Structural identification of cation binding pockets in the plasma membrane proton pump

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MEMBERS of the large family of P-type ATPases operate as pumps that transport cations across biological membranes. The transport process includes discernible catalytic intermediates termed E1, E1P, E2P, and E2, with P denoting a phosphorylated intermediate (1). Structures of the Na+/K+ -ATPase (2, 3), the sarco/plexysomal reticulum (SR) Ca2+ -ATPase (4, 5), and the plasma membrane (PM) H+ -ATPase (6) show that different members of the P-type ATPase family exhibit the same three-dimensional fold with four discrete domains involved in the basic cation transporting machinery: a nucleotide-binding domain (N), a phosphorylation domain (P), an actuator domain (A), and a transmembrane domain (M) (2, 4, 6). The three cytosolic domains are responsible for ATP hydrolysis, and the associated domain movements are coupled to cation translocation through the opening and closing of transport pathways that leads to the cation binding site(s) residing in the central part of the M domain (5, 7–9).

The plasma membrane (PM) H+ -ATPase is a central constituent of all plant and fungal cells where its ejection of protons from the cell generates the essential electrochemical gradient across the plasma membrane. The molecular mechanism of proton transport by the ATPase involves a conserved proton acceptor/donor (Asp684) in the membranous part of the protein (10). Proton inlet and exit pathways leading to and from this central proton binding site have been suggested based on structural data (6, 11). Besides protons, alkali cations, and K+ in particular, increase ATP hydrolytic activity of the PM H+ -ATPase protein (12–14). As the membrane potential approaches the reversal potential, the accumulation of the phosphorylated intermediate is likely to occur. Under these conditions, K+ might serve as an uncoupling factor by introducing a safety valve in the pump (15).

Further, direct reversible inactivation of the PM H+ -ATPase by increased cytosolic Ca2+ has been proposed to be a key step in closing of the stomatal pore in plants (13). No structural data of the proposed K+ and Ca2+ binding sites on the PM H+ -ATPase has so far been presented.

Results

The Delipidated PM H+ -ATPase Is Folded in a Catalytically Competent State. The E1 protein crystals used for localization of the ion-binding sites were produced in the absence of exogenously added lipids and likely represent the integral membrane protein in a delipidated form (6). The PM H+ -ATPase construct used for crystallization is a truncated form of the protein, which lacks the C-terminal part of its autoinhibitory domains and is consequently constitutively activated. After purification, the protein was fully functional following the addition of minor amounts of exogenous lipid (100–200 lipid molecules per protein molecule) (Fig. S1). This indicates that the crystallized PM H+ -ATPase represents a correctly folded and functional ATPase and therefore is suitable as a tool for identification of binding pockets by soaking and/or cocrystallization techniques.

Crystallographic Identification of Rb+ , Tb3+ , and Ho3+ Sites in the PM H+ -ATPase. We initially tried to obtain direct structural information of Ca2+ and K+ binding to the PM H+ -ATPase by crystal soaking and cocrystallization with Ca2+ ions and K+ ions. However, the low anomalous signal of K+ and Ca2+ did not allow for structural identification of these cations in the crystallographic data obtained. We therefore turned to Ca2+ and K+ congeners with a higher anomalous signal.

Rb+ is a congener of K+, and a Rb+ binding site was identified by replacing K+ with Rb+ in the crystallization setup. The published structure of the E1-AMPPCP form refined at 3.6-Å resolution was used as model, and anomalous difference Fourier maps were calculated based on data from 40 to 7.5 Å resolution and model phases (Table S1). Two peaks at 6.4 and 5.3 sigma (background maximum of 4.0) were identified at two sites related by noncrystallographic symmetry in the asymmetric unit containing two protein monomers. The single Rb+ ion peak observed on each protein monomer was localized to a conserved ion-binding pocket in the PM H+ -ATPase (Fig. 1 A and B). The Rb+ /K+ ion is positioned next to four backbone carbonyl groups (residues 598 to 601) and the side chain oxygen atoms from Asp617, previously demonstrated by mutagenesis to be necessary for any K+-mediated effects on the PM H+ -ATPase (15). Rb+ was the only anomalous scatter at the applied wavelength, and the anomalous difference Fourier density therefore allows us to unambiguously assign the peak densities as Rb+ ions.

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Lanthanide ions have been widely used as spectroscopic and crystallographic probes for Ca\(^{2+}\) binding sites (reviewed in ref. 16). We grew PM H\(^{+}\)-ATPase crystals and soaked them with Tb\(^{3+}\). The Tb\(^{3+}\)-ion was easily identified in both anomalous and isomorphous difference maps and gave rise to peaks at 6.8 and 6.2 Å related by noncrystallographic symmetry. The Tb\(^{3+}\) peak is located at the N domain, just above the hinge connecting the N domain to the P domain, in the binding pocket of ATP at a position next to the ribose and base of the nucleotide adjacent to the phosphate group of the bound nucleotide (Fig. 1E). Only a single negatively charged residue, the highly conserved Asp487, is in close proximity and is therefore a prime candidate for being involved in the direct binding of the Tb\(^{3+}\) ion. The positively charged residue Arg456 also resides in the vicinity and could be involved in the cation coordination of Tb\(^{3+}\)-ion through a network of water molecules.

Likewise, we used crystals soaked with Ho\(^{3+}\) to collect data to 6-Å resolution at 1.2782-Å wavelength where there is strong anomalous signal from Ho\(^{3+}\) (Table S1). Despite similar chemical properties, Tb\(^{3+}\) and Ho\(^{3+}\) do not bind at same locations in the PM H\(^{+}\)-ATPase. The Ho\(^{3+}\) signal gave rise to four clear anomalous peaks related two and two by noncrystallographic symmetry. One site is located at the interface between the N and P domains, approximately at 10-Å distance from the Tb\(^{3+}\) site, and is coordinated by well-conserved residues Asp588 and Asp329 (Fig. 1D). The second Ho\(^{3+}\) binding position is located at the extracellular side of the transmembrane domain in a groove between transmembrane segments M1, M2, M4, and M6 (Fig. 1C). It is coordinated by Asp275 (saturated in M4), Asp95 (M2), and the backbone oxygen of Ala84 (M1). Asp92 and Asp272 are situated in the vicinity of the binding site and could contribute to the local electrostatic environment. Asp92 and Asp95 are almost completely conserved (respectively, 35 and 39 of 39 PM H\(^{+}\)-ATPase sequences) in the family of PM H\(^{+}\)-ATPases.

Testing the biochemical effect of Tb\(^{3+}\) and Ho\(^{3+}\) ions on the purified PM H\(^{+}\)-ATPase, both ions were found to inhibit the catalytic turnover as well as the accumulation of the phosphorylated intermediate (EP). The apparent K\(_i\) of Tb\(^{3+}\) was found to be approximately 100 μM (Fig. 2A and Fig. S2), and the apparent K\(_i\) of Ho\(^{3+}\) was approximately 150 μM (Fig. 2B and Fig. S2).

### Table 1. Kinetic properties of PM H\(^{+}\)-ATPase mutants

<table>
<thead>
<tr>
<th>PM H(^{+})-ATPase</th>
<th>Specific activity (μmol/mg/min)</th>
<th>pH optimum</th>
<th>ATP affinity, K(_a) (µM)</th>
<th>Vanadate inhibition, IC(_{50}) (µM)</th>
<th>Proton transport, + valinomycin (%)</th>
<th>Proton transport, − valinomycin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>25.7 ± 1.8</td>
<td>~6.8</td>
<td>45 ± 5</td>
<td>~8</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>R456V</td>
<td>0.7 ± 0.1</td>
<td>&lt;5.6*</td>
<td>1428 ± 69</td>
<td>~39</td>
<td>n.d.†</td>
<td>n.d.†</td>
</tr>
<tr>
<td>D92A</td>
<td>11.8 ± 2.9</td>
<td>~6.6-6.8</td>
<td>36 ± 6</td>
<td>~7-8</td>
<td>24.7 ± 10.3</td>
<td>7.3 ± 1.9</td>
</tr>
<tr>
<td>D95A</td>
<td>16.7 ± 0.5</td>
<td>~6.8</td>
<td>39 ± 4</td>
<td>~6</td>
<td>37.6 ± 4.0</td>
<td>17.3 ± 3.6</td>
</tr>
<tr>
<td>D92A,D95A</td>
<td>12.4 ± 1.3</td>
<td>~6.6-6.8</td>
<td>35 ± 13</td>
<td>~16</td>
<td>18.2 ± 1.0</td>
<td>5.3 ± 0.2</td>
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*The pH optimum was not determined within the tested range but appears to be shifted toward more acidic values (Fig. S5).

**n.d., not determined.**

**A Single Point Mutation of Arg456 Decreases the Apparent Affinity for Tb\(^{3+}\) and Ca\(^{2+}\).** The Tb\(^{3+}\) and Ho\(^{3+}\) binding sites identified at the interface of the N and P domains are lined by highly conserved amino acid residues necessary for basic P-type ATPase function. Arg456 was suggested from the crystallographic determination to be involved in Tb\(^{3+}\) coordination (Fig. 1E). We constructed an R456V single point mutation of the PM H\(^{+}\)-ATPase in order to test its sensitivity toward Tb\(^{3+}\), Ca\(^{2+}\) and Ho\(^{3+}\) (Fig. 3A-C). The modified protein was expressed in yeast and affinity purified on the purified PM H\(^{+}\)-ATPase, both ions were found to inhibit the catalytic turnover as well as the accumulation of the phosphorylated intermediate (EP). The apparent K\(_i\) of Tb\(^{3+}\) was found to be approximately 100 μM (Fig. 2A and Fig. S2), and the apparent K\(_i\) of Ho\(^{3+}\) was approximately 150 μM (Fig. 2B and Fig. S2).

The K\(_i\) for Ca\(^{2+}\) is independent of an intact regulatory autoinhibitory domain. Ca\(^{2+}\) was found to have an inhibitory effect on ATP hydrolytic activity and EP accumulation of the purified PM H\(^{+}\)-ATPase (K\(_i\) > 500 μM) (Fig. 2C and Fig. S2). Previous studies have found a profound direct inhibitory effect of Ca\(^{2+}\) on ATPase activity of PM H\(^{+}\)-ATPase.
Fig. 2. Ca\(^{2+}\), Tb\(^{2+}\) and Ho\(^{3+}\) ions inhibit the activity of the purified PM H\(^{+}\)-ATPase independently of an intact autoinhibitory domain. ATP hydrolytic activity of the C-terminal truncated PM H\(^{+}\)-ATPase (aha2 Δ73) and the full-length PM H\(^{+}\)-ATPase (AHA2) was measured with varying concentrations of: (A) Tb\(^{2+}\) ions, (B) Ho\(^{3+}\) ions, and (C) Ca\(^{2+}\) ions. The activity without the addition of ions were set to 100% in A, B, and C. ●, aha2 Δ73; □, AHA2. All experiments are represented as ±S.D.

Fig. 3. The R456V mutant PM H\(^{+}\)-ATPase displays decreased affinity for Ca\(^{2+}\) and Tb\(^{2+}\) ions. ATP hydrolytic activity of the wild type and the R456V mutant was measured with varying concentrations of: (A) Ca\(^{2+}\) ions, (B) Tb\(^{2+}\) ions, and (C) Ho\(^{3+}\) ions. The activity without the addition of ions were set to 100% in A, B, and C. ●, wild type; □, R456V. All experiments are represented as ±S.D.

\(K_{i} \sim 1 \text{ mM}\) on the Vicia faba plant PM H\(^{+}\)-ATPase, indicating a potential important physiological function of the calcium concentration on PM H\(^{+}\)-ATPase activity (13). PM H\(^{+}\)-ATPases are subject to autoinhibition by terminal regulatory domains (17–19). The wild-type protein construct used in this study is devoid of the C-terminal part of its regulatory domain (6), and Ca\(^{2+}\) binding to the PM H\(^{+}\)-ATPase could thus potentially be dependent on an intact autoinhibitory domain. However, no difference in the effect of Ca\(^{2+}\) on the full-length and the C-terminal truncated PM H\(^{+}\)-ATPase could be detected (Fig. 2C). The fact that Ca\(^{2+}\) does not exert any significant effect on the PM H\(^{+}\)-ATPase at concentrations below 100 \(\mu\text{M}\) implies that direct inhibition of the PM H\(^{+}\)-ATPase by Ca\(^{2+}\) is unlikely to be physiologically relevant. Likewise, no significant differences between the full-length and the C-terminal truncated PM H\(^{+}\)-ATPase regarding their sensitivities to Tb\(^{3+}\) and Ho\(^{3+}\) could be detected (Fig. 2A and B).

**Calcium Does Not Regulate Activity from the Extracellular Ho\(^{3+}\) Binding Site.** The identification of a Ho\(^{3+}\) ion-binding site at the extracellular mouth of the protein could potentially signify an extracellular regulatory role of Ca\(^{2+}\) on the PM H\(^{+}\)-ATPase. Single point mutations of the acidic residues Asp87, Asp92, Asp95, Asp272, and Asp275, all located at or near the identified extracellular Ho\(^{3+}\) ion-binding site, were therefore constructed and tested for their ability to complement a conditional yeast null PM H\(^{+}\)-ATPase mutant in the presence of various concentrations of Ca\(^{2+}\) (Fig. 4A). Depending on the Ca\(^{2+}\) concentration in the growth medium, a modulatory extracellular binding site for calcium on the plant PM H\(^{+}\)-ATPase would be expected to influence the ability of plant PM H\(^{+}\)-ATPase to complement the endogenous fungal PM H\(^{+}\)-ATPase. No significant effect of calcium on the growth of yeast cells expressing the wild-type plant PM H\(^{+}\)-ATPase or any of the mutated versions were however found (Fig. 4A). Moreover, when purified to homogeneity, none of the mutant proteins displayed any significant changes in their sensitivity toward added Ca\(^{2+}\) ions compared to the wild-type PM H\(^{+}\)-ATPase (Fig. 4B). This suggests that the observed inhibitory effect of Ca\(^{2+}\) on the PM H\(^{+}\)-ATPase is mediated through binding to the identified Tb\(^{3+}\)/Ca\(^{2+}\) site located in the cytoplasmic domain.

**The Extracellular Ho\(^{3+}\) Binding Site Might Represent a Proton Exit Site.** In several proton transporting pumps a series of internal proton transfer reactions take place between charged residues along a proton transport pathway (20, 21). In order to test if the acidic cation binding pocket occupied by Ho\(^{3+}\) could be part of the proton transport pathway of the PM H\(^{+}\)-ATPase, mutants of the extracellular Ho\(^{3+}\) binding site were tested for their ability to complement the conditional yeast null PM H\(^{+}\)-ATPase mutant described previously at different proton concentrations. As shown in Fig. 5, the D87A, D272A, and D275A mutants were
able to support yeast growth to the same extent as the wild type. In contrast, the D92A and the D95A mutants displayed a decreased ability to support yeast growth at more acidic pH values (pH 4.5–3.5), indicative of the mutants being unable to pump protons against a high proton gradient. A double mutant of these two residues, D92A,D95A, was not able to complement the yeast null PM H⁺-ATPase mutant irrespective of the proton concentration (Fig. 5).

For the single and double mutants of Asp92 and Asp95, the ATPase specific activity was reduced to 45–65% of the wild-type level (Table 1). Similar Kₘ values for ATP in all mutant enzymes were found, demonstrating that the substitutions do not abrogate the ability of the enzymes to bind nucleotides and hydrolyze ATP. The pH profile of the ATP hydrolytic activity of the double mutant D92A,D95A revealed that it was largely insensitive toward the concentration of protons (Fig. S6).

The Asp92 and Asp95 mutants were reconstituted in vesicles and their ability to perform ATP-dependent proton pumping was evaluated using the ΔpH probe ACMA. The proton transport rate was severely reduced for all mutants compared to the wild type (Table 1). When a transmembrane potential was allowed to establish by the elimination of the ionophore valinomycin in the experimental setup, the capacity of the mutants to transport protons was even more inhibited relative to the wild type (Table 1).

In contrast to their different abilities to support yeast growth (Fig. 5), the catalytic activity and the proton transport rate of the D92A mutant and the D92A,D95A double mutant are at similar levels. Thus the ability to functionally complement the pma1Δ deficiency in vivo must integrate more parameters besides the ones evaluated in vitro.

**Discussion**

The presence of regulatory ion-binding pockets is a common feature of enzymes (22, 23). This study provides crystallographic evidence for three cation binding sites in the cytoplasmic domains of the PM H⁺-ATPase and an additional site in the membrane domain that is accessible from the extracellular side.

The identified Rb⁺/K⁺ ion-binding site confirms previous mutagenesis experiments (15) and corresponds to the cytoplasmic K⁺ binding site described for the Na⁺,K⁺-ATPase and for the SR Ca²⁺-ATPase (5, 24). The coordinating P-domain residues are highly conserved among P-type ATPases, and the structural identification of a K⁺ binding site in both type II (Na⁺/K⁺- and SR Ca²⁺-ATPases) and type III (H⁺) P-type ATPases hints at a fundamental function of this allosteric ion-binding site. In plant cells, the physiological concentration of K⁺ is usually constant at concentrations around 50 mM K⁺ (25). This suggests that the potassium binding site (with an apparent Kₘ for K⁺ ~1 mM) most likely is occupied by K⁺ in vivo (15). The K⁺ binding site has been suggested to be involved in the docking of the A domain during dephosphorylation of the E2P state of Na⁺,K⁺- and SR Ca²⁺,ATPase (5, 24) and allows for stimulation of uncoupled dephosphorylation of the E1P state in the PM H⁺-ATPase (15).

Intrinsic lanthanide binding sites, or novel lanthanide binding sites introduced by protein engineering, serve extensive applications in spectroscopic and crystallographic studies of proteins (16, 26, 27). The identification of highly conserved P-type ATPase lanthanide binding sites implies that they can serve as a direct tool in the biophysical and structural analysis of other members of the P-type ATPase family of proteins. Although both Tb³⁺ and Ho³⁺ were found positioned near the nucleotide at the interface of the N and the P domains, the localization of these two ions was not identical. Tb³⁺ has previously been reported to inhibit the activity of the PM H⁺-ATPase from Schizosaccharomyces pombe (12) and of the SR Ca²⁺-ATPase (28–30). However, a binding site for Tb³⁺ ions in P-type ATPases has so far not been described structurally. The Tb³⁺ ion identified in the present study is located in close vicinity of the nucleotide and could be coordinated by Asp487 and Arg456. Asp487 is part of the highly conserved D⁴⁸⁷-PPR segment of the P-type ATPase family and is located at the hinge between the N and P domains. In the Na⁺,K⁺-ATPase and the SR Ca²⁺-ATPase, the corresponding residues have been reported to be very sensitive to even conservative substitutions (31–34). Based on structural data from the SR Ca²⁺-ATPase, it was speculated that this aspartate residue has an important role in the positioning of the N domain with respect to the P domain.
to the P domain (34). Arg456 is conserved in type II and III P-type ATPases, and the corresponding residue in the $\text{Na}^+$, $\text{K}^+$-ATPase has been shown to be important for high-affinity binding of both ATP and ADP (35). The presence of a $\text{Tb}^{3+}/\text{Ca}^{2+}$ ion at the identified position will most likely influence the side chains of Arg456 and Asp487, thereby interfering with ATP binding and/or phosphoryl transfer. Indeed, the R456V mutant displayed a significant reduction in the apparent affinity for ATP. For the R456V mutant, $\text{EP}$ formation was not inhibited to the same extent by the presence of $\text{Tb}^{3+}/\text{Ca}^{2+}$ ions as the wild type, and the apparent acidic shift in the pH profile indicates that electrostatic interactions are crucial for ATP binding and/or phosphoryl transfer.

The $\text{Ho}^{2+}$ ion identified adjacent to the nucleotide at the interface between the N and the P domains resides near the canonical aspartate residue Asp329 as well as Asp588. Asp588, placed in the P-type ATPase motif TGD$^{34}$GVND, is well-conserved within the halocid dehalogenase superfamily, and for several members of this family the aspartate residue has been demonstrated to be required for $\text{Mg}^{2+}$ coordination (36). Likewise, by use of mutational analysis and peptide binding studies, the corresponding residues in the SR Ca$^{2+}$-ATPase and the $\text{Na}^+$, $\text{K}^+$-ATPase have been shown to coordinate the $\text{Mg}^{2+}$ ion during phosphoryl transfer (32, 34, 37, 38). Thus, correct positioning of this aspartate is of crucial importance for phosphorylation of Asp329, and $\text{Ho}^{2+}$ must therefore be assumed to have an inhibitory effect on the PM H$^+$-ATPase through a distortion of the $\gamma$-phosphate group away from the conserved Asp329. $\text{Ho}^{2+}$ was indeed found to inhibit $\text{EP}$ formation in both the wild-type protein and in the R456V mutant.

In this study, structural evidence for $\text{Ho}^{2+}$ binding to an extracellular negatively charged binding pocket on the PM H$^+$-ATPase was obtained. This area has been suggested to function as a proton acceptor/donor relay cluster during proton release from the intramembranous proton binding site (6). Substitution of either Asp92 or Asp95 with alanine residues led to loss of yeast complementation at high proton concentrations. This indicates that these mutants cannot sustain proton transport against a high proton electrochemical gradient. Further, the double substituted mutant (D92AD95A) was completely unable to support yeast growth. The ability of the mutants to transport protons was evaluated with and without a potential difference across the vesicle membrane, and for all the mutants tested, the proton transport rate was found to be significantly inhibited by a transmembrane potential. These results support the proposal that Asp92 and Asp95 line the proton exit pathway of the PM H$^+$-ATPase. Several of the amino acids found to coordinate $\text{Ho}^{2+}$ at the extracellular mouth of the pump are conserved in both plant and fungal PM H$^+$-ATPases, and in the Saccharomyces cerevisiae PM H$^+$-ATPase Pma1p, the corresponding aspartate residues are important for pump activity (39).

The mechanism of P-type ATPases is usually discussed in terms of the E1–E2 model developed from the Post–Albers scheme for the Na$^+$, K$^+$-ATPase (40, 41). The model proposes that P-type pumps can exist in one of two distinct forms, E1 and E2. In its original form, the model further proposes alternating exposure of the same ion-binding sites toward the intracellular and extracellular medium in the E1 and E2 conformations, respectively. While our results are compatible with the alternating-site model, the model does not predict additional sites, such as an exit site as shown in this work.

Recently, a low affinity Ca$^{2+}$ binding site was suggested in the luminal Ca$^{2+}$ exit pathway of the SR Ca$^{2+}$-ATPase (42). This site involves Glu90 of the SR Ca$^{2+}$-ATPase, which is homologous to Asp95 in the PM H$^+$-ATPase, part of the $\text{Ho}^{2+}$ cation binding site identified in this work. It therefore seems possible that all P-type pumps could have a cation leaving site. In the proton transporting bacteriorhodopsin, two acidic residues at the extracellular side of the protein form a proton release group (43–45) suggesting that a leaving site for protons could be a general feature of H$^+$ pumps. In the E1 crystal structure, the approximate distance from the Asp684 to the proton release group is approximately 15 Å. Proton transport from Asp684 could entail proton release directly to the proton exit group and the extracellular side along a hydrogen-bonded network of water upon deocclusion and opening of the release pathway. Additional studies are needed to address this and if the proton release group takes form of a H$^+$ or H$_2$O$^+$ binding site.

The proposed leaving site for transported protons identified in this work would have been difficult to locate without the use of the positively charged $\text{Ho}^{2+}$ to reveal a negative patch of residues important for proton unloading. $\text{Ho}^{2+}$ is a relatively small cation (ionic radii 0.96 Å) with a high charge density and well known to form complexes with the deprotonated form of carboxylates in protein structures (46, 47). Our structural and biochemical data identify $\text{Ho}^{2+}$ as a novel chemical tool for structural identification of proton binding sites.

Materials and Methods

Construction of Mutants. The multicopy vector Yep-351 (48) containing a modified cDNA of the Arabidopsis thaliana AHA2 plasma membrane H$^+$-ATPase isoform under the control of the PMA1 promoter (49) was used as starting point. The modified cDNA encodes the AHA2 PM H$^+$-ATPase with a C-terminal deletion of 73 amino acid residues and the insertion of a C-terminal Met-Arg-Gly-Ser-His$_6$ (MRGSH$_6$) (10), in here denoted wild type. The C-terminal deletion renders the PM H$^+$-ATPase constitutively active, while the addition of the His$_6$-tag allows for affinity purification of the constructed mutants. Site-directed mutagenesis was performed by standard procedures using polymerase chain reaction, and all mutated sequences were verified by DNA sequencing.

Expression of PM H$^+$-ATPases in Yeast. The S. cerevisiae strain RS-72 (50) was transformed and cultured essentially as described previously (51). In RS-72 (MATa ade1-100 hisd-519 leu2-3,112), the natural constitutive promoter of the yeast endogenous PM H$^+$-ATPase PMA1 has been replaced by the galactose-dependent GAL1 promoter (50).

Protein Purification. Plasma membrane enriched fractions from yeast expressing either full-length AHA2 (i.e., including the C-terminal 73 residues) or an empty vector were purified as previously described (19). The PM H$^+$-ATPases were purified by membrane solubilization using n-dodecyl-$\beta$-D-maltoside and Ni$^{2+}$-affinity chromatography according to established procedures (6).

Yeast Complementation Assay. Yeast complementation assay was performed on solid growth media (pH 5.5) as previously described (19), except that various concentration of Ca$^{2+}$ (0–20 mM) was included.

Crystallographic Identification of Cation Binding Sites. For the $\text{Ho}^{2+}$ and Tb$^{3+}$ bound form, protein crystals were grown as described previously (6) and then soaked with 10 mM $\text{HoCl}_3$ or 5 mM $\text{TbCl}_3$. For the $\text{Rb}^+$ bound form, the protein was dialyzed by 50 mM $\text{RbCl}$, 50 mM MES pH 6.5, 10% sucrose and 1xCMC C$_3$E$_8$, 5 mM Mg-AMP-PCP was added to the dialyzed protein, which was then centrifuged (70,000 rpm 10 min) and used directly at 4 °C in hanging drops by mixing equal volumes of protein and crystallization buffer [29% polyethylene glycol-400, 100 mM $\text{RbCl}$, 100 mM MES 6.0, 5% sucrose (w/v)]. Large, single crystals grew over a week and were dehydrated using the protocol previously established (6) and flash-frozen in liquid nitrogen. All diffraction data were collected at the beamline X06SA at the Swiss Light Source and were processed and merged using XDS and analyzed using programs from the CCP4 package.

Enzyme Activity Measurements. ATPase activity was determined using either plasma membrane enriched fractions or purified protein diluted in a reaction buffer as described (10). Varying concentrations of CaCl$_2$ (0–10 mM), $\text{TbCl}_3$ (0–1 mM), or $\text{HoCl}_3$ (0–630 μM) were included as indicated. Formation of the phosphorylated intermediate was performed as described (10).

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Fig. S1. The delipidated PM H\(^{+}\)-ATPase is fully functional. Activity of purified PM H\(^{+}\)-ATPase measured during the crystallization preparation protocol. Activities are expressed as specific activity (μmol Pi produced/min/mg protein). 1: Activity after purification and without any application of lipid molecules. 2: Activity after purification with inclusion of lipids. 3: Activity after a C\(_{12}\)E\(_{8}\)/Cymal-5/Sucrose step with inclusion of lipids. 4: Activity after the final ultracentrifugation step, just before crystallization set-up. (B) Time-dependent stability of the crystallization ready PM H\(^{+}\)-ATPase with respect to ATP hydrolytic activity. Protein, just before crystallization set-up, was kept at 4 °C and the activity was measured at different time intervals. (C) Lipid requirement of the purified proton pump. Different amounts of DDM solubilized lipids were added to the PM H\(^{+}\)-ATPase before activity measurements (ATP hydrolytic activity). A MW of 762 Da of the added lipids (soybean phosphatidylcholine), and a MW of 90 kDa for the PM H\(^{+}\)-ATPase were used for the calculations.

Fig. S2. Tb\(^{3+}\), Ho\(^{3+}\), and Ca\(^{2+}\) ions inhibit accumulation of the phosphorylated intermediate of the wild-type PM H\(^{+}\)-ATPase. Measurement of steady state phosphorylation levels of the purified PM H\(^{+}\)-ATPase with varying concentrations of Tb\(^{3+}\), Ho\(^{3+}\), and Ca\(^{2+}\) ions. Phosphorylation was initiated by addition of 1 μM [\(\gamma\)-\(^{32}\)P]ATP and was stopped after 20 seconds by acid quenching. The level of phosphorylated protein without the addition of cations was set to 100%. Blue, Ca\(^{2+}\); green, Tb\(^{3+}\); and red, Ho\(^{3+}\). All values are indicated ±S.D.
**Fig. S3.** The R456V single-point mutant has a reduced level of phosphoenzyme accumulation at steady state. Measurement of steady state phosphorylation levels for the wild type (black bar) and R456V mutant (white bar) PM H\(^{+}\)-ATPase. Phosphorylation was initiated by addition of 1 μM [γ\(^{32}\)P]ATP and was stopped after 20 seconds by acid quenching. The activity is reported as the mean ± S.D. and expressed as pmol EP/μg protein.

**Fig. S4.** The accumulation of the phosphorylated intermediate in the R456V mutant is less sensitive toward Tb\(^{3+}\) and Ca\(^{2+}\), but not Ho\(^{3+}\), than the wild-type protein. Measurement of steady state phosphorylation levels for the wild type and R456V mutant PM H\(^{+}\)-ATPase in the presence of no cations (white bars), Ca\(^{2+}\) (light gray), Tb\(^{3+}\) (dark gray) and Ho\(^{3+}\) (black) as described for Fig. S3. Values are reported in % relative to EP formation without addition of cations for the wild type and the R456V mutant PM H\(^{+}\)-ATPase. All values are reported as the mean ± S.E.

**Fig. S5.** The R456V mutant displays reduced ATP affinity and a shift in pH optimum with respect to catalytic activity. (A) The dependence of ATP concentration of ATP hydrolysis and (B) the pH dependence of ATP hydrolysis were measured for: ■, wild type; and □, R456V PM H\(^{+}\)-ATPases. All experiments are represented as ±S.D.
Fig. S6. The ATP hydrolytic activity of the D92A,D95A mutant is almost pH-insensitive. (A) The pH dependence of ATP hydrolysis and (B) the vanadate sensitivity of ATP hydrolysis were measured for: □, wild type; □, D92A; △, D95A; and ▽, D92A,D95A PM H⁺-ATPases. All experiments are represented as ±S.D.

<table>
<thead>
<tr>
<th>Table S1. Summary of crystallographic parameters</th>
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<tr>
<td>Form: RbCl</td>
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<td>Anomalous scatterer</td>
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<td>Method</td>
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<td>Redundancy</td>
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<td>Peak height</td>
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Highest resolution shell is shown in parentheses. Phases from the structure of the AHA2 E1-AMPPCP form (pdb-id: 3b8c) refined at 3.6-Å resolution was used to generate difference Fourier maps based on data in the given resolution ranges mentioned above.

For the Ho³⁺ peaks, the first set of peak heights denotes site 1 (at the N domain) and the second set denotes site 2 (at the extracellular side of the M domain).