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# Structure–function relationships in membrane segment 6 of the yeast plasma membrane Pma1 $H^+$-ATPase

Manuel Miranda [a,*], Juan Pablo Pardo [b], Valery V. Petrov [c]

[a] Department of Biological Sciences and Border Biomedical Research Center, University of Texas at El Paso, 500 West University Avenue, El Paso, TX 79968, USA
[b] Departamento de Bioquimica, Facultad de Medicina, Universidad Nacional Autonoma de Mexico, Ap. Postal 70159, Mexico D.F. 04510, Mexico
[c] Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, 142290 Pushchino, Russia

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ABSTRACT

The crystal structures of the $Ca^{2+}$- and $H^+$-ATPases shed light into the membrane embedded domains involved in binding and ion translocation. Consistent with site-directed mutagenesis, these structures provided additional evidence that membrane-spanning segments M4, M5, M6 and M8 are the core through which cations are pumped. In the present study, we have used alanine/serine scanning mutagenesis to study the structure–function relationships within M6 (Leu-721-Pro-742) of the yeast plasma membrane ATPase. Of the 22 mutants expressed and analyzed in secretory vesicles, alanine substitutions at two well conserved residues (Asp-730 and Asp-739) led to a complete block in biogenesis; in the mammalian P-ATPases, residues corresponding to Asp-730 are part of the cation-binding domain. Two other mutants (V723A and I736A) displayed a dramatic 20-fold increase in the $IC_{50}$ for inorganic orthovanadate compared to the wild-type control, accompanied by a significant reduction in the $K_m$ for Mg-ATP, and an alkaline shift in the pH optimum for ATP hydrolysis. This behavior is apparently due to a shift in equilibrium from the $E_2$ conformation of the ATPase towards the $E_1$ conformation. By contrast, the most striking mutants lying toward the extracellular side in a helical structure (L721A, I722A, F724A, I725A, I727A and F728A) were expressed in secretory vesicles but had a severe reduction of ATPase activity. Moreover, all of these mutants but one (F728A) were unable to support yeast growth when the wild-type chromosomal *PMA1* gene was replaced by the mutant allele. Surprisingly, in contrast to M8 where mutations S800A and E803Q (Guerra et al., Biochim. Biophys. Acta 1768: 2383–2392, 2007) led to a dramatic increase in the apparent stoichiometry of $H^+$ transport, three substitutions (A726S, A732S and T733A) in M6 showed a reduction in the apparent coupling ratio. Taken together, these results suggest that M6 residues play an important role in protein stability and function, and probably are responsible for cation binding and stoichiometry of ion transport as suggested by homology modeling.



## 1. Introduction

P-type ATPases, which are found throughout prokaryotic and eukaryotic cells, use the energy from ATP hydrolysis to pump different cations and some other substrates across biological membranes [1–3]. They have a single large catalytic subunit, embedded in the lipid bilayer by 8–10 hydrophobic α-helices, and a common reaction mechanism in which the γ-phosphoryl group of ATP is transferred to a conserved aspartate residue to form a covalent phosphorylated intermediate [1,2]. This P-ATPase family contains several subfamilies (P1 to P5); the P2 pumps, one of the largest subfamilies, include the physiologically important mammalian $Ca^{2+}$-, $Na^+$,$K^+$-, and $H^+$,$K^+$-ATPases and $H^+$-ATPases of fungi and plants. The yeast Pma1 $H^+$-ATPase, being the primary pump of the cell, belongs to this subfamily, is encoded by *PMA1* gene, and it has been shown to be essential for growth. [4].

Within the last decade, crystal structures of the mammalian $Ca^{2+}$- [5,6] and $Na^+$,$K^+$-ATPases [7–9] and fungal and plant $H^+$-ATPases [10,11] have appeared, providing a valuable framework for studying the molecular mechanism of P-type ATPases. The structure includes a cytoplasmic headpiece that is folded into three discrete domains: one (N) binds ATP; another (P) contains the phosphorylated aspartyl residue; and a third (A) may function as an "actuator" or anchor domain. A thick stalk-like region connects the cytoplasmic headpiece to the membrane domain (M), which consists of 10 α-helices (M1 to M10) with varying lengths and inclinations. Earlier site-directed mutagenesis studies of the sarcoplasmic reticulum $Ca^{2+}$- ATPase located residues essential for $Ca^{2+}$ transport in 4 of the 10 α-helices: M4 (Glu-309), M5 (Asn-768 and Glu-771), M6 (Asn-796, Thr-799, and Asp-800), and M8 (Glu-908) [12–14]. As expected, the crystal structure showed that side-chain oxygen atoms from these residues contribute to two $Ca^{2+}$-binding sites (I and II), situated in a pocket

* Corresponding author. Tel.: +1 915 747 6645; fax: +1 915 747 5808.
*E-mail address:* mmiranda3@utep.edu (M. Miranda).

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near the middle of the membrane; three additional residues from M4 (Val-304, Ala-305, and Ile-307) also furnish main-chain carbonyl oxygens to site II [5]. Similarly, the crystallographic structures of the $Na^+$,$K^+$-ATPases from pig kidney [7] and shark rectal glands [9] revealed the amino acid residues involved in the formation of two $Na^+/K^+$ binding sites. In the pig kidney enzyme, the binding site I is structured with three amino acid residues from M5 (main-chain oxygen of Thr-772 and the side-chains of Ser-775 and Asn-776) and two residues from M6 (Asp-804 and Asp-808, via a water molecule). Oxygens from main-chain of Val-322, Ala-323, Val-325 in M4, and side-chain of Asn-776 and Glu-779 in M5, Asp-804 in M6, and probably Glu-327 in M4 participate in the construction of binding site II [7,9,15].

The P-ATPase family is noteworthy for its ability to pump a wide range of cations, including $H^+$, $Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $Mn^{2+}$. There are also major differences in cation stoichiometry from one member of the family to the next, ranging from $1H^+$/ATP for the plasma membrane $H^+$-ATPase of fungi and plants [16–18] to 3 $Na^+$/2$K^+$/ATP in the $Na^+$,$K^+$-ATPase of animal cells (reviewed in [19]). Based on sequence alignments, supplemented by cryo-electron microscopy images of the *Neurospora* $H^+$-ATPase [11] and the crystal structures of the *Arabidopsis* plasma membrane $H^+$-ATPase [10] and the mammalian $Ca^{2+}$ and $Na^+$, $K^+$-ATPases [5–9], it is evident that the cation specificity and stoichiometry determinants lie in the core of membrane-spanning segments M4, M5, M6, and M8 of these cation pumps. Indeed, site-directed mutagenesis of the $Na^+$,$K^+$-ATPase identified at least eight residues in M4, M5, and M6 that are essential for cation occlusion and/or transport [20–22]. Site-directed mutagenesis of the yeast plasma membrane $H^+$-ATPase has located positions in M5 and M8 at which amino acid substitutions alter the coupling between ATP hydrolysis and $H^+$ transport [23,24].

The results described in this paper extend a systematic study of the yeast $H^+$-ATPase by focusing on M6 of that enzyme. As described above, M6 is part of the $Ca^{2+}$-binding pocket in the sarcoplasmic reticulum ATPase, contributing one residue to site I (Thr-799), a second to site II (Asn-796), and a third to both (Asp-800) [5,6]. Likewise, M6 of the $Na^+$,$K^+$-ATPase participates with two residues (Asp-804 and Asp808) in the organization of $Na^+$ binding sites I and II [7–9]. In Pma1, these residues correspond to Ala-729, Ala-726 and Asp-730, respectively. It therefore seemed worthwhile to carry out alanine-scanning mutagenesis along the complete length of M6 of the yeast $H^+$-ATPase, searching for residues that may play a role in $H^+$ transport and in any other aspect of the reaction cycle or the biogenesis and stability of the enzyme.

## 2. Materials and methods

### 2.1. Yeast strains and growth conditions

Two strains of *Saccharomyces cerevisiae* were used in this study: SY4 (*MAT*a, *ura3-52, leu2-3,112, his4-619, sec6-4$^{ts}$ GAL2, pma1::YIpGAL-PMA1*) and NY13 (*MAT*a *ura3*-52). In SY4, the chromosomal copy of the *PMA1* gene has been placed under control of the *GAL1* promotor by gene disruption [25] using the integrating plasmid, YIpGAL-PMA1 [26]. SY4 also carries the temperature-sensitive *sec6-4* mutation which, upon incubation at 37 °C, blocks the last step in plasma membrane biogenesis and leads to the accumulation of secretory vesicles in the cell [27].

### 2.2. Site-directed mutagenesis

Mutations were introduced by polymerase chain reaction into a 519 bp *Bgl*II–*Sal*I restriction fragment of the *PMA1* gene that had previously been subcloned into a modified Bluescript plasmid [28]. Each fragment was sequenced, and then moved into the full-length *PMA1* gene in plasmid pPMA1.2 for study in secretory vesicles [26] or plasmid pVP3, a wild-type version of pGW201 [24,29], for study in plasma membrane. To express the ATPase in secretory vesicles, a 3.7-kb *Hind*III to *Sac*I piece of pPMA1.2 containing the entire coding sequence of the gene was transferred to the centromeric plasmid YCp2HSE, placing the mutant *pma1* allele under control of two tandem arranged heat-shock elements; the resulting plasmid was transformed into strain SY4 [26]. To introduce the mutation into the chromosomal copy of the *PMA*1 gene for expression in plasma membranes, a 6.1-kb HindIII fragment containing the mutant allele linked to URA3 was excised from plasmid pVP3 and integrated into strain NY13 using the Alkali-Cation Yeast transformation kit (Bio 101). In all cases, PCR amplification from genomic DNA and automated DNA sequencing was repeated to confirm the identity of the mutant allele.

### 2.3. Cell fractionation and measurement of expressed ATPase

For studies in secretory vesicles, transformed SY4 cells were grown to mid-exponential phase ($A_{600}$ ~ 1) at 23 °C in supplemented minimal medium containing 2% galactose, shifted to medium containing 2% glucose for 3 h, and then heat-shocked at 37 °C for an additional 2 h. The cells were harvested, washed, and secretory vesicles were isolated by differential centrifugation, further purified by gradient centrifugation [30], and suspended in 0.8 M sorbitol, 1 mM EDTA, 10 mM triethanolamine–acetic acid, pH 7.2, containing 1 mM diisopropyl fluorophosphates (DFP), chymostatin (2 μg/ml) and leupeptin, pepstatin, and aprotinin (each 1 μg/ml) as previously described [24].

For studies on plasma membranes, NY 13-derived strains were grown to mid-exponential phase in supplemented minimal medium (YNB) containing 2% glucose, and a plasma membrane-enriched fraction was obtained by the method of Perlin et al. [31], followed by washing in 1 mM EGTA–Tris, pH 7.5, and resuspension in the same buffer containing all protease inhibitors except DFP. All preparative procedures were carried out at 0–4 °C.

The amount of expressed ATPase was measured by SDS-polyacrylamide gel electrophoresis and Western blotting, as described elsewhere [32]. Blots were incubated with affinity-purified polyclonal antibody against the closely related plasma membrane $H^+$-ATPase of *Neurospora crassa* [33]and then with $^{125}$I-protein A (ICN, Irvine, CA), and assayed by means of a PhosphorImager equipped with ImageQuant software version 5.0 (Molecular Dynamics). The expression level of mutant ATPase relative to a wild-type control was calculated from the average of three or more determinations.

### 2.4. ATP hydrolysis

Unless otherwise noted, ATP hydrolysis was assayed at 30 °C in 0.5 ml of 50 mM MES–Tris, pH 5.7, 5 mM $KN_3$, 5 mM $Na_2ATP$, 10 mM $MgCl_2$, and an ATP regenerating system (5 mM phosphoenolpyruvate and 50 μg/ml pyruvate kinase) in the presence and absence of 100 μM Na-orthovanadate. The reaction was terminated after 20–40 min and the release of inorganic phosphate from ATP was measured by the method of Fiske and Subbarow [34]. The $IC_{50}$ for vanadate inhibition was determined by measuring ATP hydrolysis in the presence of increasing concentrations of vanadate. For determination of $K_m$ values, concentration of $Na_2ATP$ was varied between 0.15 and 7.5 mM with 5 mM $MgCl_2$ excess over ATP; actual concentrations of Mg-ATP were calculated by the method of Fabiato and Fabiato [35]. To determine the pH optimum for ATP hydrolysis, the pH of the assay medium was adjusted to values between 5.2 and 7.5 with Tris base.

### 2.5. $H^+$ pumping; coupling between proton pumping and ATP hydrolysis

$H^+$ pumping into the secretory vesicles was monitored by fluorescence quenching of the pH-sensitive dye acridine orange as described previously [24,30]. Assays were carried out at 29 °C with