Evidence for a Salt Bridge between Transmembrane Segments 5 and 6 of the Yeast Plasma-membrane H\(^{+}\)-ATPase*

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The plasma-membrane H\(^{+}\)-ATPase of *Saccharomyces cerevisiae*, which belongs to the P\(_2\)_ subgroup of cation-transporting ATPases, is encoded by the *PMA1* gene and functions physiologically to pump protons out of the cell. This study has focused on hydrophobic transmembrane segments M5 and M6 of the H\(^{+}\)-ATPase. In particular, a conserved aspartate residue near the middle of M6 has been found to play a critical role in the structure and biogenesis of the ATPase. Site-directed mutations in which Asp-730 was replaced by an uncharged residue (Asn or Val) were abnormally sensitive to trypsin, consistent with the idea that the proteins were poorly folded, and immunofluorescence confocal microscopy showed them to be arrested in the endoplasmic reticulum. Similar defects are known to occur when either Arg-695 or His-701 in M5 is replaced by a neutral residue (Dutra, M. B., Ambesi, A., and Slayman, C. W. (1998) *J. Biol. Chem.* 273, 17411–17417). To search for possible charge-charge interactions between Asp-730 and Arg-695 or His-701, double mutants were constructed in which positively and negatively charged residues were swapped or eliminated. Strikingly, two of the double mutants (R695D/D730R and R695A/D730A) regained the capacity for normal biogenesis and displayed near-normal rates of ATP hydrolysis and ATP-dependent H\(^{+}\) pumping. These results demonstrate that neither Arg-695 nor Asp-730 is required for enzymatic activity or proton transport, but suggest that there is a salt bridge between the two residues, linking M5 and M6 of the 100-kDa polypeptide.

The past few years have seen steady progress toward understanding the structure and function of P\(_2\)_ type cation-transporting ATPases, including the plasma-membrane H\(^{+}\)-ATPases of *Saccharomyces cerevisiae* and *Neurospora crassa* and the Na\(^{+}\),K\(^{+}\), and Ca\(^{2+}\)-ATPases of mammalian cells (1). In particular, it now seems clear that the 100-kDa ATPase polypeptides are embedded in the lipid bilayer by 10 transmembrane segments, four at the amino-terminal end and six at the carboxyl-terminal end of the molecule. Evidence for this view came initially from a combination of indirect approaches including hydrophathy analysis, gene fusions (2), tryptic digestion (3), and *in vitro* translation of hydrophobic segments (4–7). More recently, cryo-electron microscopy of two-dimensional crystals at 8-Å resolution has provided direct images of 10 membrane-spanning \(\alpha\)-helices in the plasma-membrane H\(^{+}\)-ATPase of *N. crassa* (8) and the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (9).

Among the various membrane segments, there is particular interest in M5 and M6, which are generally connected by a hydrophilic loop of only five or six amino acid residues and thus are likely to form a hairpin in the membrane. In the mammalian P\(_2\)_ ATPases, mutagenesis studies have identified amino acid residues within M5 and M6 that appear to play a direct role in cation translocation, including Glu-771 (M5) and Asn-796, Thr-799, and Asp-800 (M6) of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (10, 11) and Glu-779 (M5) and Asp-804 and Asp-808 (M6) of the Na\(^{+}\),K\(^{+}\)-ATPase (12–14). It was therefore intriguing when Lutsenko *et al.* (15) detected a change in the state of the M5-M6 hairpin, depending upon the presence or absence of the transported cation. This finding was based on previous work by Shainskaya and Karlish (16), who demonstrated that most of the extramembranous regions of the Na\(^{+}\),K\(^{+}\)-ATPase could be removed by proteolytic digestion in the presence of K\(^+\) or Rb\(^+\), leaving a preparation still capable of occluding K\(^+\). Subsequently, Lutsenko *et al.* (15) showed that proteolytic digestion in the absence of K\(^+\) was accompanied by the disappearance of M5 and M6 from the membrane, and went on to speculate that the hairpin may move in and out of the bilayer as a normal part of the ATPase reaction cycle.

Based on recent evidence from site-directed mutagenesis, M5 and M6 are also structurally and functionally important in the Pma1 H\(^{+}\)-ATPase of *S. cerevisiae*. Several residues in both membrane segments are required for normal biogenesis of the ATPase, and others play a role in the conformational changes that accompany the reaction cycle (17, 18). Asp-730, located near the middle of M6, appears to be especially critical, since replacement by Asn or Val leads to a complete failure of newly synthesized ATPase to reach the secretory vesicles responsible for delivering it to the plasma membrane (18). To investigate this finding in greater detail, we have examined the effect of the D730N and D730V mutations on the folding and subcellular localization of the ATPase, and have gone on to search for compensatory mutations in M5. The results point to the presence of a salt bridge between Arg-695 in M5 and Asp-730 in M6, the first clearcut example of such an interaction in any of the P-type ATPases.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—*S. cerevisiae* strains SY4 (MAT a; *ura3-52; leu2-3, 112; his 4-619; sec 6-4\(_a\); GAL2; *pma1::YipGal-PMA1::URA3*) and NY805 (MAT a; *ura3-52; leu2-3, 112; GAL2*) were used in these studies. SY4 has been described in detail by Nakamoto *et al.* (19), and the sec 6-4\(_a\) mutation by Schekman and Novick (20).

**Mutagenesis**—A 519-base pair BgIII-SalI fragment of the *PMA1* gene (21), subcloned into a modified Bluescript vector (Stratagene, La Jolla, CA), was used for mutagenesis. Mutations were introduced with the Chameleon\(^\text{TM}\) Double Stranded Site-directed Mutagenesis Kit (Stratagene, New...
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agene, La Jolla, CA) and verified by automated DNA sequencing. After the BglII-SalI fragment was subcloned into plasmid pMA1-2 (19), the 3.77-kilobase HindIII-SacI fragment containing the entire ATPase gene was moved into the expression vector YCp2HSE to bring the gene under heat-shock control. Plasmids were transformed into SY4 cells by the method of Ito et al. (22).

Isolation of Secretory Vesicles—SY4 cells were grown to mid-exponential phase (A₆₀₀~1) at 23 °C in minimal medium supplemented with 2% (w/v) galactose. The cells were then shifted to minimal medium containing 2% (w/v) glucose for 3 h, and subsequently transferred to 39 °C for an additional 2 h. The cells were harvested, washed, and lysed, and secretory vesicles were isolated by sucrose density gradient centrifugation (23).

Quantification of Expressed ATPase—To assay the level of Pma1 protein expressed in secretory vesicles, isolated vesicles (5–20 μg) were subjected to SDS-gel electrophoresis followed by immunoblotting (19) with polyclonal antiserum raised against the closely related Pma1 ATPase of N. crassa (24), control experiments with partially proteolyzed preparations have shown that the antiserum recognizes epitopes scattered throughout the 100-kDa polypeptide. Expression levels were calculated relative to a wild-type control using a PhosphoImager programmed with ImageQuant Software Version 3.3 (Molecular Dynamics, Sunnyvale, CA) (25).

To determine the total amount of ATPase synthesized by the cell, SY4 cells were shifted from galactose medium at 23 °C to glucose medium at 39 °C as described above, and labeled for varying lengths of time (15, 30, 60, and 90 min) with [³⁵S]methionine (26). Total membranes were isolated and immunoprecipitated with anti-Pma1 antibody (24), and after SDS-polyacrylamide gel electrophoresis, the gels were fixed, incubated in 1 μl sodium salicylate (30 min at 23 °C), dried, and exposed to Hyperfilm-MP (Amersham, Arlington Heights, IL).

Immunofluorescence Microscopy—To determine the subcellular localization of mutant ATPases, NY605 cells were transformed with a centromeric plasmid carrying the PMA1 gene that had been tagged with c-Myc epitope in a position corresponding to the NH₂ terminus of the ATPase and placed under control of the GAL1 promoter (27). Cells were grown in 4% (w/v) raffinose, transferred to 2% (w/v) galactose and 0.5% (w/v) raffinose, and after 4 h, immunofluorescence microscopy was carried out by the method of Redding et al. (28) as modified by DeWitt et al. (27). Two different primary antibodies were used: Myc monoclonal 9E10.2 from ascites fluid (provided by H. Dohlman), diluted 1:100; and 9E10.2 from ascites fluid (provided by H. Dohlman), diluted 1:100; and

et al. (27). Two different primary antibodies were used: Myc monoclonal 9E10.2 from ascites fluid (provided by H. Dohlman), diluted 1:100; and Kar2 polyclonal antibody (provided by M. Rose), diluted 1:5000 (29).

Cells were observed with a Bio-Rad MRC-600 Scanning Confocal Microscope (Melvile, NY) using dual channel filters for simultaneous viewing of Texas Red and fluorescein isothiocyanate fluorochromes and a slit width set to provide an optical slice less than 1 μm. Images were collected and processed as described previously (27).

Tryptosynolysis—³⁵S-Labeled total membranes were diluted to 1 mg/ml in 1 mM EGTA-Tris, pH 7.5 (without protease inhibitors). Membranes (5 μg) were added to 10 μl of 20 mM Tris, 5 mM MgCl₂, pH 7.0, and after a 2-min preincubation at 30 °C, trisyl-phenylalanilichromethyl ketone-trycin (Worthington Biochemical Corp., Freehold, NJ) was added to give the desired trypsin:protein ratio in a final volume of 20 μl. After incubation for 0 to 10 min at 30 °C, the reaction was stopped by the addition of 20 μl of 2 mM diisopropyl fluorophosphate. Reaction products were analyzed by immunoprecipitation (25, 26), followed by SDS-polyacrylamide gel electrophoresis and fluorography.

Testing for Dominance or Recessiveness—NY605 cells transformed with GAL-pma1 plasmids were grown at 30 °C in synthetic medium lacking uracil and containing 2% (w/v) glucose (27). The cells were then diluted to 1000/ml in sterile deionized water and plated in 5-μl droplets onto synthetic medium, lacking uracil and containing either 2% (w/v) glucose (chromosomal PMA1 gene expressed) or 2% (w/v) galactose (both chromosomal and plasmid-borne genes expressed). The plates were incubated at 30 °C for 48 h and photographed.

ATPase Activity—ATP hydrolysis was assayed at 30 °C in buffer containing 50 mM MES, 2.5 mM NaCl, 5 mM Na₂ATP 10 mM MgCl₂, and an ATP regenerating system composed of 5 mM phosphoenolpyruvate and 50 μM pyruvate kinase, as described by Ambesi et al. (27). The specific activity was measured as the difference between hydrolysis in the presence and absence of 100 μM orthovanadate. For the determination of Km values, the Na₂ATP concentration was varied from 0.15 to 5 mM and the actual concentration of MgATP was calculated by the method of Fabiato and Fabiato (31). For the determination of V₅₀ values, the concentration of orthovanadate was varied from 0 to 100 μM. The pH optimum was determined by varying the pH from 5.0 to 8.0 with Tris base.

ATP-dependent Proton Transport—Proton transport was assayed as the initial rate of acidric orange fluorescence quenching in 0.6 μM sorbitol, 0.1 mM KC1, 20 mM HEPES/ROH, pH 6.7, Na₂ATP (0.3–3.0 mM), and MgCl₂ (5 mM excess over ATP concentration), as described by Ambesi et al. (30). Parallel measurements were made of ATP hydrolysis under the same conditions.

Protein Assay—Protein concentrations were measured by the method of Lowry et al. (32), as modified by Ambesi et al. (30).

RESULTS

Defect in Biogenesis of D730N and D730V ATPases—The starting point for this study was the finding that certain mutations of Asp-730 cause a virtual arrest of ATPase biogenesis. In the experiment of Fig. 1A, D730E, D730N, and D730V were expressed in the secretory vesicle system of Nakamoto et al. (19), which uses a temperature-sensitive allele of the sec6 gene to block the last step in the delivery of newly synthesized proteins to the plasma membrane. Shifting the cells from 23 to 39 °C led to the accumulation of secretory vesicles, which were isolated (23) and assayed by immunoblotting with anti-Pma1 polyclonal antibody. As shown in Fig. 1A, an appreciable amount of ATPase carrying the conservative D730E mutation reached the vesicles, while neither D730N nor D730V could be detected there by the antibody.

To verify that the D730N and D730V polypeptides were synthesized and to explore their stability, the cells were incubated with [³⁵S]methionine for varying lengths of time (15, 30, 60, and 90 min) at 39 °C. A total membrane fraction was then isolated, and the ATPase was immunoprecipitated with polyclonal anti-Pma1 antibody. As shown in Fig. 1B, a virtual arrest of ATPase synthesis was induced by the addition of 30 μM D730E polypeptides (Fig. 1C) appeared as a prominently labeled 100-kDa band by 15 min. The wild-type polypeptide remained stable over the entire labeling time course and D730E was nearly as stable, with only traces of a lower molecular weight band appearing over time. By contrast, D730N (Fig. 1D) was readily visible at 15 min but decreased markedly at 60 and 90 min, and D730V (Fig. 1E) became labeled more slowly, reach-

FIG. 1. Western blotting and immunoprecipitation of wild-type and mutant ATPases. Panel A, secretory vesicles were isolated from SY4 cells expressing wild-type (WT) or mutant (D730E, D730N, and D730V) ATPase and subjected to immunoblotting. Panels B–E, SY4 cells expressing wild-type or mutant (D730E, D730N, D730V) ATPase were incubated with [³⁵S]methionine for varying lengths of time (15, 30, 60, and 90 min). After glass bead lysis, a total membrane fraction was isolated, immunoprecipitated with anti-Pma1 antibody, and subjected to SDS-polyacrylamide gel electrophoresis and fluorography. Note the large proteolytic fragment that is more pronounced in the D730 mutants than in the wild-type ATPase.
Subcellular Localization of the Mutant ATPases—To pinpoint the subcellular compartment in which the mutant ATPases were arrested, the D730N and D730V genes were tagged with c-Myc epitope, placed under control of the GAL1 promoter on a centromeric plasmid, and transformed into wild-type strain NY605, which lacks the sec63a mutation and should allow newly synthesized ATPase to move all the way to the plasma membrane. The cells were grown on raffinose, shifted to galactose for 4 h to induce expression of the plasmid-encoded ATPase, fixed with formaldehyde, and processed for immunofluorescence microscopy. Labeling was carried out with c-Myc monoclonal and Kar2 polyclonal antibodies, which were detected by Texas Red and fluorescein isothiocyanate-conjugated secondary antibodies, respectively. Staining of both fluorochromes was visualized by confocal microscopy using dual channel filters, and the images were merged using Adobe Photoshop. Bar = 5 μm.

To examine the folding states of the D730N and D730V ATPases, 35S-labeled total membranes were incubated at a trypsin:protein ratio of 1:20 for varying amounts of time and then immunoprecipitated with anti-Pma1 antibody (Fig. 3). As expected, the 100-kDa wild-type polypeptide was relatively little affected by trypsin under these conditions, most of it remaining intact after 20 min of digestion (WT). By contrast, D730N was largely degraded (D730N) and D730V was barely detectable (D730V) after only 0.5 min of digestion.

Previous work has shown that the wild-type Pma1 ATPase can be digested by higher concentrations of trypsin but that it can be protected by ligands such as MgADP, MgATP, and vanadate, producing distinctive patterns of fragments that correspond to the E1 and E2 conformational states of the enzyme (e.g. Refs. 30 and 34). These patterns are illustrated in Fig. 4A, where the wild-type ATPase was treated with trypsin (1:4) for 10 min. A conspicuous 97-kDa fragment remained in the presence of 20 mM MgADP (ADP) or 20 mM MgATP (ATP), and fragments of 97 and 80 kDa, in the presence of 100, 90, and 80 kDa, in the presence of 100 μM vanadate (VO4). To ask whether the D730N and D730V ATPases might be similarly protected (Fig. 4, B and C), the trypsin:protein ratio was decreased to 1:50 to give a comparable amount of digestion in the absence of ligands. Under these conditions, there was no sign of protection by vanadate (VO4), but bands of 100, 90, and 80 kDa could be seen in the presence of MgADP (ADP) and MgATP (ATP). Thus, unlike mutants bearing substitutions at the catalytic phosphorylation site of the ATPase (D378N and D378V; Ref. 26), D730N and D730V appeared able to bind adenine nucleotides, even though they were less well protected than the wild-type enzyme and their overall conformation was clearly abnormal.

Co-expression of D730N and D730V with Wild-type PMA1—Consistent with the fact that D730N and D730V could be
D730V ATPases. Subjected to SDS-polyacrylamide gel electrophoresis and fluorography. Panel B, and D730V, panel C, panel A a trypsin:protein ratio of 1:20 for 0 to 20 min at 30 °C, and the ATPase was immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis and fluorography.

FIG. 4. Effect of ligands on trypsinolysis of wild-type, D730N, and D730V ATPases. 35S-Labeled total membranes were incubated at a trypsin:protein ratio of 1:4 (wild type/WT, panel A) or 1:50 (D730N, panel B, and D730V, panel C) for 10 min at 30 °C in the presence or absence of ligands, as described under “Experimental Procedures.” Control, no trypsin; trypsin; VO4 (100 μM vanadate); ADP, 20 mM MgADP; ATP, 20 mM MgATP. The ATPase was then immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis and fluorography.

FIG. 5. Co-expression of D730N and D730V mutant ATPases with wild-type ATPase. NY605 cells were transformed with centromeric plasmids encoding wild-type or mutant ATPase under control of the GAL1 promoter, as indicated. Each strain was diluted and 5-μl drops were placed onto solid synthetic medium containing 2% (w/v) galactose (Gal) or 2% (w/v) glucose (Glu), grown for 48 h at 30 °C, and photographed. For additional information about His-701 refer to Wach et al. (40).

Indeed, M5 contains two such residues, Arg-695 and His-701, which have themselves been shown to be required for proper biogenesis. In a recent study, when Dutra et al. (17) substituted either the Arg or His by Ala, the ATPase became highly sensitive to trypsin and was unable to reach the secretory vesicles.

To look for a possible interaction between M5 and M6, double mutants were constructed in which the positive and negative charges were swapped (R695D/D730R; H701D/D730H) or eliminated altogether (R695A/D730A; H701A/D730A). The double mutants and corresponding single mutants were transformed into strain SY4 on a centromeric plasmid under control of the heat-shock promoter, as described in the first section under “Results,” and after the cells were shifted from galactose medium at 23 °C to glucose medium at 39 °C, secretory vesicles were isolated and assayed for ATPase expression and ATP hydrolysis.

As summarized in Table I, the double substitutions H701D/D730H and H701A/D730A, like all of the single substitutions of Arg-695, His-701, and Asp-730, gave undetectable amounts of ATPase in the secretory vesicles. Strikingly, however, the R695D/D730R and R695A/D730A ATPases reached the vesicles at 50 and 85% of the level seen in the wild-type control (Table I Fig. 6), and after correction for the level of expression, were capable of nearly normal ATP hydrolysis (84 and 135%; Table I).

Enzymatic and Transport Properties of R695A/D730A and R695D/D730R—To explore the enzymatic properties of the double mutants further, measurements were made of the $K_{\text{m}}$ for MgATP, $K_{\text{s}}$ for vanadate, and pH optimum. As summarized in Table II, the values for all three parameters were essentially the same as in the wild-type ATPase. Secretory vesicles containing R695A/D730A and R695D/D730R were also assayed by means of acridine orange fluorescence quenching over a range of MgATP concentrations to see whether H⁺ transport was properly coupled to ATP hydrolysis (30). In the experiment of Fig. 7, the initial rate of ATP-dependent quenching was plotted as a function of the rate of ATP hydrolysis from 0.3 to 3.0 mM MgATP. As observed previously for the wild-type enzyme (30), there was a linear relationship between the two, with no detectable change of slope (coupling ratio) in either of the mutants. Thus, by every criterion used in the present study, charge swapping between positions 695 and 730 restored normal function, as did the elimination of charges at both positions. Although indirect effects cannot be ruled out by mutational data alone, the simplest explanation for the results is that Arg-695 interacts directly with Asp-730 to form a salt bridge that links M5 with M6.

Further Mutagenesis to Test for an Interaction between M5 and M6—In considering the structural defects that result when Asp-730 is replaced with a neutral amino acid such as Asn or Val, one possible explanation is that the negative charge of the Asp residue may interact with a nearby positive charge to stabilize the ATPase during folding and biogenesis. If so, a logical place to look for the positive charge is in M5, which is connected to M6 by a short loop of only five amino acids.
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Table I
Effect of Arg-695, His-701, and Asp-730 mutations on expression in secretory vesicles, ATP hydrolysis, and H^+ transport

| Mutation         | Expression^a | ATP hydrolysis^b | Proton transport^c |
|------------------|--------------|------------------|-------------------|
|                  | %            | Uncorrected     | Corrected | %      | Uncorrected | Corrected   |
| Wild-type        | 100          | 5.39            | 5.39      | 100    | 850         | 850         |
| Vector           | 1            | 0.08            | 12        |        |            |             |
| Single mutants   |              |                  |           |        |             |             |
| R695A^d          | 14           | 0.09            | 2         |        |            |             |
| R695D            | 8            | 0.20            | 4         |        |            |             |
| H701A            | 15           | 0.12            | 3         |        |            |             |
| H701D            | 1            | 0.04            | 1         |        |            |             |
| D730A            | 2            | 0.06            | 1         |        |            |             |
| D730R            | 1            | 0.02            | 1         |        |            |             |
| D730H            | 7            | 0.09            | 1         |        |            |             |
| Double mutants   |              |                  |           |        |             |             |
| R695A/D730A      | 85           | 3.83            | 72        | 84     | 492         | 577         |
| R695D/D730R      | 50           | 3.52            | 7.25      | 135    | 349         | 702         |
| H701A/D730A      | 7            | 0.26            | 4         |        |            |             |
| H701D/D730H      | 2            | 0.06            | 1         |        |            |             |

^a Calculated from yields of mutant and wild-type ATPase protein per mg of total secretory vesicle protein as determined by quantitative immunoblotting. Values are the mean of two determinations (single mutants) or six determinations (double mutants), with an average standard error of 15%.

^b Vanadate-sensitive ATP hydrolysis was measured as described under "Experimental Procedures." Values are the mean of two to six determinations with an average standard error of 10%. One unit is defined as 1 μmol of P_i/min.

^c The initial rate of acridine orange fluorescence quenching (H^-transport) was determined as described under "Experimental Procedures." A unit is defined as 1% of total fluorescence quenching/min. Values represent the mean of at least three determinations with a standard error less than 20%.

^d Corrections were not made for mutants with measured ATP hydrolysis below 10% of the wild-type value.

^e Proton transport was not detectable.

^f Data from Dutra et al. (17).

Discussion

Asp-730 lies near the middle of M6 and is well conserved throughout the P_e-ATPase family (Fig. 8). Consistent with this fact, strong evidence has been put forward for its functional importance in the sarcoplasmic reticulum Ca^{2+}-ATPase (where the corresponding residue is Asp-800) and the Na^+-K^-ATPase (where it is Asp-808). In the former case, replacement by Asn has been shown to abolish Ca^{2+} binding and Ca^{2+}-dependent phosphorylation from ATP (10, 11). In the latter case, replacement by a neutral amino acid permits the ATPase to fold properly and reach the cell surface, as evidenced by the binding of extracellular ouabain, but eliminates its ability to support growth (12) and to occlude K^- analogues such as Rb^- and Tl^- (14). Thus, along with several other mutationally sensitive residues in M5 and M6 (see below), Asp-800 and Asp-808 are thought to define part of the transport pathway in the Ca^{2+}- and Na^+-K^-ATPases.

The present study has shown that the corresponding aspartate plays a critical role in the yeast Pma1 H^-ATPase, but in quite a different way. When Asp-730 is replaced by an uncharged amino acid (Asn or Val), the folding of the H^-ATPase is disrupted, making the newly synthesized protein sensitive to trypsin and causing it to become arrested in the endoplasmic reticulum. To judge the severity of the folding defect, it is useful to compare the behavior of the D730N and D730V mutant ATPases with previously published data for D378N and D378V, which are parallel amino acid substitutions at the phosphorylation site of the Pma1 ATPase (26). As described above, the Asp-730 mutants were extremely sensitive to trypsin but displayed a modest amount of protection by high concentrations of MgADP or MgATP, suggesting that the central...
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**Organism** | **Gene** | **Ions** | **Loops** | **M5** | **M6**
--- | --- | --- | --- | --- | ---
*S. cerevisiae* | PMA1 | H\textsuperscript{+} | 650-S Y V V K I A L L H I F L G L W I A L L | ENSLD | ID L1 V P I A I T P A D V A T L A I A
*L. donovani* | PMA1 | H\textsuperscript{+} | 661-S Y T T R E I S A T L O U V C P F P I A C F S L | TPEKAYE5VDPHQPWSLP | V L W F M I L T I L L N D C L M T I G
*M. jannaschii* | IOG1 | H\textsuperscript{+} | 614-S Y T Y K I T T E T R T L I L F P V E C L I L | GITFPP | A L M I V L G A L N D P I L A I A
*D. ficocculata* | PMA1 | H\textsuperscript{+} | 671-S Y T S Y T I A N M T R C I G P T F G L I V T I Y | DMTFP | T I L I V I N A V P N Q G A M I A L S
*A. bahiense* | IOG1 | H\textsuperscript{+} | 644-N T I Y V E T I T T E T R T L I L F P V E C L I | EFDP | A F M Y L I A L N D P I L A I A
*Rabbit* | SRCA | Ca\textsuperscript{2+} | 759-S Q I R Y L I S S N G V G V C I P T A L A G | LFPEAL | P V Q L N V W L E V S G L P A T A L
*Sheep* | NKA | Na\textsuperscript{+},K\textsuperscript{+} | 767-K S I A T L T T G N I P T P I P F I P I A N | TPLFPLG | T V T L C I C L G T D N V P A L S L
*Human* | NKA | H\textsuperscript{+},K\textsuperscript{+} | 785-K S I A T L T T G N I P T P I P F I P I A | VPLFPLG | C I T I L F I C L C T P S P S V L

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FIG. 8. Alignment of M5 and M6 from P-type ATPases. Amino acid sequences of M5 and M6 from eight P-type ATPases were aligned using LASERGENE (DNASTAR). Arginine residue(s) in M5 and the conserved Asp-730 residue in M6 have been boxed. Residues thought to be involved in cation transport are in *ovals*. The GenBank accession numbers from top to bottom are: X00534, M17889, U67563, X73901, M241107, M12898, P04074, and P20648.

catalytic portion of the protein could fold at least partially into a functional nucleotide-binding site. In the earlier study, on the other hand, the D378N and D378V ATPases were not protected by MgADP or MgATP, indicating that these mutations had a more severe effect on the catalytic domain (20). The trypsinolysis data correlated closely with the genetic behavior of the two groups of mutants: when tested on synthetic medium, D730N and D730V acted in a recessive fashion, with little or no ability to inhibit the growth of cells co-expressing wild-type ATPase (see above), while D378A (27), D378N, and D378V behaved as dominant lethal mutations (26, 35, 36).

The most interesting part of this study has been the mutational evidence that Asp-730 forms a salt bridge with Arg-695. Previous work had shown that single neutral substitutions of Arg-695 led to defects in protein folding and biogenesis, similar to those seen with D730N and D730V. In particular, the R695A mutant ATPase was very sensitive to trypsin but could be partially protected by MgADP or MgATP; it was also blocked in the ability to inhibit the growth of cells co-expressing wild-type ATPase (37, 38), while D378N and D378V behaved as dominant lethal mutations (26, 35, 36).

It is instructive to think about the Pma1 salt bridge in the context of Fig. 8, in which M5 and M6 have been aligned for a series of P\textsubscript{2}ATPases. Unlike Asp-730, which is conserved throughout the family, Arg-695 is found only in fungal, protozoan, and archaeabacterial H\textsuperscript{+}-ATPases (*e.g.* *S. cerevisiae, Leishmania donovani*, and *Methanococcus jannaschii*, respectively). Interestingly, another Arg is located six residues further along in M5 of the algal and plant H\textsuperscript{+}-ATPases (*e.g.* *Dunaliella biculata* and *Arabidopsis thaliana*); this Arg is also found in the H\textsuperscript{+}-ATPase from *M. jannaschii*. Thus, a salt bridge could be a universal feature of H\textsuperscript{+}-translocating P\textsubscript{2}ATPases, although it would require a shift of M5 relative to M6 in the algal and plant enzymes. On the other hand, the sucrose permease of *Escherichia coli* offers a clear example of evolutionary divergence in salt bridging among members of an otherwise closely related family. In place of the interacting charged residues found in membrane segments 7 and 11 of the lactose permease (Asp and Lys; see above), the sucrose permease contains neutral residues (Asn-234 and Ser-356). Single mutations of these residues to Asp or Glu and Lys or Arg abolish function, while double mutations (for example, N234D/S356K) restore activity (39). Thus, it is likely that segments 7 and 11 lie close to one another in the sucrose permease. By analogy, helix packing is presumably conserved throughout the family of P\textsubscript{2}ATPases, even though the specific interaction between Arg-695 and Asp-730 exists in only a subset of H\textsuperscript{+}-translocating ATPases.

Finally, it is worth reviewing the functional differences between the M5 and M6 segments of the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase, Na\textsuperscript{+},K\textsuperscript{+}-ATPase, and yeast H\textsuperscript{+}-ATPase (Fig. 8). In the Ca\textsuperscript{2+}-ATPase, mutagenesis studies have implicated Glu-771 (M5) and Asn-796, Thr-799, and Asp-800 (M6) in cation liganding (10, 11), while Ser-775 (M5) and Thr-799 (M6) are implicated in Mg\textsuperscript{2+} liganding (14), while Ser-775 (M5) plays an indirect role, helping to determine the affinity for K\textsuperscript{+} (13). In the yeast H\textsuperscript{+}-ATPase, on the other hand, Asp-730 is clearly not required for H\textsuperscript{+} transport. While single mutants at this position (D730N, D730V, and D730A) could not be evaluated for function owing to the problems with biogenesis that have been discussed above, the near-normal rates of ATP hydrolysis and ATP-dependent H\textsuperscript{+} pumping seen in the R695A/D730A double mutant make it clear that Asp-730 can be replaced by a neutral residue as long as Arg-695 is replaced simultaneously.

Work is currently underway to explore the functional role of other M6 residues. In the meantime, mutagenesis of M5 has focused attention on Glu-703, where replacement by Ala has little effect (17) but replacement by Gln or Leu partially unblocks ATPase activity (40). In the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, there is evidence that Glu-779 (M5) and Asp-800 and Asp-804 (M6) bind K\textsuperscript{+} directly (14), while Ser-775 (M5) plays an indirect role, helping to determine the affinity for K\textsuperscript{+} (13).

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Evidence for a Salt Bridge between Transmembrane Segments 5 and 6 of the Yeast Plasma-membrane \( \text{H}^+ \)-ATPase

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