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Mechanism of proton transport by plant plasma membrane proton ATPases

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Abstract The mechanism of proton translocation by P-type proton ATPases is poorly defined. Asp684 in transmembrane segment M6 of the *Arabidopsis thaliana* AHA2 plasma membrane P-type proton pump is suggested to act as an essential proton acceptor during proton translocation. Arg655 in transmembrane segment M5 seems to be involved in this proton translocation too, but in contrast to Asp684, is not essential for transport. Asp684 may participate in defining the E_1 proton-binding site, which could possibly exist as a hydronium ion coordination center. A model of proton translocation of AHA2 involving the side chains of amino acids Asp684 and Arg655 is discussed.

Key words ATPase · H^+ · Plant · Plasma membrane · Proton · P-type

Introduction

Plasma membrane proton ATPases are the primary cellular motors behind the generation of the proton electrochemical gradient present across plasma membrane systems of plant and fungal cells. In this way they provide the energy source utilized by a large number of secondary transport systems present at the plasma membrane.

Plant and fungal plasma membrane H^+-ATPases belong to a large family of proteins termed P-type ATPases (Serrano et al. 1986; Pardo and Serrano 1989; Palmgren 2001), which is characterized by forming a phosphorylated intermediate during catalysis. P-type ATPases consist of only a single catalytic unit, which is responsible for both ATP hydrolysis and cation transport. The activity of some P-type ATPases is furthermore dependent on the presence of additional protein units. Members of this protein family are present in bacteria, archaea and eukaryotes, and they are found in a variety of membrane systems transporting ions such as Na^+, K^+, H^+, Ca^{2+}, Mg^{2+} and Cd^{2+} (Axelsen and Palmgren 1998). Although P-type ATPases are normally regarded as integral membrane proteins, a soluble P-type ATPase with unknown function has been identified (Ogawa et al. 2000). Besides plant and fungal H^+-ATPases, other well-characterized members of this family include the animal Na^+/K^+-ATPase, the animal sarcoplasmic reticulum (SR) Ca^{2+}-ATPase, the animal plasma membrane Ca^{2+}-ATPase and the animal H^+/K^+-ATPase. A list of identified P-type ATPases, as well as a family subdivision based on the nature of the transported species, is available in the P-type ATPase database (http://www.patbase.kvl.dk).

Although much is known about the regulation of plant and fungal plasma membrane H^+-ATPases (Serrano 1993; Morsomme and Boutry 2000; Portillo 2000; Palmgren 2001; Arango et al. 2003), one central unanswered question is how they actually transport protons. Thus, for instance, the coupling ratio (H^+/ATP), the nature of the transported species (H^+/H_3O^+) and the nature of the amino acid residues involved in the transfer are yet to be described.

Structure and function of P-type proton pumps

Crystal structures

Low-resolution structures of P-type H^+-ATPases have been obtained from 2D crystals of plasma membranes H^+-ATPases from *Neurospora crassa* (Auer et al. 1998) and from *Arabidopsis thaliana* (Jahn et al. 2001). The 8 Å structural determination of the *N. crassa* enzyme represents the best-obtained structure of a P-type H^+-ATPase (Auer et al. 1998). This structure reveals the presence of ten transmembrane helices as well as four cytoplasmic domains (Auer et al. 1998).

High-resolution structural data of a P-type ATPase has only been presented for the animal SR Ca^{2+}-ATPase

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(Toyoshima et al. 2000; Toyoshima and Nomura 2002). In the high-resolution crystal structures of the Ca^{2+}-ATPase, 10 transmembrane helices and three distinct cytoplasmic domains are revealed. The three cytoplasmic domains has been termed the anchor (A), the nucleotide (N) binding, and the phosphorylation (P) domains respectively. Three of the cytoplasmic domains seen in the 8 Å structure of the *Neurospora* proton ATPase could comprise the A-, N-, and P-domains seen in the SR Ca^{2+}-ATPase crystal structure. The fourth domain, not seen in the SR Ca^{2+}-ATPase structure, could comprise an H^+-ATPase specific C-terminal regulatory domain (Kühlbrandt et al. 2002; see below). The high-resolution crystal structures of the SR Ca^{2+}-ATPase represent a foundation for modeling of P-type H^+-ATPases (Bukrinsky et al. 2001; Kühlbrandt et al. 2002; Radresa et al. 2002). These modeling studies have suggested a relatively high level of overall structural similarity between the SR Ca^{2+}-ATPase and the plasma membrane H^+-ATPases.

Although oligomers could form in vivo, monomers of H^+-ATPases seem fully capable of conducting ATP dependent proton transport in vitro, as exemplified by fungal H^+-ATPases (Goormaghtigh et al. 1986). A high level of structural and functional conservation between plant and fungal H^+-ATPases is expected. Thus, although no high-resolution structures are available for any plasma membrane H^+-ATPases, molecular modeling of fungal and plant plasma membrane H^+-ATPases suggests a high degree of structural similarity (Bukrinsky et al. 2001). Functionally, plant plasma membrane H^+-ATPases are capable of complementing mutations in the *Saccharomyces cerevisiae* P-type H^+-ATPase PMA1 (Palmgren and Christensen 1993; d'Exaerde et al. 1995).

Enzymology of P-type H^+-ATPase

During catalysis, both plant and fungal H^+-ATPases form a phosphorylated intermediate (Amory et al. 1980; Briskin and Leonard 1982), and the catalytic cycle of plant and fungal P-type proton pumps is believed to follow the Post-Albers $E_1 \rightarrow E_2$ reaction cycle described for other P-type ATPases (Albers 1967; Post et al. 1972).

Ions to be transported out of the cell (in the case of a plasma membrane P-type ATPase) bind to the E_1 ion-binding sites. Phosphorylation from ATP represents the $E_1 \rightarrow E_1P$ conformational transition with the EP designating a phosphorylated intermediate. During an $E_1P \rightarrow E_2P$ conformational transition, the ion-binding sites are believed to change from being accessible from the cytoplasmic face of the enzyme to being accessible from the extracellular side of the enzyme. Furthermore, to ensure ion release, a significant change of the ion-binding sites with respect to affinity towards the transported species is believed to occur during the $E_1P \rightarrow E_2P$ transition. During the transfer of ions across the membrane, the ions become tightly bound (occluded) within the protein. Dephosphorylation of E_2P in combination with binding of the counter ions follows the $E_2P \rightarrow E_2$ and $E_2 \rightarrow E_1$ transitions. Modulation in geometry of the ion-binding sites during catalysis is believed to ensure alternating affinities towards the transported ions (Jørgensen and Pedersen 2001; Toyoshima and Nomura 2002).

Differences may, however, be present between the catalytic cycles of P-type proton pumps and other P-type ATPases such as the Na^+/K^+- and Ca^{2+}-ATPases. Whereas Ca^{2+} and Na^+ are believed to bind to E_1 cation-binding sites in the Ca^{2+}-ATPase and Na^+/K^+-ATPase, respectively, proton binding to H^+-ATPases could occur during the $E_2 \rightarrow E_1$ transition. Thus, measured by following changes in intrinsic fluorescence, proton binding to a yeast H^+-ATPase did accompany conformational changes compatible with an $E_2 \rightarrow E_1$ conformational transition (Blanpain et al. 1992). Furthermore, unlike the Ca^{2+}-ATPase and Na^+/K^+-ATPase, "backdoor" phosphorylation of yeast proton pumps by inorganic phosphate is undetectable (Amory et al. 1982). It is not known whether this apparent irreversibility of the catalytic cycle of P-type proton pumps could relate to the possibility that proton pumps do not perform counter transport as the Ca^{2+}- and Na^+/K^+-ATPases do.

The H^+/ATP stoichiometry of fungal and plant P-type pumps is not defined, but most studies have suggested a stoichiometry of a single H^+ pumped per ATP hydrolyzed (Perlin et al. 1986; Briskin and Reynolds-Niesman 1991). However, P-type proton pumps contain a regulatory C-terminal extension (see below), and in non-activated yeast H^+-ATPase, ATP hydrolytic activities appear to be uncoupled from proton transport (Venema and Palmgren 1995). This suggests that the H^+/ATP stoichiometry of P-type proton pumps is variable. Intrinsic uncoupling of activity has also been observed in other P-type ATPases and could represent a conserved mechanism of regulation (Berman 2001).

Regulation of the activity of plasma membrane H^+-ATPase

The C-terminal extension of plasma membrane H^+-ATPases is cytoplasmically located and has a regulatory function in both yeast and plant H^+-ATPases (Eraso and Portillo 1994; Palmgren et al. 1990, 1991). In both yeast and plant H^+-ATPases, this regulatory domain functions as an autoinhibitory domain, keeping the enzymes in a low-activity state. Several point mutations resulting in activated enzymes have been identified in both the C-terminal regions as well as in cytoplasmic domains (Eraso and Portillo 1994; Morsomme et al. 1996, 1998; Axelsen et al. 1999). Genetic evidence combined with molecular modeling could suggest that the C-terminal extensions of proton pumps interact with membrane localized amino acid residues and the P-domain (Bukrinsky et al. 2001). Another suggestion is that this regulatory domain prevents N-domain movements (Kühlbrandt et al. 2002). The basis for this suggestion was that the density in the 8 Å map of the crystallized *Neurospora* enzyme, which is likely to represent the C-terminal domain, is in close proximity to the N-domain. However, at present it remains unclear whether the crystallized pump is in an autoinhibited or activated state.

The C-terminal end of the plant H^+-ATPase harbors a binding site (Tyr-Thr-Val-COOH) that is unusual for regulatory 14-3-3 proteins. Binding is possible by a kinase medi-

ated phosphorylation of the penultimate Thr (Fuglsang et al. 1999; Svennelid et al. 1999; Madoux et al. 2000). Recently, the crystal structure of a C-terminal peptide of plant H^+-ATPases in complex with 14-3-3 protein and fusicoccin has been reported (Wurtele et al. 2003). Fusicoccin is a fungal toxin, which mimics the effect of phosphorylation. Displacement of the autoinhibitory C-terminal by the regulatory 14-3-3 proteins is believed to mediate activation of plant H^+-ATPases in vivo.

Protons and proton transport across biological membranes

Scenarios

At least two scenarios for proton transport by proton pumps such as the plant plasma membrane H^+-ATPases could be envisioned. The transported proton could somehow travel along a network of proton acceptors and proton donors through the membrane. Alternatively, a coordination center inside the membrane could constitute a single binding site for H^+ or hydronium ions.

Hydrogen ions have no free existence as naked protons in solution. Protons in aqueous solution will instead be found as hydrated proton molecules, either as a hydronium ion complex (H_3O^+) or as a dihydronium ion ($H_5O_2^+$) (Brandsburg-Zabary et al. 2000). It is generally believed that protons have a very high mobility in aqueous solution compared to the mobility of other ions. The mobility of protons in solution seems at least 7 times greater than that of Na^+. This high proton conductance of water can be ascribed a conduction of protons along the hydrogen-bonded network of water. A linear hydrogen-bonded chain of water molecules capable of conducting high-speed proton transfer is termed a proton wire.

Protons transported along hydrogen-bonded networks

The ability of protons to rapidly propagate along a hydrogen-bonded network of water molecules involves "jumping" from one water molecule to the next along the proton wire (Copeland and Chan 1989; Briskin and Hanson 1992; Pomes and Roux 1998) (Fig. 1).

Several biological membrane proton transporters have taken advantage of this capability of protons to propagate along hydrogen-bonded networks. In the high-conducting proton channel gramicidin, water molecules lining the walls of the channel provides the proton conducting machinery (DeCoursey and Cherny 2000; Pomes and Roux 1998). Also, several identified proton-pumping proteins utilize proton wires. In this case, the proton wire seems to consist of amino acid side chain groups, as well as bound water molecules that both participate in forming the hydrogen-bonded network, along which the protons can travel. However, the random diffusion of protons in water is very high. Thus, a proton can travel the length of the gramicidin channel in less than 15 ns, a time interval much shorter than the catalytic mechanism of known proton pumping proteins (Brandsburg-Zabary et al. 2000). In a protein pumping protons against an electrochemical gradient, it must be envisioned that there is a mechanism for preventing proton leakage of protons through the system. Such prevention of leakage could be imagined as accomplished either by conformational changes breaking the connectivity of the proton wire, or by the introduction of electrostatic barriers along the proton pathway (Brandsburg-Zabary et al. 2000).

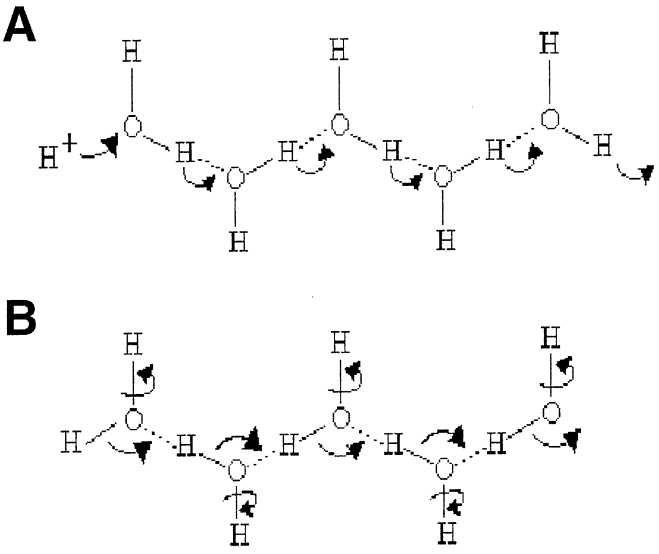

Fig. 1A,B. Proton translocation along hydrogen-bonded networks of water. **A** Rather than actual H_3O^+ diffusion, protons are transported in solution along the hydrogen-bonded network of water. Thus, the diffusion of protons in solution is mechanistically different from diffusion of cations. An excess proton enters a hydrogen-bonded network (a proton wire) at the left of the figure. After proton propagation along the wire, a proton exits at the right side of the figure. The proton leaving the wire is not the same proton that enters it. Amino acid side chains could replace water molecules in the proton wire. **B** An essential feature of sustained proton translocation along hydrogen-bonded networks is the requirement for bond turning of the involved groups. This bond turning to return to the "ground state" is illustrated

With respect to the mechanism of proton transport, the best-described proton pumps are probably bacteriorhodopsin, the F_1F_o-ATP synthase and cytochrome *c* oxidase. Structurally these proteins are very diverse. However, proton translocation in all these proteins seems to rely on proton propagation by the use of proton donors and proton acceptors along hydrogen-bonded networks (Lanyi 1997; Gennis 1998; Michel 1998; Fillingame et al. 2000; Heberle 2000; Capaldi and Aggeler 2002; Neutze et al. 2002). Thus, basic principles governing proton conduction in these proteins could be similar. In bacteriorhodopsin, for example, an ordered chain of water molecules, protonatable aspartic and glutamic acid carboxyl groups, and a prosthetic group, comprises a "proton wire". In this manner the proton pumping mechanism of bacteriorhodopsin involves propagation of protons along a chain of proton donors and proton acceptors. A timely regulation of pK_a values between the protonatable carboxyl groups and the prosthetic group

determines the direction of proton flow (Lanyi 1997; Heberle 2000; Neutze et al. 2002).

Protons transported as a cation?

The hydronium ion forms solvation structures characteristic of cationic solvation. Several biological systems transporting various cationic species have been described, and it has been suggested that transport of protons by some proton transporting systems could take place by transporting the proton as a hydronium ion rather than as a H^+ (Boyer 1988). In proton transporting systems where protons could be transported as a hydronium ion, the ion-binding site would probably be in terms of a coordination center for the hydronium ion. No hydronium coordination center in a protein has been described so far. However, high-affinity hydronium ion coordination centers can exist in small molecules such as crown ethers. Different crown ether structures, with specificity for various monovalent cations, including the hydronium ion, have been described (Behr et al. 1982; Bühl and Wipff 2002). Different crown ethers can form complexes with either H_3O^+ or H_5O^{2+}, depending on the ring size. The 18-crown-6 ether has very high affinity for the H_3O^+ ion (Bühl and Wipff 2002). In 18-crown-6 ethers, the H_3O^+ ion is coordinated by the use of hydrogen bonds (Fig. 2).

The oxygen atom of the hydronium ion does not engage in any bonding to the crown ether. The hydronium ion/crown ether complex suggests that a high affinity coordination center for a hydronium ion can be built from correctly orientated oxygen atoms. For this reason, if based on correctly orientated oxygen atoms, a high affinity hydronium ion coordination center could be imagined built into a protein structure. Backbone carbonyl oxygens, or carbonyl/carboxyl oxygens from amino acid side chains, could provide correctly orientated oxygen molecules for hydronium ion coordination. No hydronium ion proton pump has so far been described.

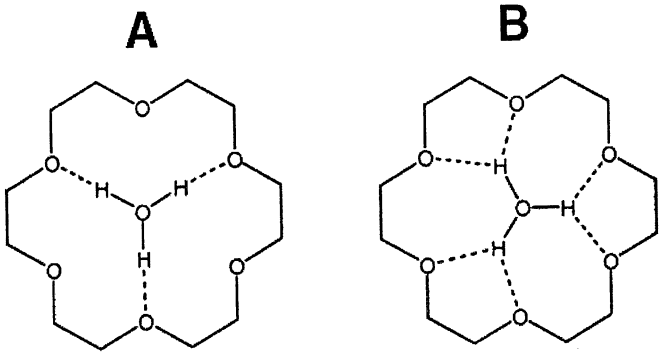

Fig. 2A,B. Coordination of hydronium ions within 18-crown-6 ethers: two possible coordination states. **A** H_3O^+ coordinated by the crown ether along three linear hydrogen bonds. **B** H_3O^+ coordinated by the crown ether by bifurcated hydrogen bonds. The two structures are believed to co-exist in H_3O^+/18-crown-6 ether structures. Figure modified from Bühl and Wipff (2000)

Mechanism of proton translocation by P-type plasma membrane proton ATPases

Conserved polar and charged residues in the membrane sector of P-type H^+-ATPases could participate in hydrogen-bonded networks and/or in hydronium ion coordination

The amino acid side chains of charged and polar residues such as serine, threonine, aspartate, asparagine, glutamate, glutamine, lysine, arginine, tyrosine and histidine all have the capacity to participate in proton transport by hydrogen-bonded networks (Copeland and Chan 1989; Briskin and Hanson 1992). Conserved charged and polar amino acid residues in the membrane sector of bacteriorhodopsin, F_1F_o ATP synthase and cytochrome *c* oxidase are engaged in the transduction chain of protons in these proteins. Thus, if proton transport by P-type proton pumps involves propagation of protons along hydrogen-bonded networks, such membrane-situated conserved charged and/or polar amino-acid residues could be expected to be involved in such types of mechanism. In the Ca^{2+}- and Na^+/K^+ P-type pumps, conserved membrane-situated polar and charged amino acids have also been found to comprise coordination centers for cations (Clarke et al. 1989; Kuntzweiler et al. 1996; Pedersen et al. 1997; Toyoshima et al. 2000). Due to the similarity between cations and H_3O^+, conserved charged and polar amino acid side chain residues could be imagined to coordinate a hydronium ion in P-type H^+-ATPase. Thus, although conserved charged and polar amino acid residues are suspected to participate in the transfer of protons in P-type proton pumps, information regarding the nature of the transported species (H^+/H_3O^+) is not necessarily coupled to the nature of the amino acid residues involved in the transfer.

Modeling of proton-binding sites in P-type H^+-ATPases

Based upon the E_1 Ca^{2+}-bound SR Ca^{2+}-ATPase crystal structure, proton-binding sites have been modeled in the *A. thaliana* AHA2 H^+-ATPase (Bukrinsky et al. 2001), the yeast *S. cerevisiae* PMA1 H^+-ATPase (Bukrinsky et al. 2001), and in the *N. crassa* PMA1 H^+-ATPase (Kühlbrandt et al. 2002).

Two ion-binding sites are present in the SR Ca^{2+}-ATPase structure (Toyoshima et al. 2000). In contrast, modeling of the AHA2 H^+-ATPase suggests that only one proton-binding site is present in this protein (Fig. 3). This proton-binding site could correspond to Ca^{2+}- binding site II of the SR Ca^{2+}-ATPase. Of the residues supposed to contribute to the proton-binding site in AHA2, only $Asp684_{AHA2}$ seems strictly conserved with the corresponding amino acid residues in the SR Ca^{2+}-ATPase (Bukrinsky et al. 2001).

The side chain of $Asp684_{AHA2}$ (TM6), as well as backbone carbonyls of amino acid residues in transmembrane helix 4 (from Ile282, Gly283 and Ile285) could provide liganding groups for a high-affinity hydronium ion coordination site in the AHA2 P-type proton pump (Fig. 3). Recent mutagen-

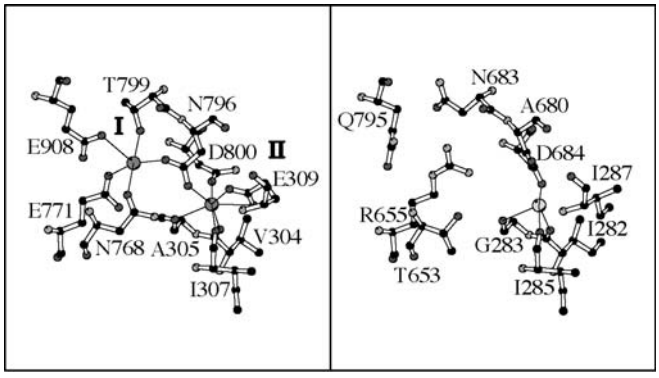

Fig. 3. Model of the proton-binding site in AHA2 plant plasma membrane H^+-ATPase. To the *left* are the E_1 calcium ion-binding sites of the SR Ca^{2+}-ATPase. Two Ca^{2+} ion-binding sites are present in the E_1 calcium-bound structures of the SR Ca^{2+}-ATPase as presented by Toyoshima et al. (2000). The two sites are termed calcium-binding site I and calcium-binding site II. Site I (*I*) is seen at the left of the figure, site II (*II*) at the right. To the *right*, a putative H_3O^+ proton-binding site modeled into the amino acid sequence of the plant AHA2 H^+-ATPase. The area of the putative proton-binding site in AHA2 corresponds to calcium binding site II. The figures were modified from Bukrinsky et al. (2001)

esis experiments support such a model (see below). $Asp684_{AHA2}$ is highly conserved in P-type ATPases (http://www.patbase.kvl.dk), and aligns with $Asp730_{PMA1}$, $Asp800_{Serca1a}$ and $Asp808_{Na/K}$. In the Ca^{2+}-bound crystal structure of the SR Ca^{2+}-ATPase, $Asp800_{Serca1a}$ is involved in coordination of both of the calcium ions (Toyoshima et al. 2000). Mutagenesis studies have similarly led to the suggestion that the corresponding residue $Asp808_{Na/K}$ is involved in Na^+ and K^+ binding (Kuntzweiler et al. 1996; Pedersen et al. 1997).

Based upon the modeling of AHA2, a positively charged amino acid residue of transmembrane segment M5 ($Arg655_{AHA2}$) was suggested to serve as an inbound cation in a region corresponding to Ca^{2+}- binding site I of the SR Ca^{2+}-ATPase (Bukrinsky et al. 2001). Both plant and fungal P-type H^+-ATPases have a conserved, positively charged arginine situated in the putative transmembrane segment M5 (http://www.patbase.kvl.dk). Thus, among plant (AHA2) and yeast (PMA1) plasma membrane H^+-ATPases, $Arg655_{AHA2}$ and $Arg695_{PMA1}$ are completely conserved, respectively. In homology models of fungal and plant H^+-ATPases, this arginine could be orientated in a place corresponding to Ca^{2+}-binding site I of the SR Ca^{2+}-ATPase, in close contact with the $Asp684_{AHA2}$/$Asp730_{fungal}$ side chains (Bukrinsky et al. 2001, Kühlbrandt et al. 2002). However, $Arg655_{AHA2}$ aligns with $His701_{PMA1}$ whereas $Arg695_{PMA1}$ aligns with $Ala649_{AHA2}$ (http://www.patbase.kvl.dk). This makes it unclear whether $Arg655_{AHA2}$ should be regarded as a residue homologous to $Arg695_{PMA1}$ or $His701_{PMA1}$.

In the model of the *Neurospora* PMA1 protein, $Asp730_{PMA1}$, $Arg695_{PMA1}$ and $Glu805_{PMA1}$ were suggested to comprise the proton-binding site. As in the model by Bukrinsky et al. (2001), Kühlbrandt et al. (2002) also modeled backbone carbonyl oxygen atoms from transmembrane M4 in this area. Thus, proton-binding sites modeled in $PMA1_{yeast}$ and AHA2 (Bukrinsky et al. 2001) seem more or less in accordance with the proton-binding site modeled over the $PMA1_{Neurospora}$ enzyme (Kühlbrandt et al. 2002).

Asp684 of AHA2 defines the proton-binding site in the plant plasma membrane H^+-ATPase

Proton binding to the H^+-ATPase enzyme is taken as a prerequisite for ATP hydrolysis and $E_1 \rightarrow E_1P$ conformational transitions. In catalytic models of P-type ATPases, the ion to be transported must be bound before the $E_1 \rightarrow E_1P$ conformational change and any ATP hydrolysis can take place. In structural model of P-type proton pumps, $Asp684_{AHA2}$/$Asp730_{PMA1}$ reside in the middle of the membrane sector (Bukrinsky et al. 2001; Kühlbrandt et al. 2002; Radresa et al. 2002).

This location of the conserved $Asp684_{AHA2}$ in the middle of the membrane sector in combination with data derived from mutagenesis of this residue seems to define $Asp684_{AHA2}$ as an essential proton acceptor along the proton translocation pathway. Furthermore, several lines of evidence suggest that the function of $Asp684_{AHA2}$ could be many-sided. Thus, although both oxygens of the $Asp684_{AHA2}$ side chain seem to participate in the H^+- transporting mechanism, it seems that these oxygens can be discriminated with respect to function. After removing the potential protonation group (D684N, D684A, D684V and D684R) of the $Asp684_{AHA2}$ side chain, no E_1P–E_2P enzyme conformational changes or proton transport are associated with measured ATP hydrolytic activity (Buch-Pedersen et al. 2000; Buch-Pedersen and Palmgren 2003) (Table 1). Hence, the negative charge of the $Asp684_{AHA2}$ carboxylate moiety appears to act as a proton acceptor during $E_1P \rightarrow E_2P$ transitions. The $Asp684_{AHA2}$ side-chain oxygens seem also to influence the rate of initial proton binding to the E_1 proton-binding site. $Asp684_{AHA2}$ can be replaced with asparagine with apparently little effect upon initial binding. However, removing the side chain oxygens completely seems to lower the rate of initial proton binding markedly. Thus, the side chain of the amino acid at position 684 must also be ascribed a role in initial proton binding. This discrimination between the two oxygens of $Asp684_{AHA2}$ into a proton-binding carbonyl oxygen and a proton-transporting negatively charged oxygen requires that the amino acid side chain is to be found to be asymmetric in the E_1 conformational state of the H^+-ATPase.

Arg655 is involved in the $E_1P \rightarrow E_2P$ transition

The pK_a value of arginine residues in solution is of the order of 12. It must therefore be expected that the guanidium moiety of $Arg655_{AHA2}$ is protonated under most circumstances. After replacing $Arg655_{AHA2}$ with a lysine (R655K), the substituted H^+-ATPase was able to complement a PMA1 deficient yeast strain effectively relative to wild-type plant H^+-ATPase (Buch-Pedersen and Palmgren 2003).

Table 1. Biochemical characteristics of affinity-purified wild type and R655 or D684 single-point substituted H^+-ATPase

H^+-ATPase protein	ATP hydrolysis[a]	Proton-pumping (%)[b]
Wild type	25.7 ± 1.8	100%
R655K	13.6 ± 1.0	ca 45
R655A	3.7 ± 0.1	ca 6
R655D	4.1 ± 0.1	ca 10
D684E	3.9 ± 0.1	ca 8
D684N	5.4 ± 0.3	0%
D684A	2.8 ± 0.1	0%
D684V	3.4 ± 0.2	0%
D684R	0.13 ± 0.03	0%

[a]ATP hydrolysis expressed as production of inorganic phosphate (μmol min^{-1} mg $protein^{-1}$), measured at 3 mM ATP, 4 mM $MgSO_4$, pH 6.5
[b]Values for H^+-pumping relative to the wild type AHA2 protein

Replacing $Arg655_{AHA2}$ with alanine (R655A) or by aspartate (R655D) left the mutated enzymes with little, though some, capability in complementing the PMA1 deficient yeast strain.

Reconstitution of affinity-purified $Arg655_{AHA2}$-substituted enzymes into artificial liposomes made it possible to demonstrate that all were capable of conducting proton transport (Table 1). This indicates that the presence of the positively charged $Arg655_{AHA2}$ is not essential for proton conduction by the AHA2 H^+-ATPase. However, whereas the $R655K_{AHA2}$-substituted protein was almost as efficient in performing ATP hydrolysis and H^+ transport as the wild-type plant H^+-ATPase, the $R655A_{AHA2}$ and $R655D_{AHA2}$ proteins were severely inhibited in both ATP hydrolysis and proton transport. Both R655A and R655D AHA2 mutants accumulated as the phosphorylated intermediate at levels significantly higher (approximately 400%) than the wild-type protein, and this phosphorylated intermediate was demonstrated to be ascribed the E_1P conformation (Buch-Pedersen and Palmgren 2003). Thus, the positively charged $Arg655_{AHA2}$ must be ascribed a role in the $E_1P \rightarrow E_2P$ transition. Neither of the $Arg655_{AHA2}$-substituted enzymes seemed to have any significantly changed pH dependency in activity. This in combination with the high level of E_1P enzyme accumulating in these substituted mutants suggest that $Arg655_{AHA2}$ does not have a significant (or any) role in the critical proton-binding event allowing E_1P formation to occur.

A model of the E_1 proton-binding site allowing $E_1 \rightarrow E_1P$ transition

The fact that $Arg655_{AHA2}$ does not appear to be involved in the specific proton-binding event allowing the $E_1 \rightarrow E_1P$ transition to proceed seems to exclude the idea that $Arg655_{AHA2}$ acts as proton donor during this step.

The negative charge of $Asp684_{AHA2}$ is absolutely essential for proton transport to occur. It is, however, puzzling that the $D684N/A/V/R_{AHA2}$ substitutions are able to hydrolyze ATP, albeit the ATPase activity in these mutants is completely uncoupled from proton pumping. ATP hydrolysis is normally accompanied by binding of the ion to be transported, suggesting that the proton is bound to the proton-binding site in the $D684N/A/V/R_{AHA2}$ substituted enzymes. The possibility that removal of the negative charge at position Asp684 mimics the protonated Asp cannot be excluded, however.

It is tempting to speculate that the proton-binding event allowing E_1P formation to proceed consists of the binding of a hydronium (or the protonation of a pre-bound water molecule) in a cavity lined by $Asp684_{AHA2}$. Backbone carbonyl oxygens from the non-helical segment of transmembrane M4 are in the E_1 structure of the Ca^{2+}-ATPase exposed into Ca^{2+}-binding site II (Toyoshima et al. 2000). Here they provide coordinating groups for Ca^{2+}-binding. Structural changes in the non-helical region of M4 during enzyme conformational changes combined with the high degree of structural conservation of this segment in P-type ATPases have led to the suggestion that this region could be directly involved in signaling cation binding in P-type ATPases to the rest of the molecule (Xu et al. 2002).

In the homology model of fungal and plant plasma membrane H^+-ATPase, backbone carbonyls of M4 are exposed in a cavity also lined by the conserved aspartate in TM6 ($Asp684_{AHA2}$/$Asp730_{PMA1}$). This area is mutation-sensitive in both the yeast H^+-ATPase (Ambesi et al. 1996) and in the plant H^+-ATPase (S Svensson, A Møller, MJ Buch-Pedersen, MG Palmgren, unpublished results). In the fungal H^+-ATPase, a cavity in this area has, based on modeling, been suggested to contain a bound water molecule (Radresa et al. 2002).

The region around $Asp684_{AHA2}$ in the AHA2 homology model (Bukrinsky et al. 2001) seems to include other well-conserved amino acid residues, and besides $Asp684_{AHA2}$, $Asn106_{AHA2}$, $Tyr648_{AHA2}$, $Thr653_{AHA2}$, $Arg695_{AHA2}$ and $Thr799_{AHA2}$ could occupy central positions in this region of P-type proton pumps.

Proton transport and the $E_1P \rightarrow E_2P$ transition

During the $E_1P \rightarrow E_2P$ transition, the proton is transferred from the E_1P proton binding in a protonation reaction of $Asp684_{AHA2}$. Although the E_1 proton-binding site is speculated to consist of a H_3O^+ coordination center, the necessity for a proton acceptor group in the proton transport process probably suggest that H^+ also should be regarded as a possible transported species.

Analysis of a large number of mutant AHA2 plant H^+-ATPases in which $Arg655_{AHA2}$ and $Asp684_{AHA2}$ have been doubly substituted, has led to the suggestion that these residues are not involved in a structural salt bridge (Buch-Pedersen and Palmgren 2003). Furthermore, characterization of Arg655/Asp684 double mutants could suggest that the role of $Arg655_{AHA2}$ is later in the proton conduction mechanism (Buch-Pedersen and Palmgren 2003). A role for the conserved $Arg655_{AHA2}$ could perhaps be to provide positive charge to the proton-binding site, possibly polarizing the $Asp684_{AHA2}$ side chain and modulating its pK_a.

Mutagenesis experiments do not suggest a role for $Asp730_{PMA1}$ and the conserved arginine in TM5

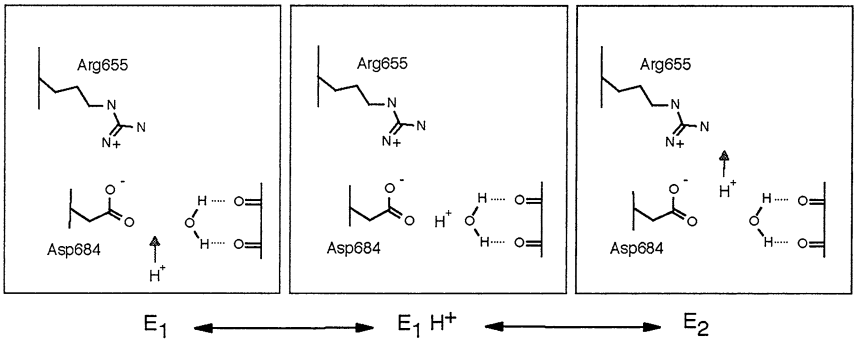

Fig. 4. Model for the proton transportation mechanism of the plant plasma membrane H^+-ATPase AHA2. During the $E_1 \rightarrow E_1(H^+)$ transition, a proton in the form of a H^+ arrives at the cation coordination center, either at a pre-bound water molecule coordinated to a conserved region of transmembrane segment M4, with the possible aid of other groups, or at the carboxyl group of Asp684. Auto-phosphorylation of the ATPase defines the $E_1(H^+) \rightarrow E_1P(H^+)$ transition. Alternatively, the side chain carboxylate of Asp684 first becomes protonated during the $E_1P(H^+) \rightarrow E_2P(H^+)$ transition. Protonation of Asp684 is speculated to constitute an essential step of the proton translocation pathway. The positive charge of Arg655 is suggested to control the pK_a of Asp684. Removal of the negative charge of $Asp684_{AHA2}$ by mutagenic substitution mimics the protonation event of $Asp684_{AHA2}$ and allows the enzyme to proceed into the E_1P conformation where it remains blocked

($Arg695_{PMA1}$) in the proton translocation mechanism of the *S. cerevisiae* PMA1 (Gupta et al. 1998). Whereas $R695_{PMA1}$ and $D730_{PMA1}$ singly substituted proteins appear abnormally folded and extremely protease sensitive (Dutra et al. 1998; Gupta et al. 1998; Petrov et al. 2000), a double substituted R695D/D730R was capable of conducting ATP-dependent proton transport (Gupta et al. 1998).

As $Arg695_{PMA1}$ and $Asp730_{PMA1}$ might be in close proximity in fungal H^+-ATPases (Bukrinsky et al. 2001; Kühlbrandt et al. 2002; Radresa et al. 2002), it could be speculated that the proton-binding site of the yeast H^+-ATPase exhibits some flexibility with respect to exact location, as has been demonstrated for residues contributing the proton-binding site of the F_1F_o ATP synthase (Miller et al. 1990; Rastogi and Girvin 1999; Dimitriev et al. 2002). However, in *S. cerevisiae* PMA1, both $Arg695_{PMA1}$ and $Asp730_{PMA1}$ can be substituted with alanine in an R695A/D730A double substituted mutant with retention of function (Gupta et al. 1998). An active R695A/D730A PMA1 mutant is difficult to explain in the context of $Asp730_{PMA1}$ being an absolutely essential proton acceptor during proton transport. Rather, available evidence points to $Arg695_{PMA1}$ and $Asp730_{PMA1}$ as being engaged in an essential structural salt-bridge (Gupta et al. 1998).

Conclusions about the proton translocation mechanism of plant plasma membrane H^+-ATPases

A description of the proton translocation pathway through the plant plasma membrane H^+-ATPase must include a conserved Asp in M6 corresponding to $Asp684_{AHA2}$. This residue is conserved in Na^+/K^+- and Ca^{2+}-ATPases as well. Conserved charged and/or polar residues in the surrounding of $Asp684_{AHA2}$ could be imagined to provide liganding groups for high-affinity hydronium ion coordination, or they could perhaps participate in defining the acidity or geometry of the proton-binding site. Further studies are needed to reveal this point. Nevertheless, $Asp684_{AHA2}$ seems to constitute at least part of the active proton translocation site. Minimal models of the involvement of $Asp684_{AHA2}$ and $Arg655_{AHA2}$ in the proton translocation pathway of the plant H^+-ATPase are provided in Fig. 4.

The proton pumping mechanism of the plant plasma membrane H^+-ATPase seems perhaps best described by a mixture of known proton translocation proteins and other P-type ATPases. Thus, the transduction of protons seems dependent upon protonation/deprotonation reactions of charged amino acid residues (in a minimal scheme, $Asp684_{AHA2}$) as in bacteriorhodopsin, F_1F_o ATP synthase and cytochrome *c* oxidase. Such a mechanism of transport must be envisioned to involve changes in pK_a values of the involved residues as demonstrated in other proton transporting proteins. However, the E_1 proton-binding site could resemble a typical P-type ATPase cation-binding site in the sense that a hydronium ion could be the initial bound (occluded) species.

The domain movement identified in the membranous region of the SR Ca^{2+}-ATPase during catalysis (Toyoshima and Nomura 2002) is likely to apply to other P-type ATPases as well. Such domain movement during catalysis could account for necessary pK_a changes and furthermore assure that proton leaks do not occur during the proton transfer event.

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