A Conserved Asparagine in a P-type Proton Pump Is Required for Efficient Gating of Protons

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Running title: Gating residue in a P-type proton pump

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**Background:** P-type proton pumps work against large electrochemical gradients, but how gating is controlled is poorly understood. **Results:** Substitution of Asn106 in transmembrane segment M2 significantly reduces the ability of the pump to perform uphill proton transport. **Conclusion:** Asn106 fulfills several criteria for being a gating residue in P-type proton pumps. **Significance:** The presence of a gating residue provides a mechanism for preventing backflow of protons during pumping.

The minimal proton pumping machinery of the Arabidopsis thaliana P-type plasma membrane H⁺-ATPase isoform 2 (AHA2) consists of an aspartate residue serving as key proton donor/acceptor (Asp684) and an arginine residue controlling the pKₐ of the aspartate. However, other important aspects of the proton transport mechanism such as gating, and the ability to occlude protons, are still unclear. An asparagine residue (Asn106) in transmembrane segment 2 of AHA2 is conserved in all P-type plasma membrane H⁺-ATPases. In the crystal structure of the plant plasma membrane H⁺-ATPase this residue is located in the putative ligand entrance pathway, in close proximity to the central proton donor/acceptor Asp684. Substitution of Asn106 resulted in mutant enzymes with significantly reduced ability to transport protons against a membrane potential. Sensitivity towards orthovanadate was increased when Asn106 was substituted with an aspartate residue, but decreased in mutants with alanine, lysine, glutamine, or threonine replacement of Asn106. The apparent proton affinity was decreased for all mutants, most likely due to a perturbation of the local environment of Asp684. Altogether, our results demonstrate that Asn106 is important for closure of the proton entrance pathway prior to proton translocation across the membrane.

Proton pumps establish proton gradients across biological membranes that represent a form of energy that can be tapped to perform work such as secondary active transport across membranes, ATP synthesis in mitochondria and chloroplasts, and flagellar motions (1,2). Despite progress in understanding the mechanism of transport of protons by biological pumps, it still remains unclear how protons are restricted from sliding backwards when transported against electrochemical gradients (3-5). Protons move extremely fast in water as protons tunnel from one water molecule to the next via hydrogen bonding between water molecules and, likewise, protons might cross biological membranes through networks of hydrogen bonds (6). The unique properties of protons caused Nagle and Morowitz (7) to predict that the path for protons in a hydrogen-bonded chain could involve water molecules.
within the protein to complete a proton relay system (8,9). No matter the nature of the hydrogen bonded chain, the chain defining the proton transport pathway has to be efficiently interrupted at least once along the transport pathway in order to prevent back-slipping of protons, which is a requirement for uphill transport of protons.

P-type ATPases are a family of cation transporters found in all eukaryotes that share the same overall structural fold (10-13). Prominent members of the P-type ATPase family are the well-studied Na\(^+\),K\(^+\)-ATPase and the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). P-type plasma membrane (PM) H\(^+\)-ATPases are responsible for generating the essential electrochemical gradient across the plasma membrane of fungi and plants, and in these organisms they are the physiological equivalent of the Na\(^+\),K\(^+\)-ATPase in animals (14). The PM H\(^+\)-ATPase transports one proton per ATP hydrolyzed (15,16), and pumps protons from the cytosol to the extracellular space apparently without any ion being counter-transported. Consequently, P-type PM H\(^+\)-ATPases maintain high electrochemical gradients across the fungal and the plant plasma membranes (up to -300 mV and -200 mV, respectively (17,18); much higher than the approximately -70 mV reported for the Na\(^+\),K\(^+\)-ATPase).

Diverse proton transporters such as bacteriorhodopsin, the F\(_{1}\)F\(_{0}\) ATP synthase, and the PM H\(^+\)-ATPase appear to employ a common proton transport mechanism that involves a central aspartate residue serving as key proton donor/acceptor and an arginine residue controlling the pK\(_{a}\) of that aspartate residue as the minimal proton pumping apparatus (19). For the plant PM H\(^+\)-ATPase, the central proton donor/acceptor has been shown to be the essential residue Asp684, and, in addition, the nearby Arg655 has been shown to be important, though not essential, for proton transport in this protein (20,21). Besides the involvement of these two residues and the recent identification of a proton release group (22), not much is known about how proton transport is achieved by PM H\(^+\)-ATPases.

Recently, the first three-dimensional structure of a P-type H\(^+\)-ATPase was published, showing the plant PM H\(^+\)-ATPase in a quasi-occluded \(E_1\) conformation at a resolution of 3.6 Å (12). Although this structure has been an important step forward in the understanding of the PM H\(^+\)-ATPase, the resolution is too low to unambiguously determine the orientation of amino acid side chains or the presence of water molecules, and can thus only provide indications for the proton translocation mechanism of the PM H\(^+\)-ATPase.

In the Na\(^+\),K\(^+\)-ATPase, the cytosolic cation entrance pathway has been proposed to be closed by movement of transmembrane segment 1 (M1), thereby creating a hydrophobic plug that restricts the movement of the side chain of Glu329, a residue in M4 involved in liganding and gating (23,24). However, the residues implicated in the occlusion mechanism of the Na\(^+\),K\(^+\)-ATPase are not conserved in the PM H\(^+\)-ATPase. Furthermore, the PM H\(^+\)-ATPase faces other challenges such as the very high proton mobility. The structural basis for the higher efficiency of the PM H\(^+\)-ATPase compared to the Na\(^+\),K\(^+\)-ATPase is not known. In this study, we have investigated the role of an asparagine residue, Asn106, in transmembrane segment 2 (M2) of the plant PM H\(^+\)-ATPase, which is completely conserved in all PM H\(^+\)-ATPases. Our results suggest that this residue is important for proton transport in P-type PM H\(^+\)-ATPases and could serve to ensure closure of the proton entrance pathway.

**MATERIALS AND METHODS**

**Construction of Mutants** - The multi-copy vector Yep-351 (25) containing a modified cDNA of the Arabidopsis thaliana AHA2 PM H\(^+\)-ATPase isoform under the control of the PMA1 promoter (26) was used for the cloning of the Asn106 mutants. 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$_6$) tag (20). 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 in Yeast** - The *Saccharomyces cerevisiae* strain RS-72 (27) was transformed and cultured essentially as described previously (28). In RS-72 (MATa adel-100 his4-519 leu2-3,112), the natural constitutive promoter of the yeast endogenous PM H$^+$-ATPase *PMA1* has been replaced by the galactose-dependent GAL1 promoter (27). As *PMA1* is essential for yeast growth, RS-72 is only able to grow on galactose-containing medium. Using this strain, plasmid-borne plant PM H$^+$-ATPases under the control of the constitutive *PMA1* promoter can be tested for their ability to rescue *pma1* mutants on glucose-containing medium. Plasma membrane enriched fractions from yeast expressing the wild type or the various Asn106 mutants were purified as previously described (29).

**Yeast Complementation Assay** - Yeast was grown for 3 days at 30 °C in liquid medium containing 2% galactose. Approximately 10$^3$ cells in 10 µl were spotted onto solid synthetic minimal medium containing either 2% galactose at pH 5.5 or 2% glucose at pH values of 6.5, 5.5, 4.5, and 3.5, respectively. Growth was recorded after incubation for 3 days at 30 °C.

**Gel Electrophoresis and Western Blotting** - Membrane fractions or purified proteins were separated by SDS-PAGE on 10% acrylamide. After electrophoresis of the proteins to a nitrocellulose membrane (Millipore), protein blots were probed with an antibody directed against Dpm1p, Pma1p or the MRSGH$_6$ epitope present at the C terminus (Qiagen, Chatsworth, CA).

**Protein Determination** - Protein concentrations were determined by the method of Bradford (30) employing bovine serum albumin as a standard.

**Purification and Reconstitution of ATPase** - Growth of yeast for protein purification was the same as described (20). The transformed yeast cells were harvested, and the PM H$^+$-ATPases were purified by membrane solubilization using n-dodecyl-β-D-maltoside and Ni$^{2+}$-NTA-affinity chromatography according to established procedures (12). Artificial liposomes were prepared from soybean lecithin or dioleoylphosphatidylcholine (DOPC) by extrusion through a 100 nm filter. Protein was reconstituted into the liposomes at a lipid to protein ratio (w/w) of 200:1 as described previously (31). Reconstitution was performed in a buffer containing 10 mM MES-KOH, pH 6.5, 50 mM K$_2$SO$_4$, and 20% (v/v) glycerol.

**ATPase and Phosphorylation Assays** - ATPase activity was determined as described previously (32) using purified protein in a reactivation buffer (20% (v/v) glycerol, 100 mM MES, pH 6.5, 1 mM EDTA, 1 mM dithiothreitol, 10 µg/µl soybean L-α-phosphatidylcholine (lecithin), 0.7% (w/v) n-dodecyl-β-D-maltoside). Phosphorylation by [$\gamma$-32P]ATP was performed as described previously (20).

**Measurement of Proton Transport** - Proton transport into DOPC or lecithin liposomes was measured as described (33) by monitoring fluorescence quenching of ACMA. The reaction medium contained 10 mM MES-KOH 6.5, 50 mM K$_2$SO$_4$, 10% glycerol, 1 mM ATP, 1 µM ACMA, and 125 nM valinomycin. Proton pumping reactions were started by the addition of MgSO$_4$ to a final concentration of 2 mM, and the proton gradient was collapsed by the addition of carbonyl cyanide-m-chlorophenylhydrazone (CCCP) to a final concentration of 125 nM.

**Measurement of Membrane Potential** - The buildup of membrane potential in reconstituted DOPC proteoliposomes was measured using Oxonol VI (33). The reaction medium had a final volume of 3 mL and contained 10 mM Mes-KOH 6.5, 50 mM K$_2$SO$_4$, 10% glycerol, 1 mM ATP, and 50 nM Oxonol VI. The measurements were performed
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at 10 °C to allow the formation of a stable steady-state fluorescence level. After 5 minutes pre-equilibration, reactions were initiated by the addition of MgSO₄ to a final concentration of 2 mM. Fluorescence was followed until steady state, and the membrane potential was collapsed by the addition of valinomycin to a final concentration of 125 nM.

RESULTS

Asn106 Is a Candidate Gating Residue in P-type Proton Pumps - In the only available crystal structure of a P-type PM H⁺-ATPase, the 3.6 Å structure of the plant PM H⁺-ATPase AHA2 (12), it seems that Asn106, located in M2, is within hydrogen bonding distance of the central proton donor/acceptor Asp684 (Fig. 1A). Cavities likely to be filled with water are seen on both sides of the Asn106-Asp684 pair in the quasi-occluded E₁ state. Asn106 is completely conserved in all P-type H⁺-ATPases suggesting an important function, but no mutagenesis data have been presented to support this assumption. To probe the role of Asn106, this residue was replaced with alanine (short, hydrophobic residue), aspartate (negatively charged residue), lysine (positively charged residue), glutamine, or threonine (longer or shorter polar residue), giving rise to constructs N106A/D/K/Q/T, respectively. Mutations at Asn106 were introduced in a truncated version of AHA2 lacking 73 C-terminal residues comprising most of its regulatory domain (aha2Δ73; termed wild type in this study for clarity) and thereby rendered constitutively active (20,29).

Mutations at Asn106 Abolish the Ability of the Plant PM H⁺-ATPase to Complement pma1 - When situated in the plasma membrane of a living cell, the PM H⁺-ATPase faces the challenge of pumping protons against a steep electrochemical gradient involving both a pH gradient and a membrane potential (positive on the outside). A heterologous yeast complementation assay at different medium pH values was performed to test the activity of the Asn106 mutants in vivo. We employed the yeast strain RS-72, in which the gene encoding the endogenous yeast PM H⁺-ATPase, PMA1, has been placed under the control of a galactose promoter and, as a consequence, the strain is only capable of growing on galactose-containing medium unless the pma1 deficiency is complemented (27). The ability of the plant PM H⁺-ATPase and mutants hereof to complement the function of Pma1p was tested on glucose-containing medium. Under these conditions, the wild type plant PM H⁺-ATPase readily complemented pma1. However, none of the Asn106 mutants were able to support yeast growth in the pH range from pH 3.5 to 6.5 (Fig. 2). Both wild type and the Asn106 mutants were found to co-localize with Pma1p in the plasma membrane enriched fraction, and similar expression levels were observed for the wild type and mutant proteins (Fig. 3A). This demonstrates that Asn106 mutants cannot operate under in vivo conditions characterized by the presence of an electrochemical gradient.

Expression of the Wild Type and the Asn106 Mutant PM H⁺-ATPases - The inability of the mutants to support yeast growth prompted us to further characterize the kinetic properties of the pumps. Each mutant enzyme expressed in yeast strain RS-72 was purified to homogeneity (Fig. 3B). The ATPase kinetic properties of the wild type and the Asn106 mutant enzymes were evaluated in a detergent solubilized and partially lipidated state to describe any qualitative changes in the Asn106 mutant enzymes (Table I). All of the enzymes showed ATP dependent phosphate release with apparent ATP affinities in the range of 0.07 to 0.25 mM. Thus, the Asn106 mutant proteins have retained the ability to bind and hydrolyze ATP.

ATP Affinity, pH Dependence and Vanadate Sensitivity of the Asn106 Mutants Indicate Shift in the Conformational Equilibrium of the Mutant Enzymes - In general, P-type ATPases are characterized by two major conformations, E₁ and E₂. The first state, E₁, shows a high affinity for the exported ion (H⁺), and is phosphorylated by ATP to form E₁P, which in turn is sensitive to dephosphorylation by ADP, and for the PM H⁺-ATPase, K⁺. The second state, E₂, is characterized by a low
affinity for the exported ion and high affinity for the imported ion, is insensitive to ADP, and is inhibited by the phosphate analogue orthovanadate (34). The observed increase in apparent ATP affinity for the Asn106 mutants (Table I) could indicate a shift in the conformational equilibrium of the Asn106 mutants. To investigate this we analyzed the effect of proton concentration (pH) and vanadate on the catalytic activity of the mutant proteins.

Increasing the proton concentration has a stimulatory effect on PM H⁺-ATPase activity, similar to the effect observed for other P-type ATPases by increasing the concentration of the transported cation(s); therefore, the pH dependence of ATP hydrolysis by P-type H⁺-ATPases is, at least partially, assumed to reflect their substrate affinity (19). All of the Asn106 mutants showed a shift in the pH dependence towards more acidic values, indicative of a lower proton affinity for the mutant enzymes when compared with the wild type (Fig. 4A, Table I).

The sensitivity towards vanadate inhibition was reduced 20-fold or more for four out of five mutant enzymes (N106A, K, Q, and T) compared to the wild type, with the N106K mutant being almost completely insensitive towards vanadate (Fig. 4B, Table I). This indicates that these four mutants have difficulties in proceeding from the E1 to E2. In contrast, the N106D mutant enzyme displayed a significantly increased sensitivity towards vanadate, indicative of this mutant being arrested in the E2 conformation (Fig. 4B, Table I). The shift of the N106D mutant enzyme towards the E2 state is also supported by its significantly reduced apparent ATP affinity and its lowered pH optimum.

**The Asn106 Mutants Are Arrested in Different Conformational States -** While the above data suggest that the N106D mutant PM H⁺-ATPase accumulates in the E2 conformation, they do not lend themselves toward any conclusion on the conformational state of the other Asn106 mutants. To further investigate the conformational shift of the Asn106 mutants we studied the two phosphorylated conformational states, E1P and E2P, by measuring phosphorylation levels and sensitivity towards potassium. The amount of phosphorylated intermediate was measured under steady-state conditions for the wild type and the Asn106 mutants (Fig. 5A). All of the mutants showed a shift in the steady-state phosphorylated intermediate levels (EP) compared to the wild type. The N106D mutant PM H⁺-ATPase accumulated significantly lower levels of phosphorylated intermediate (approximately one quarter) than the wild type, which supports the notion of this mutant being arrested in the E2 conformation. Interestingly, the other four mutants did not accumulate equal amounts of phosphorylated intermediate. Thus, the N106K and N106T mutant enzymes showed reduced EP levels compared to the wild type (approximately one quarter) (Fig. 5A), which in combination with their decreased vanadate sensitivity could suggest that these mutants are stalled in the E1 position of the catalytic cycle. In contrast, the N106A and N106Q mutant PM H⁺-ATPases accumulated significantly higher EP levels than the wild type (approximately two-fold) (Fig. 5A).

Potassium ions stimulate allosterically the hydrolytic activity of the PM H⁺-ATPase by promoting dephosphorylation of the E1P conformation of the pump (35). To assess whether the phosphorylated intermediate observed for the N106A and N106Q mutant PM H⁺-ATPases accumulated significantly higher EP levels than the wild type (approximately two-fold) (Fig. 5A).

A slight but significant decrease in the hydrolytic activity was observed for both the
N106K and N106T mutant enzymes in the presence of Na\(^+\), and for the N106K mutant in the presence of K\(^+\) as well. This decrease is most likely a consequence of the E1 conformational state of these mutant enzymes in which it is possible for the monovalent cations to disrupt the proton transfer reactions in the cytosolic entrance pathway (36).

Asn106 Mutants Transport Protons at Reduced Rates - To test whether the Asn106 mutants were capable of transporting protons, the wild type and the mutant proteins were reconstituted into artificial liposomes and proton transport into the proteoliposomes was measured using the ΔpH probe 9-amino-6-chloro-2-methoxyacridine (ACMA). All the Asn106 mutants were capable of performing proton transport into soybean lecithin or dioleoylphosphatidylcholine (DOPC) liposomes, albeit at reduced rates compared to the wild type (Table II). The observed proton transport rates in lecithin vesicles were found to correlate with the ATPase activity for all Asn106 mutants (Table I). When proton pumping was tested in the absence of the ionophore valinomycin, thus allowing for the buildup of a membrane potential, all of the PM \(\text{H}^+\)-ATPase proteins showed reduced activity (Fig. 6A, Table II). This indicates that the establishment of a membrane potential slows the PM \(\text{H}^+\)-ATPase in general and the Asn106 mutants in particular. This phenomenon was especially noticeable in DOPC liposomes, where proton pumping above the background could only be detected for the wild type.

In lecithin liposomes, the N106D mutant had a higher initial proton transport activity than the wild type in the presence of valinomycin, consistent with its pH optimum and wild type-like maximal activity. However, the rate of proton pumping for the N106D mutant declined more rapidly than the rate observed for the wild type (Fig. 6A), suggesting that this mutant is strongly inhibited by the electrochemical gradient. When reconstituted in DOPC vesicles, the N106D mutant displayed a lower rate of proton pumping compared to the wild type (Fig. 6B), and this rate of proton pumping was coupled to a lower ATPase activity in the DOPC liposomes (data not shown). This could suggest that DOPC liposomes are tighter towards protons and therefore build up higher electrochemical gradients than lecithin liposomes.

Asn106 Is Important For Membrane Potential Formation - The wild type pump was assayed for its ability to generate a membrane potential \textit{in vitro} using the probe oxonol VI. Membrane potential formation could successfully be measured in DOPC proteoliposomes (Fig. 7A), but not in lecithin proteoliposomes. Membrane potential formation was fast and a maximum value was approached in less than a minute. Asn106 mutant enzymes could all establish membrane potentials (Fig. 7B) but were much slower at establishing the steady-state level (Fig. 7A,C). The rates of membrane potential formation were comparable to the proton transport rates measured in DOPC vesicles in the presence of valinomycin (Table II).

DISCUSSION

Asn106 Is Important for Uphill Proton Translocation - Asn106 is completely conserved in all P-type PM \(\text{H}^+\)-ATPases but no mutational study involving this residue has previously been reported. The data presented here suggest an important role for Asn106 in uphill proton transport.

Substituting Asn106 resulted in a marked reduction in the ability of the modified pumps to transport protons except for the N106D mutant (Table II). We noted that not only was the initial proton transport rate affected, but the transport rate seemed to decline more rapidly for the Asn106 mutants than observed for the wild type (Fig. 6A). Removal of valinomycin from the assay, thus allowing for the build-up of a membrane potential, had a more pronounced effect on the Asn106 mutants than observed for the wild type (Fig. 6A). Removal of valinomycin from the assay, thus allowing for the build-up of a membrane potential, had a more pronounced effect on the Asn106 mutants than observed for the wild type. The inability of the mutants to pump
protons against a potential gradient is consistent with their inability to complement the function of the yeast PM H\(^+\)-ATPase in vivo.

Asn106 is a Gatekeeping Residue in the Cytosolic Proton Entrance Pathway - All Asn106 mutant enzymes showed a shift in their pH optimum, a property that in proton pumps is correlated with substrate affinity. Thus, the reduced ability of the Asn106 mutant PM H\(^+\)-ATPases to pump protons could be the result of an interruption of the proton binding event, either by decreasing the affinity of the site for the proton itself, or by allowing a greater degree of diffusion out of the site. If the binding event itself was disrupted, we would expect the pump to be stalled in the same conformational state for all mutants, namely in the E\(_1\)-E\(_{1P}\) transition which is coupled to the binding event. However, our results show that the Asn106 mutant PM H\(^+\)-ATPases are blocked in different catalytic steps of the reaction cycle: In the reversion of the E\(_2\) state back to E\(_1\) (N106D), in the phosphorylation step associated with binding and occlusion (N106K/T), or in the transition to the E\(_2P\) state (N106A/Q). This strongly supports the notion that Asn106 is implicated in events that are associated with major conformational rearrangements such as substrate binding and occlusion.

In SERCA, the residue corresponding to Asn106 is Asn101, and during the catalytic cycle the position of this asparagine residue undergoes large displacements (Fig. 1B). P-type ATPases show a high degree of overall structural similarity, and therefore it seems reasonable to assume that Asn106 in the PM H\(^+\)-ATPase will be subjected to similar movements as Asn101 in SERCA during the catalytic cycle. According to this assumption, the conformational changes accompanying the E\(_2\)-E\(_1\) and the E\(_1\)-E\(_{1P}\) transitions would shift Asn106 into the proximity of Asp684, and it can be speculated that hydrogen bonding between Asn106 and the protonated Asp684 would lead to occlusion of the binding site and lock the bound proton to Asp684. Mutagenesis of Asn106 would thus perturb the local environment of Asp684, thereby explaining the observed decrease in apparent proton affinity — and consequently the apparent ATP affinity as well - for all the Asn106 mutants. Interestingly, mutagenesis of Asn106 only affects the conformational steps that bring Asn106 into the vicinity of Asp684, supporting the hypothesis that Asn106 acts as a gatekeeper in cooperation with Asp684 to ensure efficient vectorial transport of protons in the PM H\(^+\)-ATPase.

A Common Theme for Gating in Proton Pumps? - Proton transport is inherently quite different from that of other cations which could suggest that specific mechanistic features must be present in proton transporters. In other proton transporters such as the CLCec Cl\(^-\)/H\(^+\) exchanger and cytochrome c oxidase, gating has been suggested to be dependent on only a single amino acid residue. Thus, in the CLCec1 Cl\(^-\)/H\(^+\) exchanger, gating of the proton pathway is achieved by the rotation of the side chain of a single glutamate residue, located at the beginning of the pathway (37,38). The uptake of protons in cytochrome c oxidase occurs through an entrance pathway named the D pathway, a 25 Å long cavity which consists of a network of polar residues and at least 11 crystallographically identified water molecules. However, in all the available crystallographic data, the pathway is interrupted shortly after the entrance by an asparagine residue, and molecular modeling has suggested that this asparagine residue serves as a gate that controls the polarity of the water chain within the D pathway through rotation of its side chain (39). Mutagenesis of this asparagine residue to an aspartate, a cysteine or a threonine residue leads to a complete abolishment of proton pumping while retaining the oxidase activity and proton uptake for the chemical reaction whereas mutagenesis of the asparagine residue to an alanine or a valine abolishes proton pumping and reduces the oxidase activity (40-43).

In the proton translocating F\(_{1}\)F\(_{0}\) ATP synthase, a completely conserved asparagine residue is located in M4 of the a subunit, and mutagenesis of this residue results in only small reductions in the hydrolytic activity, while proton transport is reduced to 5-50% (44).
Interestingly, the modeling of a stretch of M4 from the α subunit with the c ring structure was recently performed for the Na⁺-transporting F₁F₀ ATP synthase from *Ilyobacter tartaricus*, which showed that the conserved asparagine residue is located at the periphery of the binding site in correlation with cross-linking studies in both *I. tartaricus* and the proton translocating F₁F₀ ATP synthase from *E. coli* (45,46). Furthermore, cross-linking studies showed that the protonation status of the stator arginine significantly influences the cross-linking pattern of this asparagine residue (45). Recently, chemical reactivity studies have indicated that this asparagine residue is accessible from the periplasmic half-channel, and it was suggested that the asparagine could be involved in gating H⁺ access from the periplasmic half-channel to the binding site (47).

In conclusion, we here provide evidence that Asn106 functions as a gatekeeper residue in the PM H⁺-ATPase. This gating mechanism is distinct from that found for the SERCA Ca²⁺-ATPase. However, because of a similar placement of a conserved asparagine in a number of structurally divergent proton pumps, we speculate that gating by an asparagine residue is a common theme among proton pumps in nature.

REFERENCES


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FOOTNOTES

The abbreviations used are: ACMA, 9-amino-6-chloro-2-methoxyacridine; AMPPCP, adenosine 5'- (β,γ-methylene)-triphosphate; CCCP, carbonyl cyanide-m-chlorophenylhydrazone; DOPC, dioleoylphosphatidylcholine; EP, phosphorylated intermediate; ER, endoplasmic reticulum; M1-M6, transmembrane segments 1-6, respectively; MES, 4-morpholineethanesulfonic acid; PM, plasma membrane; SERCA, sarco(endo)plasmic reticulum Ca²⁺-ATPase.

FIGURE LEGENDS

Figure 1. Asn106 in AHA2 is located close to Asp684 in the E1 state, and the corresponding residue in the SR Ca²⁺-ATPase, Asn101, undergoes large displacements during the transition from the E1 to the E2 conformation. A, the crystal structure of AHA2 (PDB ID: 3B8C, green) is superimposed on the structure of SERCA in the Ca²⁺-bound E1 state with bound AMPPCP (PDB ID: 1T5S, magenta). N101 and D800 from SERCA, and N106 and D684 from AHA2 are shown as sticks. B, superposition of the SERCA in the Ca²⁺-bound E1 state with bound AMPPCP (PDB ID: 1T5S, magenta), the E2P state with bound BeF₃⁻ (PDB ID: 3B9B, cyan), and the proton bound E₂ state with bound AMPPCP and thapsigargin (PDB ID: 2C8K, yellow). N101 (*) and D800 (#) are shown as sticks. Viewpoint is the same as in A.

Figure 2. Mutagenesis of Asn106 abolishes the ability of the PM H⁺-ATPase to complement pma1. Yeast complementation assay was performed for no PM H⁺-ATPase (empty vector), wild type PM H⁺-ATPase and the various Asn106 mutant PM H⁺-ATPases. In the yeast strain RS-72, the endogenous yeast PM H⁺-ATPase, Pma1p, has been placed under the control of a galactose promoter, whereas the introduced, plasmid-borne plant PM H⁺-ATPases are under the control of the constitutive PMA1 promoter (27). Yeast growth on glucose is therefore dependent on a functional plasmid-borne PM H⁺-ATPase. Transformed yeast cells, RS-72, were spotted on either galactose-containing medium (Gal) at pH 5.5 or glucose-containing media (Glu) at different pH values. Growth was recorded after three days.

Figure 3. Heterologously expressed wild type and Asn106 mutants of AHA2 are located in the yeast plasma membrane. A, Total membranes (Total) from yeast expressing wild type or Asn106 mutated PM H⁺-ATPases were subjected to sucrose step gradient fractionation. The proteins present in each fraction were determined by protein blot analysis. Membrane marker proteins are as follows: Dpm1p, ER; Pma1p, PM. B, Coomassie staining of affinity purified PM H⁺-ATPase proteins.

Figure 4. Asn106 mutant PM H⁺-ATPases show reduced affinity for protons and altered vanadate sensitivity. A, representative example of the pH dependence of ATP hydrolysis by the wild type and the Asn106 mutant PM H⁺-ATPases. The data shown is representative of 3 independent experiments. B, vanadate sensitivity of ATP hydrolysis by the wild type and the Asn106 mutant PM H⁺-ATPases at pH 6.5. Values are presented ± S.D. Legends are the same as in A.
Figure 5. Asn106 mutant PM H⁺-ATPases are halted in different conformational states. A, measurement of steady-state phosphorylation levels at pH 6.5 of wild type and Asn106 mutant PM H⁺-ATPases. Level of steady-state phosphorylated intermediate are given relative to wild type (WT). B, ATP hydrolytic activity of the wild type and the Asn106 mutants was measured without monovalent ions (black) or with the addition of 50 mM Li⁺ (dark grey), Na⁺ (light grey), or K⁺ (white). The activity without monovalent ions for each protein was set to 100%. The data presented are the mean of at least 3 independent measurements ± SD.

Figure 6. Proton transport by the Asn106 mutant PM H⁺-ATPases is inhibited by the formation of a membrane potential. A, proton transport was initiated by the addition of MgSO₄ to reconstituted lecithin proteoliposomes containing either wild type PM H⁺-ATPase (blue) or the mutant enzymes, here N106D (green). Proton pumping was assayed both in the presence (dark colours) and absence (light colours) of 125 nM valinomycin. The decrease in ACMA fluorescence reflecting proton accumulation in the vesicle lumen was abolished by the addition of the protonophore CCCP to 125 nM. The experiment shown is representative for 3-4 independent experiments. B, proton transport measured as above for wild type (blue), N106A (pink) or N106D (green) PM H⁺-ATPases reconstituted into DOPC vesicles in the presence of 125 nM valinomycin. The experiment shown is representative for 3-4 independent experiments.

Figure 7. Asn106 mutant PM H⁺-ATPases show delayed formation of steady-state membrane potential. A, typical membrane potential measurements using Oxonol VI for the wild type or Asn106 mutants reconstituted into DOPC vesicles. Proton pumping was initiated by addition of MgSO₄. After a steady state was reached, valinomycin was added to collapse the membrane potential. The experiment shown is representative for several independent experiments. B, peak fluorescence obtained by the Asn106 mutant enzymes in A, relative to the level in the wild type. Results shown are the mean of 3-4 independent experiments ± S.D. C, the time dependency, given as t½, of the wild type and the Asn106 mutants. t½ represents the time required to achieve 50% of the fluorescence steady-state level. Results shown are the mean of 3-4 independent experiments ± S.D.
Table I

Kinetic data of purified wild type AHA2 PM H⁺-ATPase and Asn106 mutant enzymes

<table>
<thead>
<tr>
<th>PM H⁺-ATPase</th>
<th>Specific Activity (µmol Pi min⁻¹ mg⁻¹)</th>
<th>pH optimum</th>
<th>Apparent ATP affinity (µM)</th>
<th>Vanadate sensitivity (IC₅₀) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>25.7 ± 1.8</td>
<td>7.1 ± 0.1</td>
<td>45 ± 5</td>
<td>8.7 ± 1.1</td>
</tr>
<tr>
<td>N106A</td>
<td>3.6 ± 1.1 *</td>
<td>6.6 ± 0.1*</td>
<td>69 ± 7*</td>
<td>305 ± 69*</td>
</tr>
<tr>
<td>N106D</td>
<td>18 ± 9.6</td>
<td>6.5 ± 0.1*</td>
<td>255 ± 22*</td>
<td>2.6 ± 1.0*</td>
</tr>
<tr>
<td>N106K</td>
<td>1.7 ± 1.0 *</td>
<td>6.4 ± 0.1*</td>
<td>155 ± 39*</td>
<td>2037 ± 201*</td>
</tr>
<tr>
<td>N106Q</td>
<td>3.9 ± 0.5 *</td>
<td>6.6 ± 0.1*</td>
<td>65 ± 11*</td>
<td>162 ± 32*</td>
</tr>
<tr>
<td>N106T</td>
<td>3.6 ± 0.4 *</td>
<td>6.4 ± 0.2*</td>
<td>102 ± 26*</td>
<td>332 ± 43*</td>
</tr>
</tbody>
</table>

Values are presented as the mean of at least 3 independent experiments ± S.D. Asterisks denote significant difference from the wild type (two-tailed P<0.05).
## Table II

Proton pumping efficiency of purified wild type AHA2 PM H\(^+\)-ATPase and Asn106 mutant enzymes reconstituted into liposomes composed of either soybean lecithin or dioleoyl-phosphatidylcholine (DOPC)

<table>
<thead>
<tr>
<th>PM-H(^+)-ATPase</th>
<th>Lecithin</th>
<th>DOPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Valinomycin</td>
<td>- Valinomycin</td>
</tr>
<tr>
<td>wild type</td>
<td>100 ± 12 (1.8% s(^{-1}))</td>
<td>100 ± 8 (0.9% s(^{-1}))</td>
</tr>
<tr>
<td>N106A</td>
<td>12 ± 7*</td>
<td>5 ± 3*</td>
</tr>
<tr>
<td>N106D</td>
<td>131 ± 21</td>
<td>74 ± 21</td>
</tr>
<tr>
<td>N106K</td>
<td>3 ± 1*</td>
<td>2 ± 1*</td>
</tr>
<tr>
<td>N106Q</td>
<td>12 ± 6*</td>
<td>8 ± 5*</td>
</tr>
<tr>
<td>N106T</td>
<td>22 ± 14*</td>
<td>13 ± 5*</td>
</tr>
</tbody>
</table>

Values are presented as the mean of at least 3 independent experiments ± S.D. Numbers in parentheses represent the observed rates expressed as decrease in fluorescence per second.

* Asterisks denote significant difference from the wild type (two-tailed \(P<0.05\)).

* nd: not detected. No proton pumping significantly above the level of empty liposomes could be measured.
Figure 1

A

B

Gating residue in a P-type proton pump
Figure 2
Figure 3

A

<table>
<thead>
<tr>
<th>% (w/w) sucrose</th>
<th>29/33</th>
<th>33/43</th>
<th>43/53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dpm1p</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pma1p</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N106A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N106D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N106K</td>
<td></td>
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<td></td>
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<tr>
<td>N106Q</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N106T</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

wild type       N106A N106D N106K N106Q N106T
Figure 4

A

![Graph A](image)

B

![Graph B](image)
Figure 5

A

\[
\text{EP/EP}_{\text{wt}} \text{ (\%)}
\]

*P<0.05 to WT

B

\[
\text{Activity (\%)}
\]

*P<0.05 to No Ion
Figure 6

A

B

Fluorescence (%)

Time (s)

WT +val

WT

WT-val

N106D -val

N106D +val

MgSO₄

CCCP

Fluorescence (%)

Time (s)

N106A

N106D

MgSO₄

CCCP
Figure 7

A

MgSO_4

Valinomycin

Peak Fluorescence (% of WT)

Time (s)

B

*P<0.05 to WT

C

*P<0.05 to WT

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A conserved asparagine in a P-type proton pump is required for efficient gating of protons

Kira Ekberg, Alex G. Wielandt, Morten J. Buch-Pedersen and Michael G. Palmgren

J. Biol. Chem. published online February 18, 2013

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