Homology models of plasma membrane H\textsuperscript{+}-ATPase (Bukrinsky, J. T., Buch-Pedersen, M. J., Larsen, S., and Palmgren, M. G. (2001) FEBS Lett. 494, 6–10) has pointed to residues in transmembrane segment M4 as being important for proton translocation by P-type proton pumps. To test this model, alanine-scanning mutagenesis was carried out through 12 residues in the M4 of the plant plasma membrane H\textsuperscript{+}-ATPase AHA2. An I282A mutation showed apparent reduced H\textsuperscript{+} affinity, and this residue was subsequently substituted with all other naturally occurring amino acids by saturation mutagenesis. The ability of mutant enzymes to substitute for the yeast proton pump PMA1 was found to correlate with the size of the side chain rather than its chemical nature. Thus, smaller side chains (Gly, Ala, and Ser) at this position resulted in lower H\textsuperscript{+} affinity and lowered levels of H\textsuperscript{+} transport \textit{in vivo}, whereas substitution with side chains of similar and larger size resulted in only minor effects. Substitutions of Ile-282 had only minor effects on ATP affinity and sensitivity toward vanadate, ruling out an indirect effect through changes in the enzyme conformational equilibrium. These results are consistent with a model in which the backbone carbonyl oxygen of Ile-282 contributes directly to proton translocation.

Plasma membrane proton ATPases are vital proteins for plant and fungal life. These pumps are responsible for ATP-fueled ejection of protons out of the cell and establish the essential proton and electrical gradient across plant and fungal plasma membranes. Phylogenetically, plasma membrane proton ATPases belong to a large group of proteins termed P-type ATPases. Common for P-type ATPases is the formation of a phosphorylated intermediate during catalysis, inhibition by vanadate, and a common domain structure with typically 10 transmembrane helices and a large cytoplasmic extension (1). Cation transport by the membranous part of the protein is coupled to ATP splitting taking place in the cytoplasmic part of the protein. Members of the P-type ATPase family are localized in a variety of membrane systems and transport cations such as Na\textsuperscript{+}, K\textsuperscript{+}, H\textsuperscript{+}, Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and Cd\textsuperscript{2+} (2). The mammalian Na\textsuperscript{+}/K\textsuperscript{+}- and Ca\textsuperscript{2+}-ATPases, together with plant and fungal plasma membrane H\textsuperscript{+}-ATPases, are among the best investigated P-type ATPases.

In addition to the plant and fungal plasma membrane proton ATPases, other P-type pumps can catalyze proton translocation. Thus, both Ca\textsuperscript{2+}-ATPases (3) and H\textsuperscript{+}/K\textsuperscript{+}-ATPases (4) transport protons during their catalytic cycle; the H\textsuperscript{+}/K\textsuperscript{+}-ATPases transport in the same direction as the plasma membrane H\textsuperscript{+}-ATPases, and the Ca\textsuperscript{2+}-ATPases transport the opposite way. Still, the mechanism of proton transport and the nature of the transported species (H\textsuperscript{+}/H\textsubscript{3}O\textsuperscript{+}) is yet to be described for any proton-translocating P-type ATPase (5).

Hydrogen ions differ qualitatively from the other cations transported by P-type ATPases (6). First, hydrogen ions have no free existence as naked ions in solution where they are found as hydrated proton molecules in the form of the hydronium ion complex (H\textsubscript{3}O\textsuperscript{+}). Second, protons have a very high mobility in aqueous solution along proton wires, hydrogen-bonded networks of water molecules allowing for fast proton conductance. Currently described proton-translocating proteins have taken advantage of this capability of protons to propagate along hydrogen-bonded networks involving e.g. an ordered chain of protonable amino acid side chain groups, prosthetic groups, and/or water molecules (7–9).

The transport mechanism of P-type ATPases has been described as an alternating access mechanism in which one or more cation coordination centers change affinity for the transported ion(s) (1). In these coordination centers, the transported cations get tightly bound within the membrane sector of the protein during catalysis in a process termed occlusion. The nature of such a P-type ATPase coordination center is most clearly seen in the high resolution structure of the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase in which oxygen atoms from both the side chains and the main chain carbonyls provide liganding groups for the coordination of two calcium ions (10).

Provided that all P-type ATPases share a common mechanism for transport of ions, it would be expected that proton-translocating P-type ATPases form proton coordination centers during catalysis. In such a scenario, the transported proton is likely to get occluded during catalysis in the form of a hydronium ion in a specific high affinity hydronium ion coordination center (5). No hydronium coordination center in a protein has been described to date, but high affinity hydronium ion coordination centers have been demonstrated in specific crown ether structures (11, 12) in which the hydronium ion is coordinated by the use of hydrogen bonds.

Based upon the Ca\textsuperscript{2+}-bound sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase crystal structure, homology models of the Arabidopsis thaliana AHA2 H\textsuperscript{+}-ATPase (13), the yeast Saccharomyces cerevisiae PMA1 H\textsuperscript{+}-ATPase (13), and the Neurospora crassa PMA1 H\textsuperscript{+}-ATPase (14) have been presented. In the region corresponding to the calcium binding sites of the Ca\textsuperscript{2+}-ATPase, only a single charged amino acid residue seems to be strictly conserved between these pumps (Asp-684\textsubscript{AHA2}, Asp-730\textsubscript{PMA1},...
Proton Pumping Mechanism of Plasma Membrane H⁺-ATPase

EXPERIMENTAL PROCEDURES

Expression in Yeast—The S. cerevisiae strain RS-72 was transformed and cultured essentially as described previously (17). In RS-72 the natural constitutive promoter of the endogenous yeast plasma membrane H⁺-ATPase, PMA1, has been replaced by the galactose-dependent promoter of GAL1. Because PMA1 is essential for yeast growth, this strain only grows on galactose medium. By using the RS-72 yeast strain, we can test plant H⁺-ATPases brought under the control of the constitutive PMA1 promoter for their ability to rescue pma1 mutants on glucose medium. In a study a plant H⁺-ATPase devoid of 73 COOH-terminal amino acids, aha2Δ73, was used because it is constitutively active and complements pma1 mutations well (18). Complementation tests were as reported previously (17). Yeast cells were grown and harvested, and microsomal membranes were isolated as described (17).

Pma1 Complementation Test in Yeast—Yeast was grown for 3 days at 30 °C in liquid medium containing 2% galactose. Approximately 250 cells in 10 μl were spotted onto minimal media plates containing either 2% galactose (Gal) at pH 5.5 or 2% glucose (Glu) at pH values of 6.5, 5.5, and 4.5. Growth was recorded after incubation for 3 days at 30 °C. Very similar results were seen for three independent complementation experiments.

Gel Electrophoresis and Western Blotting—Membrane fractions were separated by electrophoresis on 10% SDS-polyacrylamide gels. After electrotransfer of the proteins to an Immobilon-P membrane (Milli-}

and Asp-800SERCA1a) (13). The carboxylate side chain of Asp-684AHA2 has previously been demonstrated to be essential for translocation of protons (15), and in the sarcoplasmic reticulum Ca⁺⁺-ATPase the corresponding residue is liganding both of the bound calcium ions (10).

In the structure of Ca⁺⁺-ATPase, a region of M4 is unwound that allows for liganding backbone carboxyl oxygens to be provided to calcium binding site IIA (10). Unwinding of M4, supported by conserved Pro residues, has been proposed to be a characteristic feature of P-type ATPases (16). In the homology model of the plasma membrane H⁺-ATPase AHA2, this proposed unwound part of M4 seems spatially close to the essential Asp-684AHA2 (13). This raises the question as to whether backbone carboxyl groups of M4 in the P-type H⁺-ATPases, in combination with other liganding groups such as Asp-684AHA2, participate directly in the proton transport process. In an effort to illuminate this question, the aim of the present study was to investigate the role in proton transport of residues in M4 of the plant plasma membrane H⁺-ATPase.

Fig. 1. Amino acid sequence alignment of transmembrane-spanning helix M of the SERCA1a Ca⁺⁺-ATPase (Swiss-Prot accession number P04159) and the plant H⁺-ATPase AHA2 (Swiss-Prot accession number P19456). The alignment was performed using ClustalW (www.ebi.ac.uk/clustalw) employing default parameters. The two unnumbered lines refer to conserved residues between H⁺-ATPases and Ca⁺⁺-ATPases, respectively. Conserved residues between all aligned ATPases are indicated by a light gray background, and the residues from M4 involved in ion coordination in SERCA1 are marked with a black background. Residues from Leu-278AHA2 to Met-289AHA2 subjected to alanine-scanning mutagenesis are indicated by a horizontal line. The M4 region of AHA2 is completely conserved between plant H⁺-ATPases (www.fasta.embnet.nl).

Fig. 2. Ability of plant plasma membrane H⁺-ATPase M4 substitutions to complement a pma1 yeast mutant. Yeast cells transformed with an empty vector were used as a negative control. Approximately 250 cells in 10 μl were spotted onto minimal media plates containing either 2% galactose (Gal) at pH 5.5 or 2% glucose (Glu) at pH values of 6.5, 5.5, and 4.5. Growth was recorded after incubation for 3 days at 30 °C. Very similar results were seen for three independent complementation experiments.

Fig. 3. ATP hydrolytic activity as a function of pH for wild type and mutant plant H⁺-ATPases. ATP hydrolysis was measured on proteoliposomes reconstituted with a microsomal protein isolated from yeast cells expressing plant H⁺-ATPase. Before normalization, the background ATPase activity of membranes from cells transformed with empty vector was subtracted. The result was typical for three independent membrane isolations.

pore), protein blots were probed with an antibody against the MRGSH₆ tag present at the COOH terminus (Qiagen, Chatsworth, CA).

Protein Determination—Protein concentrations were determined by the method of Bradford (21), using bovine serum albumin as a standard.

Glucose-dependent H⁺ Extrusion from Yeast Cells—Proton efflux from yeast cells expressing different H⁺-ATPases was measured by means of a computer-controlled pH-stat system (22, 23).

RESULTS

Alanine Scanning of Transmembrane Segment 4—A typical feature of the SERCA1a Ca⁺⁺-ATPase is that M4 is par-
for yeast growth, RS-72 can only grow on a galactose medium.

Three independent membrane isolations.

Type enzyme. The result was typical for expression levels as compared with the wild type enzyme. ATPases of H\(^+\)-ATPases of ~92 kDa; percentage sign, expression levels as compared with the wild type enzyme. The result was typical for three independent membrane isolations.

H\(^+\)-ATPase (Fig. 3). A G284A mutation did not show any activity for vanadate for the unsubstituted protein and the mutant protein hydrolyzes ATP to 22% of the wild type enzyme and has a very low level of proton pumping (27). As the phenotype of P286A mutation could result from indirect effects due to the special properties of Pro, this mutation was not analyzed further.

When pH dependence profiles for G284A and I282A mutants were investigated, only the I282A mutant demonstrated a changed pH dependence compared with that of the wild type H\(^+\)-ATPase (Fig. 3). A G284A mutation did not show any apparent change in H\(^+\) dependence (Fig. 3). The fact that a higher proton concentration was required for optimal activity could imply that the affinity of the enzyme for the transported H\(^+\) has decreased. To further investigate the role of Ile-282 in the proton pumping mechanism of AHA2, this residue was chosen for further study. It seemed unlikely that the side chain of this residue could be involved in proton coordination. Homology models (13) have suggested that the carboxyl oxygen of Ile-282 is part of a proton binding site. Because it is not possible by a conventional mutagenesis approach to modify carboxyl oxygens, a saturated mutagenesis analysis approach was chosen.

Expression of Ile-282 Substituted H\(^+\)-ATPases in Yeast—Ile-282 was substituted with all other naturally occurring amino acids, and the resulting mutant proteins were expressed in yeast (Fig. 4). To compare the expression levels of the different Ile-282 substituted H\(^+\)-ATPases, yeast microsomal proteins were separated on polyacrylamide gels (Fig. 4A) and immunoblotted for specific detection of the heterologous plant membrane protein (Fig. 4B). All recombinant ATPases were found to be expressed, but lower accumulation levels were repeatedly seen for the I282D, I282P, I282H, I282K, and I282R mutant proteins compared with the wild type protein (Fig. 4B). Replacement of Ile-282 with Gly, Ala, Ser, and Cys, the pH optimum was determined to be 6.3 (Fig. 6A). In cases where Ile-282 had been replaced by a

The pH Dependence of ATP Hydrolysis for the Plant H\(^+\)-ATPase Shows More Acidic pH Profiles When Ile-282 Is Replaced With Smaller Amino Acid Residues—To investigate the pH dependence for Ile-282 substituted H\(^+\)-ATPases, the level of ATP hydrolysis in the microsomal membrane fraction was studied between pH 6.0 and 7.7. As is characteristic for P-type H\(^+\)-ATPases, the pH profiles of ATP hydrolysis were bell-shaped (Fig. 6). The unsubstituted plant H\(^+\)-ATPase had a pH optimum of ~6.9 (Fig. 6A). In cases where Ile-282 had been replaced by a smaller amino acid residue (Gly, Ala, Ser, and Cys), the pH optimum was shifted toward a more acidic value of 6.6 (Fig. 6A). For H\(^+\)-ATPases with a slightly larger amino acid residue (Val or Thr) in place of Ile-282, the pH optimum was determined to be 6.7 (Fig. 6A). The pH optima for ATP hydrolysis of other

FIG. 4. Expression of wild type and mutant plant H\(^+\)-ATPases in yeast. Six micrograms of microsomal protein of proteoliposomes was loaded into each lane. A, 10% acryl amide gels stained with Coomassie Blue following SDS-PAGE. B, protein blot following immunodecoration with an antibody recognizing recombinant H\(^+\)-ATPase. The single letter amino acid abbreviations indicate the Ile-282 substitutions; ctr, control; arrows, the position of the Ile-282 and mutant plant H\(^+\)-ATPases of ~92 kDa; percentage sign, expression levels as compared with the wild type enzyme. The result was typical for three independent membrane isolations.

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tigated Ile-282 substituted H\(^+/\)ATPases were similar to that of the wild type H\(^+/\)ATPase (Figs. 6, B and C). Thus, the pH dependence of proteins substituted at position Ile-282 and, thus, their proton affinity seems to be related to the size of the side chain rather than its chemical nature.

**FIG. 5.** ATP dependence and vanadate sensitivity of wild type and Ile-282 single point-substituted plant H\(^+/\)ATPases. All measurements were carried out at pH 7.0. Before normalization, the background ATPase activity of membranes from cells transformed with empty vector was subtracted. For \(K_m\) (ATP) and IC\(_{50}\) values, see Table I. A, determination of affinities for ATP. B, determination of vanadate sensitivity of ATP hydrolysis. The result was typical for three independent membrane isolations.

**FIG. 6.** pH dependence of ATP hydrolysis by Ile-282 single point-substituted plant H\(^+/\)ATPases (A–C). Before normalization, the background ATPase activity of membranes from cells transformed with empty vector was subtracted. The result was typical for three independent membrane isolations.

**Ability of H\(^+/\)ATPase Ile-282 Substitutions to Complement pma1 Yeast Mutants—**The Ile-282 substitutions were investigated in a growth test of transformed pma1 yeast mutants (Fig. 7). No complementation was observed when Ile-282 was substituted with a Pro residue (Fig. 7). In addition, positively charged amino acid residues introduced at this position eliminated the ability of the mutant enzymes to substitute for PMA1 (Fig. 7). Substitution with the negatively charged residue Asp likewise had a strong negative effect on yeast growth in this system. Furthermore, relatively small amino acid residues (Gly, Ala or Ser) as replacements for Ile-282 caused less efficient complementation of pma1 by AHA2. All other Ile-282

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**TABLE I**

| H\(^+/\)ATPase protein | Specific activity \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) protein | Vanadate sensitivity \(\mu\)M | \(K_m\) (ATP) \(\mu\)M |
|------------------------|--------------------------|-----------------|----------------|
| I282                   | 0.31 ± 0.10              | 2.4 ± 0.10      | 130 ± 15       |
| I282G                  | 0.06                     | 4.0             | 220            |
| I282A                  | 0.47                     | 3.1             | 220            |
| I282S                  | 0.43                     | 2.3             | 240            |
| I282C                  | 0.33                     | 2.1             | 250            |
| I282V                  | 0.31                     | 1.8             | 150            |
| I282T                  | 0.35                     | 1.3             | 200            |
| I282L                  | 0.07                     | 5.8             | 140            |
| I282N                  | 0.49                     | 1.3             | 150            |
| I282E                  | 0.21                     | 1.7             | 78             |
| I282Q                  | 0.44                     | 1.5             | 170            |
| I282M                  | 0.40                     | 1.1             | 120            |
| I282F                  | 0.12                     | 4.5             | 100            |
| I282Y                  | 0.13                     | 9.5             | 110            |
| I282W                  | 0.24                     | 5.1             | <50            |

*IC\(_{50}\) values were determined for vanadate sensitivity of ATP hydrolysis."
substitutions were able to support yeast growth in the absence of PMA1 (Fig. 7). The specific H⁺-ATPase activity of isolated membranes was typically 0.3 μmol min⁻¹ mg⁻¹ protein for Ile-282 with comparable activities for mutant enzymes, except for Lys, Asp, Arg, Leu, Gly, Pro, and His substitutions that reproducibly showed reduced activity. There was, in general, good correlation between the ATP hydrolytic activities measured in isolated membranes and the ability of mutant pumps to substitute for yeast Pma1p. However, the 1282L and 1282G substitutions supported yeast growth well (Fig. 7) but showed very little ATPase activity in vitro (Table I). The reason for this discrepancy is unclear but could be related to reduced stability of these mutants during the membrane purification process.

The Lowered Proton Affinity of H⁺-ATPases With Small Amino Acids Introduced at Position Ile-282 Is Accompanied by Lowered Proton-transporting Capabilities—To investigate the correlation between the biochemical assays performed with the reconstituted protein and the ability to complement pma1, we measured proton extrusion from intact yeast cells expressing plant 1282G, 1282A, and 1282S H⁺-ATPases (Fig. 8). Compared with the 1282 enzyme, lower rates of H⁺ efflux were observed for the 1282G, 1282A, and 1282S proton pumps (Fig. 8) even though kinetic parameters were difficult to ascertain in this assay.

DISCUSSION

Alanine-scanning of Residues in M4 of the Plant Plasma Membrane H⁺-ATPase—In the investigated region of M4 of the plant plasma membrane H⁺-ATPase AHA2 that stretches from Leu-278 to Met-289 (Fig. 1), only amino acid residues, Gly 284, Pro-286, and Ile-282, seemed to influence proton pumping (Fig. 2). Among these residues, Ile-282 appeared as a potential proton-coordinating candidate, because the 1282A substitution has apparent reduced H⁺ affinity (Fig. 3). Consequently, this residue was investigated in more detail. To more deeply investigate the role of Ile-282 in the proton pumping mechanism of AHA2, we substituted this residue with all naturally occurring amino acids. Yeast cells expressing H⁺-ATPases with a small amino acid residue (Gly, Ala, or Ser) as a replacement for Ile-282 showed less efficient complementation as compared with the wild type H⁺-ATPase, and purified recombinant mutants showed a shift toward more acidic pH profiles for ATP hydrolysis, as expected for a decreased affinity for protons. From our data it appears that at this position the size of the side chain, rather than its chemical properties, determines proton affinity. These substitutions do not exhibit any large change in conformational equilibrium, which would suggest that the effects on H⁺ affinities are direct rather than indirect.

Role of Homologous Residues in Other P-type ATPases—Our analysis has identified Ile-282AHA2 in the plant plasma membrane H⁺-ATPase as belonging to a novel mutant phenotype with decreased H⁺ affinity but no indication of a shift in the Eᵢ-Eₑ conformational equilibrium. The residue has not been systematically analyzed in other P-type pumps, but structure-function studies have been initiated. In the rabbit muscle sarco(endo)plasmic reticulum, Ca²⁺-ATPase SERCA1 mutations of the residue corresponding to Ile-282AHA2 Val-304SERCA1 resulted in mutant proteins with either decreased (V304LSERCA1) or increased (V304ASERCA1) Ca²⁺ affinities (28). Alanine-scanning mutagenesis of M4 in the yeast plasma membrane H⁺-ATPase Pma1p resulted in the identification of three groups of mutated proteins with altered properties (27). Group 1 consists of mutant enzymes defective in biogenesis, group 2 encompasses mutants with reduced ATPase but no apparent changes in kinetic parameters, and
group 3 involves mutant enzymes with a shift in the conformational equilibrium toward the $E_1$ form with high affinity for ATP and an alkaline pH optimum. The residue in Pma1p corresponding to Ile-282AHA2 is Ile-331PMA1, which was classified as a group 2 enzyme (27). We have no explanation for this apparent discrepancy, although it has to be stressed that in Pma1p only an I331A mutant was analyzed. In the Na$^+/\mathrm{K}^+/\mathrm{H}^+$-ATPase the corresponding residue, Val-329NaK, could be altered to a Cys without any detectable changes in protein function (29).

How Could Ile-282 of the Plant $H^+/\mathrm{K}^+$-ATPase Be Involved in Proton Translocation?—The three-dimensional model of the putative $H_3O^+$ coordination center in AHA2 indicates the involvement of three backbone carbonyl oxygen atoms of M4, namely those originating from Ile-282, Gly-283, and Ile-285 (13). Concerning Gly-283 and Ile-285, further investigations are required, but this study demonstrates that Ile-282 could indeed be involved in the proton translocation mechanism of AHA2. The fact that the size rather than the chemical nature of the side chain at position Ile-282 influences the ability of the pump to transport protons is in good agreement with the role of backbone carbonyl oxygen of Ile-282 in determining the proton affinity of the enzyme. The mutational evidence presented in this paper will serve as an important tool for future interpretations of a high resolution crystal structure of a plasma membrane $H^+$-ATPase.

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