Structure-Function Relationships in Membrane Segment 5 of the Yeast Pma1 H⁺-ATPase*

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Membrane segment 5 (M5) is thought to play a direct role in cation transport by the sarcoplasmic reticulum Ca²⁺-ATPase and the Na⁺,K⁺-ATPase of animal cells. In this study, we have examined M5 of the yeast plasma membrane H⁺-ATPase by alanine-scanning mutagenesis. Mutant enzymes were expressed behind an inducible heat-shock promoter in yeast secretory vesicles as described previously (Nakamoto, R. K., Rao, R., and Slayman, C. W. (1991) J. Biol. Chem. 266, 7940–7949). Three substitutions (R695A, H701A, and L706A) led to misfolding of the H⁺-ATPase as evidenced by extreme sensitivity to trypsin; the altered proteins were arrested in biogenesis, and the mutations behaved genetically as dominant lethals. The remaining mutants reached the secretory vesicles in sufficient amounts to be characterized in detail. One of them (Y691A) had no detectable ATPase activity and appeared, based on trypsinolysis in the presence and absence of ligands, to be blocked in the E₁-E₂ step of the reaction cycle. Alanine substitution at an adjacent position (V692A) had substantial ATPase activity (54%), but was likewise affected in the E₁-E₂ step, as evidenced by shifts in its apparent affinity for ATP, H⁺, and orthovanadate. Among the mutants that were sufficiently active to be assayed for ATP-dependent H⁺ transport by acridine orange fluorescence quenching, none showed an appreciable defect in the coupling of transport to ATP hydrolysis. The only residue for which the data pointed to a possible role in cation liganding was Ser-699, where removal of the hydroxyl group (S699A and S699C) led to a modest acid shift in the pH dependence of the ATPase. This change was substantially smaller than the 13–30-fold decrease in the K₁⁄₂ values for both Na⁺ and K⁺ countertransport, based on changes in 7-(diethylamino)coumarin, causing disruption of K⁺ binding. Arguello and Kaplan (8) first drew attention to this residue by demonstrating that it reacts with 4-(diazomethyl)-7-(diethylamino)coumarin, causing disruption of K⁺ and Na⁺ occlusion. Thus, there was reason to think that Glu-771 in the Na⁺,K⁺ pump, like its close relative in the Ca²⁺ pump, might be a cation-liganding residue. Subsequently, however, mutagenesis studies have shown that this simple interpretation cannot be correct. Whereas replacement of the glutamate in M5 by Leu does inactivate the Na⁺,K⁺-ATPase, replacement by Gln, Ala, or Arg leaves a functional enzyme with relatively normal K₁⁄₂ values for both Na⁺ and K⁺ (10–12). Arguello et al. (13) have suggested that Glu-771 may instead be part of the voltage-dependent cation access channel, based on the voltage independence of the pump current in the E779A mutant. In parallel, Arguello and Lingrel (14) have implicated a nearby residue, Ser-775, in K⁺ binding by describing mutants (S775A and S775C) with large increases in the K₁⁄₂ for stimulation of ATPase activity by K⁺.

Parallel studies of the animal cell Na⁺,K⁺-ATPase have been of interest since it also contains a glutamate at the corresponding point in M5 (1) (Glu-779 in the canine and sheep α₁ isoforms). Arguello and Kaplan (8) drew first attention to this residue by demonstrating that it reacts with 4-(diazomethyl)-7-(diethylamino)coumarin, causing disruption of K⁺ and Na⁺ occlusion. There was reason to think that Glu-771 in the Na⁺,K⁺ pump, like its close relative in the Ca²⁺ pump, might be a cation-liganding residue. Subsequently, however, mutagenesis studies have shown that this simple interpretation cannot be correct. Whereas replacement of the glutamate in M5 by Leu does inactivate the Na⁺,K⁺-ATPase, replacement by Gln, Ala, or Arg leaves a functional enzyme with relatively normal K₁⁄₂ values for both Na⁺ and K⁺ (10–12). Arguello et al. (13) have suggested that Glu-779 may instead be part of the voltage-dependent cation access channel, based on the voltage independence of the pump current in the E779A mutant. In parallel, Arguello and Lingrel (14) have implicated a nearby residue, Ser-775, in K⁺ binding by describing mutants (S775A and S775C) with large increases in the K₁⁄₂ for stimulation of ATPase activity by K⁺.

Given the intriguing differences in M5 between the Ca²⁺- and Na⁺,K⁺-ATPases, we set out recently to map structure-function relationships in M5 of a third, phylogenetically distant member of the P-type ATPase family, the yeast plasma mem-

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1 The abbreviations used are: M5, membrane segment 5; MES, 2-(N-morpholino)ethane sulfonic acid; SERCA, sarcoplasmic reticulum ATPase.
brane H⁺-ATPase. This enzyme is encoded by the PMA1 gene, accounts for 10% of plasma membrane protein, and splits as much as one-quarter of the ATP produced by the cell (reviewed in Ref. 15). It generates the proton electrochemical gradient that underlies nutrient uptake and, consistent with its key physiological role, is essential for cell viability. While the mechanism of proton transport by the yeast ATPase has not yet been studied in detail, work on a very closely related pump (the Pma1 ATPase of the filamentous fungus Neurospora crassa) provides evidence for a simple stoichiometry of 1 H⁺ translocated per ATP split (16).

In this study, we have carried out alanine-scanning mutagenesis along the full length of M5 in the yeast ATPase. The results have identified five amino acid residues that play a significant role in the reaction cycle, along with three others that are required for proper protein folding and transit through the secretory pathway.

EXPERIMENTAL PROCEDURES

Yeast Strains—Two related strains of Saccharomyces cerevisiae were used in this study: SY4 (MATα, ura3-32, leu2-3,112, his3-11,15, sec6-4, GAL1-, YIPGAL-PMA1) and NY605 (MATα, ura3-32, leu2-3,112, GAL2). In strain SY4, the chromosomal copy of the PMA1 gene has been placed under control of the GAL1 promoter by gene disruption (17) using the integrating plasmid YIPGAL-PMA1 (18). SY4 also carries the temperature-sensitive sec6-4 mutation, which, upon incubation at 37 °C, blocks the fusion of secretory vesicles with the plasma membrane (19). NY605 was generously provided by Dr. Peter Novick (Department of Cell Biology, Yale School of Medicine).

Mutagenesis—Mutagenesis (20) was performed on a 519-base pair BglII-SalI restriction fragment subcloned into a modified Bluescript plasmid (Stratagene, La Jolla, CA). Following DNA sequencing, the BglII-SalI fragment carrying the mutation was moved into plasmid pPMMA1.2 (18). The 3.8-kilobase HindIII-Sacl fragment, which contains the entire pma1 coding region, was cloned into the yeast expression vector YCP2HSE (18), placing the mutant allele under heat-shock control. Plasmids were then transformed into yeast according to the method of Ito et al. (21).

Isolation of Secretory Vesicles and Measurement of Expressed ATPase—Transformed SY4 cells were grown to mid-exponential phase (A600 ~ 1) at 23 °C in supplemented minimal medium containing 2% galactose, shifted to medium containing 2% glucose for 9 h, and then heat-shocked at 39 °C for an additional 2 h. The cells were harvested and washed, and the secretory vesicles were isolated as described previously (22). To determine the level of expressed Pma1 protein relative to a wild-type control, secretory vesicles (5–20 μg) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-Pma1 antibody (26), and after SDS-polyacrylamide gel electrophoresis, the gels were fixed, incubated in 1 M sodium salicylate (30 min at 23 °C), dried, and exposed to Hyperfilm-MP (Amersham Pharmacia Biotech). Immunoprecipitation was performed on both isolated secretory vesicles and 35S-labeled total membranes. Vesicles or membranes were diluted into 1 ml EGTA/Tris, pH 7.5; centrifuged at 100,000 × g for 35 min; and suspended at 0.5 mg/ml in 20 mM Tris-HCl, pH 7.0, and 5 mM MgCl2. Following preincubation in the absence or presence of 100 μM orthovanadate, 10 mM MgADP, or 10 mM MgATP at 30 °C for 5 min, tosylated aXyl chloromethyl ketone-treated trypsin was added (trypsin/protein ratio of 1:4 for secretory vesicles or 1:20 for total membranes), and the incubation was continued for 0.5–20 min. The reaction was terminated by the addition of 1 mM diisopropyl fluorophosphate. Reaction products were analyzed either by immunoblotting (secretory vesicles) or by immunoprecipitation and fluorography (total membranes).

Protein Determination—Protein concentrations were determined by a modification of the method of Lowry et al. (27) as described by Ambesi et al. (22) using bovine serum albumin as a standard.

RESULTS

Expression and ATP Hydrolysis—In this study, alanine-scanning mutagenesis was used to examine the structural and functional role of amino acids in M5 of the yeast plasma membrane H⁺-ATPase. Residues Ser-690 to Leu-713 were included based on hydropathy analysis of the Pma1 protein sequence (reviewed in Ref. 15). All but two of the residues were replaced with alanine, whereas alanines at positions 697 and 711 were replaced with serine. The mutant alleles were transformed into yeast strain SY4, expressed under control of an inducible heat-shock promoter, and secretory vesicles were isolated and characterized with respect to expression and ATP hydrolysis.

Most substitutions allowed reasonable amounts of the ATPase to reach the secretory vesicles, but there were three cases (R695A, H701A, and L706A) in which little or no Pma1 protein could be detected in the vesicles (Fig. 1A and Table I, part A). Immunoprecipitation from 35S-labeled total membranes revealed that each of these mutant proteins was synthesized, but became arrested in an earlier compartment of the secretory pathway, presumably the endoplasmic reticulum (Fig. 1B).

The remaining 21 mutant ATPases reached the secretory vesicles in amounts ranging from 39 to 99% of the wild-type control (Table I, part A). When these mutants were assayed for their ability to hydrolyze ATP, 17 of them had activities of 37% or better after correction for the level of expression in the secretory vesicle preparations. Three showed significant reductions in activity: Y984A to 22%, W709A to 21%, and S699A to 8% of the wild-type control. One mutant ATPase (Y691A) was completely inactive. Y691A and S699A, along with R695A, H701A, and L706A, which were blocked in biogenesis, were later examined for structural defects by limited trypsinolysis, as described below.

Kinetic Properties—The mutant ATPases were next assayed for vanadate sensitivity, MgATP dependence, and the effect of

FIG. 1. Western analysis and immunoprecipitation of Pma1 mutants. A, secretory vesicles were isolated from yeast expressing either wild-type (WT) or mutant (R695A, H701A, and L706A) ATPase and subjected to immunoblotting. B, alternatively, Pma1 was immunoprecipitated from 35S-labeled total membranes and subjected to SDS-polyacrylamide gel electrophoresis and fluorography.

Y691A and S699A, along with R695A, H701A, and L706A, which were blocked in biogenesis, were later examined for structural defects by limited trypsinolysis, as described below.
TABLE I
Effect of pma1 mutations on expression, ATP hydrolysis, and proton transport
Values represent the mean of at least three determinations with an average S.E. of 11% or less.

| Mutation | Expression | ATP hydrolysis | Proton transport |
|----------|------------|---------------|-----------------|
|          |            | Uncorrected   | Corrected       | Uncorrected     | Corrected     |
|          |            | units/mg      | %               | units/mg        | %             |
| Part A   |            |               |                 |                 |
| Wild type| 100        | 3.60          | 3.60            | 100             | 954           | 954           | 100           |
| Vector   | 7          | 0.09          | NDc             | ND              | ND            | ND            | ND            |
| S690A    | 93         | 4.15          | 4.46            | 124             | 1069          | 1149          | 120           |
| Y691A    | 92         | 0.09          | ND              | ND              | ND            | ND            | ND            |
| V693A    | 77         | 1.50          | 1.95            | 54              | 903           | 1172          | 123           |
| V695A    | 79         | 2.57          | 3.25            | 90              | 834           | 1055          | 110           |
| Y694A    | 40         | 0.32          | 0.80            | 22              | ND            | ND            | ND            |
| R695A    | 14         | 0.09          | ND              | ND              | ND            | ND            | ND            |
| I696A    | 84         | 1.13          | 1.34            | 37              | 270           | 321           | 33            |
| A697A    | 76         | 1.65          | 2.17            | 60              | 568           | 747           | 78            |
| L698A    | 90         | 2.28          | 2.53            | 70              | 569           | 652           | 66            |
| S699A    | 90         | 0.27          | 0.30            | 8               | ND            | ND            | ND            |
| L700A    | 84         | 1.62          | 1.93            | 53              | 310           | 369           | 39            |
| H701A    | 15         | 0.59          | 1.51            | 42              | 125           | 320           | 33            |
| L702A    | 39         | 3.58          | 3.77            | 105             | 833           | 877           | 92            |
| E703A    | 95         | 3.58          | 3.77            | 105             | 833           | 877           | 92            |
| I704A    | 98         | 3.04          | 3.10            | 86              | 709           | 723           | 76            |
| F705A    | 73         | 2.00          | 2.74            | 76              | 461           | 631           | 66            |
| L706A    | 7            | 0.05         | ND              | ND              | ND            | ND            | ND            |
| G707A    | 62         | 1.53          | 2.47            | 68              | 272           | 439           | 46            |
| L708A    | 64         | 1.54          | 2.40            | 66              | 309           | 483           | 50            |
| W709A    | 41         | 0.32          | 0.78            | 21              | ND            | ND            | ND            |
| I71A     | 75         | 1.72          | 2.20            | 61              | 350           | 449           | 47            |
| A711S    | 99         | 2.02          | 2.04            | 56              | 385           | 389           | 41            |
| I712A    | 73         | 1.86          | 2.54            | 70              | 328           | 449           | 47            |
| L713A    | 76         | 1.96          | 2.44            | 68              | 332           | 437           | 46            |
| Part B   |            |               |                 |                 |               |               |               |
| Wild type| 100        | 4.43          | 4.43            | 100             | 609           | 609           | 100           |
| Y694G    | 64         | 0.78          | 1.22            | 28              | 48            | 75             | 12            |
| S690C    | 79         | 0.56          | 0.71            | 16              | 55            | 73             | 12            |
| S699T    | 94         | 3.99          | 4.24            | 96              | 573           | 610           | 100           |
| E703D    | 56         | 0.96          | 1.71            | 39              | 135           | 241           | 40            |

a Calculations were made from yields of mutant and wild-type H+-ATPase protein/mg of total secretory vesicle protein, as determined by quantitative immunoblotting (see “Experimental Procedures”).

b Vanadate-sensitive ATP hydrolysis was measured as described under “Experimental Procedures.” One unit is defined as 1 μmol of Pi/min.

c The initial rate of fluorescence quenching (proton transport) was determined as previously described (25). One unit is defined as 1% of total fluorescence quenching/min.

ND, not determined. Corrections for expression have not been made for mutants with measured ATPase activities below 3% of the wild-type value, but have been made for mutants with measured activities between 3 and 10%. In both cases, proton transport could not be detected by the acridine orange assay.

pH on the rate of ATP hydrolysis. In all but one case, the kinetic properties of the mutants proved to be essentially normal (Table II, part A). The exception was V692A, which displayed an increased K_m for vanadate (11 μM compared with 1.8 μM for the wild-type control), a decreased K_m for MgATP (0.1 mM compared with 1.1 mM for the wild type), and a relatively alkaline pH optimum (pH 6.4 compared with pH 5.7 for the wild type). Because Val-692 is presumably buried in the membrane, it is unlikely to contribute in a direct way to the vanadate- and MgATP-binding sites. Rather, the increased K_m and altered pH optimum can more reasonably be accounted for by a slowing of the E_1P-to-E_2P conformational change; as a result, the ATPase accumulates in E_1, which has a relatively high affinity for ATP and protons and a relatively low affinity for orthovanadate. This idea is supported by the fact that mutations at three positions in M4 lead to a similar set of kinetic changes (25).

ATP-dependent Proton Transport—Given the fact that membrane segment 5 is believed to play a direct role in cation translocation in the Ca^2+- and Na^+K+-ATPases, it was of particular interest to explore the proton-pumping ability of the yeast M5 mutants. For this purpose, secretory vesicle preparations were assayed for ATP-dependent quenching of acridine orange fluorescence. In seven of the mutants (Y691A, V694A, R695A, S699A, H701A, L706A, and W709A), ATPase activities were below the limit at which associated proton pumping could have been reliably detected by the acridine orange assay. With one exception, all of the remaining mutants showed a reasonable correlation between the initial rate of ATP-dependent quenching and the rate of ATP hydrolysis (Table I, part A). In V692A, the rate of acridine orange quenching (123% of the wild-type control) appeared to exceed the rate of ATP hydrolysis (54% of the wild-type control). However, separate measurements indicated that the apparent discrepancy could be accounted for by the pH difference between the hydrolysis assay and the quenching assay, together with the above-mentioned alkaline shift in pH optimum. Thus, at pH 6.7, the V692A enzyme split ATP at 119% of the wild-type rate, completely consistent with its relative rate of acridine orange quenching at the same pH (123%; see Table I, part A). There was no evidence for abnormal ATP-dependent proton transport in any of the other 16 mutants that were studied (Table I, part A).

Further Study of Tyr-694, Ser-699, and Glu-703—Based on comparison with other P-type ATPases, it seemed useful to make additional amino acid replacements at three positions: Tyr-694, Ser-699, and Glu-703. In the case of Tyr-694, Andersen (28) has reported that mutation of the corresponding residue to glycine leads to uncoupling of the SERCA Ca^2+-ATPase; the other two residues are believed to play a role in cation binding in the Na^+K^- and SERCA Ca^2+-ATPases,
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Kinetic properties for each mutant were determined under standard assay conditions (50 mM MES/Tris, pH 5.7, 5 mM Na₂ATP, 10 mM MgCl₂, 5 mM KN₃, 5 mM phosphoenolpyruvate, and 50 mg/ml pyruvate kinase at 30 °C) with the following exceptions. The Kᵢ was determined by varying Na₂ATP from 0.15 to 7.5 mM (MgCl₂ in excess of ATP by 5 mM); the Kᵢ was determined by varying the concentration of vanadate from 0 to 200 μM; and the pH optimum was calculated from assays carried out over a pH range of 5.2–7.5 (see “Experimental Procedures”). Values represent the mean of at least three determinations with an average S.E. of <7%.

| Mutation | Kᵢ(MgATP) mm | Kᵢ(van) μM | pH optimum |
|----------|--------------|------------|------------|
| Part A   |              |            |            |
| Wild type| 1.1          | 1.8        | 5.7        |
| S690A    | 0.7          | 1.4        | 5.8        |
| V692A    | 0.1          | 10.9       | 6.4        |
| V693A    | 0.7          | 1.8        | 5.8        |
| I696A    | 0.6          | 2.8        | 5.7        |
| A697A    | 1.0          | 1.8        | 5.6        |
| L698A    | 0.9          | 1.3        | 5.7        |
| L700A    | 1.0          | 2.1        | 5.5        |
| L702A    | 0.7          | 5.3        | 5.7        |
| E703A    | 1.3          | 2.2        | 5.7        |
| I704A    | 1.3          | 2.2        | 5.6        |
| F705A    | 1.1          | 2.8        | 5.7        |
| G707A    | 1.2          | 1.9        | 5.7        |
| L708A    | 1.0          | 2.1        | 5.7        |
| I710A    | 1.4          | 2.5        | 5.6        |
| A711S    | 1.3          | 2.0        | 5.6        |
| I712A    | 1.0          | 2.3        | 5.7        |
| L713A    | 1.0          | 2.2        | 5.7        |
| Part B   |              |            |            |
| Wild type| 1.1          | 1.8        | 5.7        |
| Y694G    | 0.9          | 59.6       | 5.4        |
| S699C    | 0.5          | 7.5        | 5.4        |
| S699T    | 1.8          | 3.6        | 5.7        |
| E703D    | 0.8          | 7.3        | 5.7        |

* van, vanadate.

expression, 39% hydrolysis, 40% transport) was less well tolerated than the substitution by Ala (95% expression, 105% hydrolysis, 92% transport), there was sufficient activity in both cases to carry out a quantitative study of H⁺ pumping over a range of MgATP concentrations. The results are illustrated in Fig. 3. As reported earlier (25), the relationship between the initial rate of acridine orange fluorescence quenching and the rate of hydrolysis was approximately linear in the wild-type control. Significantly, the data points for E703A and E703D fell along the same straight line, consistent with normal coupling between H⁺ pumping and ATP hydrolysis.

Conformational Analysis by Limited Trypsinolysis—As described above, three mutations in M5 led to a virtually complete block in Pma1 biogenesis: R695A, H701A, and L706A. Because it seemed possible that these proteins might be structurally abnormal, they were examined by limited trypptic digestion. Total membranes were isolated from labeled cells expressing either mutant or wild-type ATPase and incubated at a trypsin/protein ratio of 1:20 for 0, 1, or 5 min (Fig. 4). Under these conditions, the 100-kDa wild-type enzyme underwent minor proteolytic cleavage, yielding a 97-kDa band, whereas the three mutants were completely degraded in <1 min. Thus, alanine substitutions of Arg-695, His-701, and Leu-706 appear to cause severe misfolding, interfering with the ability of the newly synthesized ATPase to move through the secretory pathway.

Two other mutants were studied by limited trypsinoysis: Y691A and S699A. Both were well expressed in secretory vesicles (92 and 90%, respectively), but Y691A had no detectable ATPase activity, and S699A had very low activity (8%). To examine the folding of the mutant proteins, vesicles were incubated at a trypsin/protein ratio of 1:4 in the absence of ligands (Fig. 5A). Under these conditions, the 100-kDa wild-type ATPase was rapidly converted to the 97-kDa form, which in turn was digested to smaller fragments. Significantly, Y691A and S699A were no more trypsin-sensitive than the wild-type enzyme (Fig. 5A), consistent with their ability to undergo normal trafficking. However, a difference between the two mutants became apparent in the experiment of Fig. 5B, which explored the ability of ligands to protect against trypptic degradation. MgADP and MgATP (which should hold the enzyme in the E₁ conformation) protected 100-, 97-, and 62-kDa protein fragments in the wild-type and both mutant ATPases, whereas vanadate (which should pull the enzyme into the E₂ conformation) protected 97- and 80-kDa fragments in the wild-type ATPase activity.
type and S699A ATPases, but not in Y691A. Thus, like its neighbor (V692A), Y691A appears to be defective in the E1-to-E2 conformational change.

Coexpression of M5 Mutants with Wild-type PMA1—Wach et al. (29) have previously shown that Asp, Gln, and Arg substitutions of His-701 behave as dominant lethal mutations. In a similar series of experiments, we subcloned H701A, R695A, and L706A into plasmid YCplac33 (30) and placed the mutant alleles under GAL1 control. Each of the plasmids was then transformed into yeast strain NY605, which carries a wild-type PMA1 gene on the chromosome. When the resulting cells were plated on glucose-containing medium, where only the constitutive wild-type gene could be expressed, all of them grew normally. By contrast, on galactose-containing medium, where the mutant gene was also expressed, no growth was observed for cells transformed with H701A, R695A, or L706A. This dominant lethal phenotype indicates that the abnormal proteins interfere with the processing of coexpressed wild-type ATPase, as has previously been shown for H701D, H701Q, H701R, and a number of other PMA1 mutations (26, 29, 31–33).

DISCUSSION

In analyzing the data from this study, the sequence alignment of Fig. 6 can serve as a useful guide. It illustrates a modest degree of evolutionary conservation along membrane segment 5 of the P-ATPases and highlights six residues that are discussed in detail below.

Tyr-694 lies near the cytoplasmic boundary of M5 and is present in all known P-type H+ pumps from fungi, algae, higher plants, and protozoans. Other members of the family have a similar amino acid residue at this position (usually Tyr or Phe; occasionally Trp, Met, or Leu), except for the Cta3 Ca2+-ATPase of Schizosaccharomyces pombe, where there is a His. It is now clear that Tyr-694 plays a key role in the reaction cycle of the yeast Pma1 H+ ATPase since ATP hydrolysis decreased substantially in Y694A and Y694G, and ATP-dependent H+ transport was barely detectable. In the mammalian SERCA ATPase, as mentioned above, mutation of the corresponding Tyr to Gly led to the uncoupling of Ca2+ transport from ATP hydrolysis (28); and in the Na+,K+-ATPase, the same substitution led to inactivation of the enzyme (34). Thus, there appears to be a nearly uniform requirement for a bulky, hydrophobic amino acid at the position of Tyr-694.

Ser-699 is another residue displaying significant, although not complete, evolutionary conservation (Fig. 6). All of the known P-type H+ ATPases contain either Ser or Thr at this position, and there is a Ser only one residue away in the mammalian Na+,K+- and SERCA Ca2+-ATPases, the MgtB ATPase of Salmonella typhimurium, and the Cta3 ATPase of S. pombe. Clear evidence has been put forward for the functional importance of this Ser in the Na+,K+-ATPase, where mutation to Ala or Cys produced a severalfold decrease in the Vmax for ATP hydrolysis and a 13–31-fold increase in the Km for K+; as a result, cells expressing the altered pump required higher than normal extracellular K+ for growth (14). By contrast, Ser-to-Ala and Ser-to-Cys mutations had little effect on either the activity or the Ca2+ affinity of the SERCA ATPase (35). In this study, yeast Pma1 ATPase containing the S699A mutation traveled efficiently to the secretory vesicles, and based on limited trypsinolysis, it was well folded and able to bind MgATP.

![FIG. 3. Energy coupling in Glu-703 mutants. The initial rate of fluorescence quenching (proton transport) was determined over a range of ATP concentrations (0.3–3.0 mM) and is plotted as a function of vanadate-sensitive ATPase activity (ATP hydrolysis) measured under the same conditions as described previously (25).](image)

![FIG. 4. Trypsinolysis of R695A, H701A, and L706A. 35S-Labeled total membranes were incubated at a trypsin/protein ratio of 1:20 for 0–5 min at 30 °C. The reaction was terminated by the addition of 1 mM diisopropyl fluorophosphate. Pma1 protein was immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis and fluorography. WT, wild type.](image)

![FIG. 5. Trypsinolysis of Y691A and S699A. Secretory vesicles were incubated at a trypsin/protein ratio of 1:4 at 30 °C, followed by SDS-polyacrylamide gel electrophoresis and immunoblotting. A, time course of trypsinolysis from 0 to 20 min. B, effect of 100 μM vanadate, 10 mM MgADP, or 10 mM MgATP on trypsinolysis (carried out for 20 min). WT, wild type.](image)
MgADP, and orthovanadate; however, it had exceedingly low ATPase activity and no detectable ATP-dependent proton pumping. The S699C and S699T enzymes were more active, allowing them to be studied in greater detail. S699T appeared normal in every respect, but S699C displayed a modest acid shift in the pH dependence of ATP hydrolysis (0.2 units). While the shift does not necessarily reflect a lowered affinity for H⁺ at a cation-liganding site in the translocation pathway, this is clearly one possibility. If so, the effect is substantially smaller than the change seen in the Na⁺,K⁺-ATPase, the corresponding glutamate (either Glu-779 or Glu-781, depending upon the species) plays a less critical role since substitution by Ala, Asp, Gln, or Arg failed to disrupt two sequentially filled Ca²⁺-binding sites (7, 38). In the Na⁺,K⁺-ATPase (14), where recent work by Blostein et al. (36) has provided direct evidence that Ser-775 is either a cation-liganding residue or part of a gating structure close to the liganding sites. The difference between the two enzymes may be related to the immediate downstream sequences, which are LHLE in the case of the H⁺,K⁺-ATPase and NlPE in the Na⁺,K⁺-ATPase. As emphasized in a recent study (37), a proline residue leaves the backbone carbonyl at position 4 without a hydrogen donor and introduces a kink that disrupts hydrogen bonding between position 3 (Ser-775) and position +1. Thus, the presence of a Pro in the Na⁺,K⁺-ATPase would free up backbone carbonyls that might contribute to cation binding; it would also increase the local flexibility of the polypeptide chain, which in turn could influence conformational interactions with other parts of the protein.

Glu-703 is a third residue that deserves careful attention. In the mammalian SERCA ATPase, replacement of the corresponding glutamate (Glu-771) by an uncharged amino acid (Glu-779, Glu-781) results in a lower affinity for H⁺ at a cation-liganding site (38). In the Na⁺,K⁺-ATPase, the corresponding glutamate (either Glu-779 or Glu-781, depending upon the species) plays a less critical role since substitution by Ala, Asp, Gln, or Arg failed to disrupt function (10–12). However, the Ala mutant did display a severalfold decrease in the apparent affinity for K⁺ and Na⁺ (11, 12), along with the disappearance of voltage dependence in patch clamp experiments, suggesting that this residue may line the cation access channel (13). In the yeast Pma1 H⁺-ATPase, on the other hand, there is no demonstrable role for Glu-703. Replacement by an uncharged amino acid (E703A) had little or no effect on biogenesis, ATPase activity, or the rate of ATP-dependent proton pumping. Furthermore, a detailed look at the relationship between H⁺ pumping and ATP hydrolysis in E703A and E703D gave no evidence for a defect in coupling. It may therefore be significant that Glu-703 is replaced by Val or Ala in heavy metal-transporting ATPases (Fig. 6).

Finally, Arg-695, His-701, and Leu-706 are at least structurally important for the Pma1 ATPase since replacement by Ala led to trypsin sensitivity (misfolding), failure to move to the secretory vesicles, and a dominant lethal phenotype. In the case of His-701, this result corroborates earlier work by Wach et al. (29), who showed that H701R, H701Q, and H701E behave...
as dominant lethal mutations. Furthermore, although Arg-695, His-701, and Leu-706 are poorly conserved among the P-type ATPases, position 706 is occupied by Ile in the SERCA ATPase, where mutation to Ala has been reported to produce a nonfunctional enzyme (35).

To place these and the rest of the data in a useful context, Fig. 7 provides a helical wheel diagram for M5 of the yeast H\(^{+}\)-ATPase. It is perhaps significant that the structurally and functionally important amino acids identified in this study are seen to lie along one face of the putative α-helix. Included are three residues required for proper ATPase folding and biogenesis (Arg-695, His-701, and Leu-706), three residues at which Ala substitutions inactivate the ATPase (Tyr-694, Trp-709, and Ser-699), and two residues at which mutations lead to complex changes in ligand binding and/or the equilibrium between \(E_1\) and \(E_2\) conformations (Tyr-691, based on the inability of vanadate to protect against trypsinolysis, and Val-692, based on simultaneous shifts in several kinetic parameters).

Taken together, although the results of this study give no firm evidence for a transport site in M5 of the yeast ATPase, they do indicate that M5 plays a significant role in the reaction cycle. As progress is made toward solving the structure of the closely related Neurospora H\(^{+}\)-ATPase (40), the mutational data should provide helpful insight into structure-function relationships.

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