the substrate-binding function, and Asp253 and Glu263 are essential for the Mg2+ binding function (Fig. 6). All aspartate residues (Asp253, Asp279, Asp283, Asp287, Asp287, Asp277, and Asp271) in the PPi-binding motif and two acidic regions may be involved in interaction with the substrate. It has also been reported that Glu305 and Asp504 of Arabidopsis V-PPase, Glu301 and Asp500 for mung bean V-PPase, respectively, are essential for PPi hydrolysis activity (18). Thus, we propose that these two acidic regions (279–287 and 723–731) and a common DXADLVGKXE (253–263) motif form a core catalytic domain of the V-PPase together with a few other motifs. To verify our model, we need to perform a high resolution crystallographic study to determine the structure.

Acknowledgments—We thank Drs. K. Nakamura, A. Morikami, K. Matsuoka, and H. Ueoka-Nakanishi of Nagoya University for stimulating discussions.

REFERENCES

1. Rea, P. A., and Poole, R. J. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 157–180
2. Baltscheffsky, M., Schultz, A., and Baltscheffsky, H. (1999) FEBS Lett. 457, 527–33
3. Maeshima, M. (2000) Biochim. Biophys. Acta 1465, 37–51
4. Zhen, R. G., Kim, E. J., and Rea, P. A. (1992) Adv. Bot. Res. 18, 195–202
5. Gordon-Weeks, R., Parmar, S., Davies, T. G., and Leigh, R. A. (1999) Biochem. J. 337, 373–377
6. Kirsch, R. D., and Joly, E. (1998) Nuc. Acids Res. 26, 1848–1850
7. Vartanian, J. P., Henry, M., and Wain-Hobson, S. (1996) Nucleic Acids Res. 24, 2627–2631
8. Kim, E. J., Zhen, R. G., and Rea, P. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6128–6132
9. Sherman, F., Fink, G. R., and Lawrence, C. W. (1979) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
11. Rosenberg, S., Coit, D., and Tekamp-Olson, P. (1990) Methods Enzymol. 185, 341–351
12. Rea, P. A., Kim, Y., Sarafian, V., Poole, R. J., Davies, J. M., and Sanders, D. (1999) Trends Biochem. Sci. 24, 548–351
13. Williams, L. E., Pittman, J. K., Hall, J. L. (2000) Biochim. Biophys. Acta 1452, 104–126
14. Stokes, D. L., Wagenknecht, T. (2000) Eur. J. Biochem. 267, 5274–5279
15. Toyoshima, C., Nakazato, M., Nomura, H., Ogawa, H. (2000) Nature 405, 647–655
16. Aravind, L., Galperin, M. Y., Koonin, E. V. (1998) Trends Biochem. Sci. 23, 127–129
17. Hoffman, K., and Steffel, O. (1993) Biol. Chem. Hoppe-Seyler 374, 166 (abstr.)