Functional Role of Charged Residues in the Transmembrane Segments of the Yeast Plasma Membrane H\(^+\)-ATPase*

Received for publication, January 20, 2000, and in revised form, February 10, 2000
Published, JBC Papers in Press, March 9, 2000, DOI 10.1074/jbc.M000546200

Valery V. Petrov‡, Kristine P. Padmanabha, Robert K. Nakamoto§, Kenneth E. Allen, and Carolyn W. Slayman

From the Departments of Genetics and Cellular & Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06510

As defined by hydropathy analysis, the membrane-spanning segments of the yeast plasma membrane H\(^+\)-ATPase contain seven negatively charged amino acids (Asp and Glu) and four positively charged amino acids (Arg and His). To explore the functional role of these residues, site-directed mutants at all 11 positions and at Glu-288, located near the cytoplasmic end of M3, have been constructed and expressed in yeast secretory vesicles. Substitutions at four of the positions (Glu-129, Glu-288, Asp-833, and Arg-857) had no significant effect on ATP hydrolysis or ATP-dependent proton pumping, substitutions at five additional positions (Arg-695, His-701, Asp-730, Asp-739, and Arg-811) led to misfolding of the ATPase and blockage at an early stage of biogenesis, and substitutions of Asp-143 allowed measurable bio­genesis but nearly abolished ATP hydrolysis and proton transport. Of greatest interest were mutations of Glu-703 in M5 and Glu-803 in M8, which altered the apparent coupling between hydrolysis and transport. Three Glu-703 mutants (E703Q, E703L, E703D) showed significantly reduced pumping over a wide range of hydrolysis values and thus appeared to be partially uncoupled. At Glu-803, by contrast, one mutant (E803N) was almost completely uncoupled, while another (E803Q) pumped protons at an enhanced rate relative to the rate of ATP hydrolysis. Both Glu-703 and Glu-803 occupy positions at which amino acid substitutions have been shown to affect transport by mammalian P-ATPases. Taken together, the results provide growing evidence that residues in membrane segments 5 and 8 of the P-ATPases contribute to the cation transport pathway and that the fundamental mechanism of transport has been conserved throughout the group.

The plasma membrane H\(^+\)-ATPase of yeast, which is encoded by the PMA1 gene (1), belongs to the large and physio­logically versatile family of P-type ATPases found throughout prokaryotic and eukaryotic evolution (12). Thus, there are ample structural differences in the membrane-embedded portion of the P-ATPases that may underlie the known functional role of charged residues within the 10 transmembrane segments of the yeast plasma membrane H\(^+\)-ATPase. Multiple substitutions were made at each of these positions by site-directed mutagenesis. The resulting ATPases were then expressed in yeast secretory vesicles (13) and examined for the ability to undergo proper biogenesis, hydrolyze ATP, and pump protons. Nearly half of the mutant ATPases proved to be misfolded, as judged by limited trypsinolysis, and were arrested at an early point in the secretory pathway. Of the remainder, many were functionally normal, but mutants of Glu-703 in membrane segment 5 and Glu-803 in membrane segment 8 displayed apparent alterations in the coupling between ATP hydrolysis and H\(^+\) pumping. As described below, these two residues are conserved in other P-ATPases, where mutational evidence also suggests a role in the cation transport pathway.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Both the Amersham kit for oligonucleotide-directed mutagenesis (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) and the polymerase chain reaction (14) were used to introduce mutations into PMA1 gene fragments (596-bp Clai to BstEII; 615-bp BstEII to EcoRI; 519-bp BgIII to Sall; 1769-bp BgIII to

1 The abbreviations used are: bp, base pair(s); TEA, triethanolamine-acetic acid; DCCD, N,N\(^{-}\)-dicyclohexylcarbodiimide; MES, 4-morpholineethanesulfonic acid.
SacI; Ref. 1) that had previously been subcloned into a modified version of Bluescript (Stratagene, La Jolla, CA). Each fragment was sequenced to verify the presence of the mutation and the absence of unwanted base changes, and moved into the full-length PMA1 gene in plasmid pPM1A1.2 (13). A 3770-bp HindIII to SacI piece containing the entire coding sequence of the gene was then transferred to the centromeric plasmid YCp2HSE, placing the mutated gene under heat shock control; the resulting plasmids were transformed into strain SY4 of Saccharomyces cerevisiae (13).

Expression Studies: Preparation of Secretory Vesicles—In strain SY4 (MATa, ura3–52, leu2–3, 112, his3–11, 19, ade2–0, 1;Gal, pma1::YEpGAL-PMA1; Ref. 13), the chromosomal PMA1 gene has been placed under control of the GAL1 promoter (pGAL-PMA1) by the gene disruption strategy of Cid et al. (15). SY4 also carries a temperature-sensitive mutation in the SEC6 gene, blocking the last step in plasma membrane biogenesis and leading to the accumulation of secretory vesicles (16). For expression studies, SY4 cells transformed with the desired plasmid were grown to mid-exponential phase (A600 = 0.7–1.2) on supplemented minimal medium containing 2% galactose at 23 °C, shifted to medium containing 2% glucose for 3 h to turn off expression of the chromosomal copy of the PMA1 gene, and then shifted to 37 °C for 2 h to turn on expression of the plasmid-borne allele.

Secretory vesicles containing newly synthesized plasmid-encoded ATPase were isolated by differential centrifugation and further purified by gel filtration (13, 17) or gradient centrifugation (18, 19). In both cases, cells were converted to spheroplasts and lysed by homogenization in 0.4 M sorbitol, 10 mM triethanolamine-acetic acid (TEA), pH 7.2, 1 mM EDTA, 1 mM diisopropyl fluorophosphate, 2 μg/ml chymostatin, and 1 μg/ml leupeptin, pepstatin, and aprotinin (for gel filtration or sorbitol gradient centrifugation) or in the same buffer containing 12.5% sucrose (for sucrose gradient centrifugation). After centrifugation at 14,500 × g for 10 min to remove debris, the supernatant fraction was spun at 160,000 × g for 35 min to collect a crude secretory vesicle fraction, which was suspended in 0.8M sorbitol, 10 mM TEA, pH 7.2, 1 mM EDTA (for gel filtration or sorbitol gradient centrifugation) or 12.5% sucrose, 10 mM TEA, pH 7.2, 1 mM EDTA (for sucrose gradient centrifugation).

Both resuspension buffers contained protease inhibitors (10 mM NaF, 0.75 mM EDTA, 0.7–1.2 M acrylamide, acrylamide, and ATP (0.2–3.0 mM), and after stabilization of base-line fluorescence (120–150 s), proton pumping was initiated by the addition of MgCl2 (5.2–8.0 mM). Parallel measurement of ATPase activity under the same conditions was performed in 100–150 μl of 0.6 M sorbitol, 20 mM HEPES-KOH, pH 6.7, 100 mM KCl, 2 μM acridine orange, and ATP (0.2–3.0 mM), and after stabilization of base-line fluorescence (120–150 s), proton pumping was initiated by the addition of MgCl2 (5.2–8.0 mM). The reaction was stopped with 1 ml of 1.25% trichloroacetic acid, and inorganic phosphate was measured as above.

RESULTS

Choice of Residues to Be Studied—Fig. 1 illustrates the 10 postulated transmembrane segments of the yeast plasma membrane H+–ATPase. The present study has focused on seven negatively charged residues (Glu-129, Asp-143, Glu-703, Asp-739, Asp-803, Asp-833, and Arg-857) and four positively charged residues (Arg-625, His-701, Arg-811, Arg-857) located within these segments. Glu-288, which lies close to the cytoplasmic end of M3, was also included. In each case, site-directed mutagenesis was used to replace the charged amino acid by two different neutral amino acids of roughly equivalent size (Asp by Asn or Val; Glu by Gln or Leu; Arg by Leu or Met; His by Gln or Leu), and the resulting ATPase was expressed in yeast secretory vesicles (13).
Expression and ATP Hydrolysis—Because it seemed likely that some of the mutant ATPases might be structurally altered in a way that would prevent normal biogenesis, isolated secretory vesicles were examined by quantitative immunoblotting with anti-ATPase antibody. Indeed, of the 24 mutants examined in the initial set, 11 showed major defects in biogenesis (R695L, R695M, H701L, H701Q, D730N, D730V, D739N, D739V, E803L, R811L, and R811M), with ATPase protein reaching the vesicles at only 1–13% of the level observed for the wild-type enzyme (Table I).

Previous work has revealed that mutations at two of these positions, Arg-695 and Asp-730, lead to severe misfolding of the ATPase, greatly increasing the sensitivity of the 100-kDa protein to trypsin (27). As illustrated in Fig. 2, mutations at His-701 and Asp-739 had a similar effect. In this experiment, 35S-labeled total membranes were incubated at a trypsin:protein ratio of 1:20 and immunoprecipitated with anti-ATPase antibody. By contrast with the wild-type ATPase, in which a major 97-kDa fragment appeared at 0.5 min and remained at 1.0 min, H701Q and D739V were completely degraded by 0.5 min, with no trace of the 97-kDa band; similar results were obtained for H701L and D739N (data not shown). Thus, as with Arg-695 and Asp-730, His-701 and Asp-739 appear to play a critical role in protein folding.

Of the remaining mutants, three (D143N, D143V, D833N) reached the secretory vesicles in reduced but measurable amounts and gave very low rates of ATP hydrolysis (8–13%). It is interesting to note that at least two members of this group were relatively resistant to limited trypsinolysis, even though the 97-kDa band was not seen (D143V, illustrated in Fig. 2; D143N, data not shown).

The rest of the mutants (E129Q, E129L, E288Q, E288L, E703Q, E703L, E803Q, D833V, R857M, R857L) were well expressed in the vesicles (71–111%) and hydrolyzed ATP at a sufficient rate (37–123%) to be studied in detail.

Kinetic Properties—A simple way to screen for qualitative changes in mutant H^+-ATPases is to measure several standard kinetic properties including the $K_m$ for MgATP, $IC_{50}$ for orthovanadate, and pH optimum. When this was done for the 10

![Fig. 2. Limited trypsinolysis of wild-type (WT) and mutant ATPases.](image)

$^{*}$ The specific expression of 100-kDa ATPase protein was calculated by quantitative immunoblotting as described under “Experimental Procedures.”  

$^a$ ATP hydrolysis was measured as outlined under “Experimental Procedures.” This series of experiments included 62 wild-type preparations with an average ATPase activity of 4.25 ± 0.21 µmol of P/min · mg. Data for mutant ATPases are the average of 4–12 determinations except in the case of D730N, D730V, D739N, and D769V, for which there were two determinations; each mutant value was corrected for expression relative to a wild-type control run in parallel on the same day.

$^b$ Secretory vesicles were isolated from cells containing the expression plasmid YCp2HSE with the wild-type PMA1 gene (positive control).

$^c$ Secretory vesicles came from cells carrying the plasmid with no PMA1 gene (negative control). Asterisks indicate that no corrections were made for preparations with low expression and/or ATPase activity.
mutants listed above, only minor differences were observed from the wild-type control (Table II). Thus, there is no evidence for significant defects in the ATP or vanadate binding sites, which are presumably located in the cytoplasmic portion of the molecule. Equally important, there does not seem to be any appreciable shift in the equilibrium between the $E_1$ and $E_2$ conformational states of the enzyme, since such a shift would be expected to result in a coordinated change of $K_m$, $K_v$, and pH optimum (for further discussion, see Ref. 28).

$H^+$ Pumping—To ask whether any of the charged residues are essential for proton pumping, the 10 enzymatically active mutants were further assayed for their ability to carry out ATP-dependent $H^+$ transport. In each case, the initial rate of acridine orange fluorescence quenching was measured over a range of MgATP concentrations, and the rate of quenching was plotted as a function of the rate of ATP hydrolysis, measured in parallel under the same conditions.

For the wild type ATPase, as observed previously (28), there was a linear relationship between the two rates (Fig. 3), consistent with the idea that the stoichiometry of proton transport remained roughly constant over the entire range of pump velocities. Seven of the mutants gave data points that superimposed with those seen in the wild-type control (Fig. 3). For E703Q and E703L, however, the slope of quenching versus ATPase activity was significantly lower than in the wild type, suggesting that proton transport was partially uncoupled from ATP hydrolysis (Fig. 4). Surprisingly, one mutant (E803Q) gave an increased slope, consistent with the idea that proton transport was enhanced relative to the rate of hydrolysis (Fig. 4).

It was important to ask whether any difference in proton leak could be detected in secretory vesicles containing the Glu-703 and Glu-803 mutant ATPases. To address this question, the MgATP concentration was adjusted to give approximately equal maximal values of $p$H in E803Q and wild-type vesicles (1 and 3 mM MgATP, respectively) and in E703Q and wild-type vesicles (3 and 1 mM MgATP, respectively), and the time courses of ATP hydrolysis and acridine orange fluorescence quenching were compared in detail. In each case, hydrolysis was linear for at least 12 min (Fig. 5, bottom panels), while quenching reached a maximum within 1–2 min (Fig. 5, top panels); at this point, the inward pumping of protons was presumably balanced by the outward leak. At the steady state, the pump was then turned off by addition of 250 $\mu M$ vanadate (Fig. 5, top) or 0.5 mg of hexokinase + 1% glucose (data not shown). In both mutants, the pH gradient decayed with a time course very similar to that seen in the corresponding wild-type control. Thus, there is no evidence that the passive permeability of the secretory vesicle membrane is different in the mutants; rather, the pumps themselves appear to be altered in the coupling between proton transport and ATP hydrolysis, with “undercoupling” in E703Q and “overcoupling” in E803Q.

**Additional Mutations of Glu-703, Asp-730, and Glu-803—** From the data described thus far, Glu-803 clearly emerged as an intriguing residue; replacement by Gln produced an enzyme with enhanced proton pumping, while replacement by Leu blocked biogenesis. To gain a better understanding of structure-function relationships at this position, five additional mutants were constructed and analyzed (Table III). The ability of the mutants to be expressed in secretory vesicles ranged widely, from 8% in E803S to 24% in E803D, 45% in E803C, 82% in E803N, and 93% in E803R, showing no obvious correlation with the size, charge, or hydrophobicity of the substituted amino acid. In the mutants that were expressed sufficiently well to allow a reliable assay of ATP hydrolysis, activity was undetectable in E803R, barely detectable in E803C, and reasonably good in E803N (45% of the wild-type control after correction for expression; Table III). There were no significant abnormalities of $K_m$ or pH optimum in E803N (Table II). Interestingly, however, the comparison of acridine orange fluorescence quenching with ATP hydrolysis over a range of MgATP concentrations revealed that E803N was very poorly coupled (Fig. 4). Thus, Glu-803 is a residue at which substitutions can either increase or decrease the apparent coupling between hydrolysis and transport.

For Glu-703, two previously constructed mutants (E703D and E703A) were examined. Like E703Q and E703L, E703D appeared partially uncoupled (Fig. 4). Significantly, however, E703A was capable of normal rates of proton transport throughout the entire range of MgATP concentrations.

An additional mutant was also constructed at position 730, where replacement of Asp by Asn or Val had led to a complete block in biogenesis. As shown in Table III, replacement by Glu (D730E) allowed a measurable amount of ATPase (48%) to reach the secretory vesicles, but the rate of ATP hydrolysis was not high enough to permit a detailed study of kinetic parameters or proton pumping.

**DISCUSSION**

As described in the Introduction, the goal of the present study was to determine the functional role of charged residues in the transmembrane segments of the yeast PMA1 $H^+\text{-ATPase.}$ The results provide helpful insights into the mechanism by which protons are pumped across the membrane and also into the folding and biogenesis of the ATPase polypeptide; both topics will be discussed in turn.

**Mechanism of Proton Transport: Role of Charged Residues in M4, M5, M6, and M8—** Several laboratories including our own are making vigorous efforts to map the transport pathway of representative P-ATPases and to understand the determinants of cation specificity and stoichiometry. Although neither question has yet been answered in detail, there has been encouraging progress. As described in the Introduction, cryoelectron microscopy of the Neurospora $H^+\text{-ATPase}$ (10) and the sarcoplasmic reticulum Ca$^{2+}\text{-ATPase}$ (11) has led to the visualization of 10 membrane-spanning $\alpha$-helices, tilted at angles of $<5^\circ$ to $34^\circ$ from the plane of the lipid bilayer. Indeed, the arrangement of helices is very similar in the two enzymes, even though the $H^+\text{-}$ and Ca$^{2+}\text{-ATPases}$ share only 27% sequence identity (29). High resolution structures are expected soon and will allow the membrane-spanning helices to be identified directly. In the meantime, mutagenesis data from the sarcoplasmic reticulum Ca$^{2+}\text{-ATPase}$ and the Na$^+\text{-K}^+\text{-ATPase}$ make it likely that M4, M5, M6, and M8 actually flank the cation transport...
pathway (29) since they contain polar residues that are required for high affinity cation binding/occlusion and cation transport (30–43). Three of the required residues (Glu in M4, Glu in M5, Asp in M6) are shared by the Na\(^+\),K\(^+\) - and Ca\(^{2+}\)-ATPases, while a second Asp in M6 is found only in the former enzyme and a Glu in M8, only in the latter.

Fig. 6 compares the yeast H\(^+\)-ATPase with its mammalian counterparts at each of these positions. The most conspicuous difference is in M4, where a valine (Val-336) appears in the yeast enzyme at the position occupied by Glu-327/Glu-309 in the Na\(^+\),K\(^+\)- and Ca\(^{2+}\)-ATPases. When the functional role of Val-336 was explored as part of a previous study of the M4 segment (28), substitution by Ala led to an ensemble of kinetic changes that could most easily be explained by a shift in equilibrium from the \(E_2\) to the \(E_1\) conformation. Proton transport was normally coupled to ATP hydrolysis in V336A, however, and two other mutants, V336L and V336E, showed no significant abnormalities in kinetic behavior or in coupling. Thus, although Val-336 may affect the overall reaction mechanism of the H\(^+\)-ATPase (28), it does not play an integral role in proton transport.

In M5, Glu-703 of the yeast H\(^+\)-ATPase is homologous to Glu-779/Glu-771 of the mammalian ATPases. As described above, substitutions of Glu-703 by Gln, Leu, or Asp led to partial uncoupling of the yeast ATPase, although ATP-dependent proton pumping could still be detected; substitution by Ala had no measurable effect. Thus, Glu-703 is not absolutely required for transport, but it has a significant influence on the functioning of the transport pathway. The homologous glutamyl residue plays a more critical role in the Na\(^+\),K\(^+\)- and sarcoplasmic reticulum Ca\(^{2+}\)-ATPases. Studies on the former enzyme have shown that the carboxyl-selective reagent 4-(diazo- methyl)-7-(diethylamino)-coumarin disrupts K\(^+\) and Na\(^+\) binding upon reaction with Glu-779 (44), that mutation of Glu-779 to Ala greatly reduces the voltage dependence of the Na\(^+\),K\(^+\)-ATPase (38), and that mutation to Asp eliminates high affinity Rb\(^+\) and Tl\(^+\) occlusion (42). In the latter enzyme, mu-
1.37 under "Experimental Procedures." Rates of fluorescence quenching, as described was assayed at 29 °C under the conditions E803Q, and E703Q ATPases. Hydrolysis course of ATP hydrolysis by wild-type, (cretory vesicles containing wild-type ing by wild-type and E803Q secretory ves-"m atoms, or by wild-type and E703Q secretory vesicles at 1 and 3 mM ATP, respec-

tively (left), or by wild-type and E703Q secretory vesicles at 1 and 3 mM ATP, respec-
tively (right). At the steady state, the pump was turned off by the addition of 250 μm vanadate. Bottom panels, time course of ATP hydrolysis by wild-type, E803Q, and E703Q ATPases. Hydrolysis was assayed at 29 °C under the conditions of fluorescence quenching, as described under "Experimental Procedures." Rates of ATP hydrolysis were as follows: left, 1.37 μmol of P/min/mg for E803Q at 1 mM MgATP, and 2.07 μmol of P/min/mg for wild type at 3 mM MgATP; right, 0.77 μmol of P/min/mg for wild type at 1 mM MgATP, and 1.00 μmol of P/min/mg for E703Q at 3 mM MgATP.

**FIG. 5. Passive permeability of secretory vesicles containing wild-type (WT) and mutant ATPases. Upper panels, acridine orange fluorescence quenching by wild-type and E803Q secretory vesicles at 3 and 1 mM ATP, respectively (left), or by wild-type and E703Q secretory vesicles at 1 and 3 mM ATP, respectively (right). At the steady state, the pump was turned off by the addition of 250 μm vanadate. Bottom panels, time course of ATP hydrolysis by wild-type, E803Q, and E703Q ATPases. Hydrolysis was assayed at 29 °C under the conditions of fluorescence quenching, as described under "Experimental Procedures." Rates of ATP hydrolysis were as follows: left, 1.37 μmol of P/min/mg for E803Q at 1 mM MgATP, and 2.07 μmol of P/min/mg for wild type at 3 mM MgATP; right, 0.77 μmol of P/min/mg for wild type at 1 mM MgATP, and 1.00 μmol of P/min/mg for E703Q at 3 mM MgATP.**

**TABLE III**

| Mutation | Membrane segment | Expression | ATP hydrolysis† | Uncorrected | Corrected |
|----------|------------------|------------|----------------|-------------|-----------|
| Wild type | M6              | 100        | 4.64          | 100         | 100       |
| D730E    | M6              | 48         | 0.47          | 15          | *         |
| E803D    | M8              | 24         | 0.38          | 9           | *         |
| E803N    | M8              | 82         | 2.09          | 37          | 45        |
| E803S    | M8              | 45         | 0.22          | 5           | *         |
| E803C    | M8              | 45         | 1.05          | 14          | *         |
| E803R    | M8              | 93         | 0.38          | 5           | *         |

†This series of experiments included 21 preparations of wild-type vesicles with an average ATPase activity of 4.64 ± 0.26 μmol of P/min⋅mg. Data for mutant ATPases are the average of two determinations except in the case of D730E and E803N, for which there were 7–10 determinations; each mutant value was corrected for expression relative to a wild-type control run in parallel on the same day. Asterisks indicate no corrections were made for preparations with low expression and/or ATPase activity. For other details, see Table I and "Experimental Procedures."
Charge residues in transmembrane segments of P-type ATPases. Included are the yeast plasma membrane H^+ -ATPase, the sheep Na^+ ,K^+ -ATPase, and the rabbit sarcoplasmic reticulum Ca^{2+} -ATPase (Gen-Bank accession nos. X03534, P20648, and M12898, respectively). The circled residues have been implicated in cation transport by the Na^+ ,K^+ -ATPase (32–37, 41, 42), Ca^{2+} -ATPase (30, 31, 40, 43, 45), and H^+ -ATPase (this study).

Functional Role of Charged Residues in Other Membrane Segments—The present study has shown that four other charged residues (Glu-129 in M1; Glu-288, close to the cytosolic end of M3; Asp-833 in M9; and Arg-857 in M10) are clearly not required for ATP-dependent proton pumping. Among this group, Glu-129 deserves special mention. In previous work on the closely related plasma membrane H^+ -ATPase of N. crassa, Sussman and Slayman (50) found that the enzyme was inactivated by the hydrophobic carboxyl reagent N,N'-dicyclohexyl-carbodiimide (DCCD) at an apparent stochiometry of 0.4 mol/ mol of polypeptide, consistent with the idea that the inhibitor was reacting with single amino acid residue. In a subsequent study, the site of inhibition was provisionally identified as Glu-129 in M1, based on labeling with [14C]DCCD (51). To follow up these results, we have recently examined the sensitivity of the yeast PMA1 H^+ -ATPase to DCCD, taking advantage of the above-described mutants in which Asp and Glu residues throughout the transmembrane segments were substituted one at a time by neutral amino acids (52). The pattern of DCCD sensitivity proved to be quite complex in the mutants, with fractional reductions in rate constants in E129Q and E129L, D143S, D143V, and E703Q and E703L. The simplest explanation was that DCCD could react in parallel at three sites to inhibit ATPase activity. As described above, the homologue of Glu-703 in the Na^+ ,K^+ -ATPase (Glu-779) has been shown by Arguello and Kaplan (44) to be labeled by the fluorescent carbonyl-selective reagent, 4-(diazomethyl)-7-(diethylamino)-coumarin. Until similar labeling studies are carried out for the yeast H^+ -ATPase, it remains possible that Glu-129, Asp-143, and Glu-703 are not themselves targeted by carboxyl reagents, but that mutations at these positions reduce reactivity at one or more sites elsewhere in the enzyme. In any event, it seems clear that the fungal P-type H^+ -ATPases interact with DCCD in a considerably more complex way than the structurally unrelated F_o,F_1 ATPases, where Fillingame and others (53, 54) have established that the aspartyl residue involved in proton transport (Asp-61 of subunit c) is also the major site of inhibition by DCCD.

Charged Residues Involved in Protein Folding and Biogenesis—Not unexpectedly, five of the residues examined in the present study (Arg-695, His-701, Asp-730, and Arg-811) proved to be necessary for proper protein folding and biogenesis. Replacement with a neutral amino acid at any of these positions resulted in a polypeptide that was highly sensitive to trypsin and unable to move along the secretory pathway. Consistent findings for His-701 have been reported by Wach et al. (55), who showed that H701D, H701Q, and H701R behaved genetically as lethal mutations.

As mentioned above, the basis for the folding defect is clear for mutants at two of the five positions. A previous study from our laboratory has provided evidence that Arg-695 in M5 and Asp-730 in M6 form a salt bridge, presumably helping to stabilize the M5-M6 hairpin during its insertion into the membrane (27). Single substitutions of either Arg-695 or Asp-730 gave a trypsin-sensitive, nonfunctional protein, but double mutants in which the two charges had been swapped (R695D/D730R) or eliminated (R695A/D730A) were once again resistant to trypsin, traveled normally to the secretory vesicles, and displayed near-normal rates of ATP hydrolysis and ATP-dependent proton pumping. Early in the present study, inspection of the topological model in Fig. 1 raised the possibility that Asp-739 in M6 might form a similar charge pair with Arg-811 in M8. In this case, however, additional experiments (data not shown) revealed that the corresponding double mutants (D739R/R811D and D739A/R811A) were still blocked in their ability to move to the secretory vesicles, indicating that the folding problem had not been overcome. Thus, based on presently available data, there is no evidence for a salt bridge between M6 and M8 of the yeast ATPase, and the structural role of Asp-739 and Arg-811 (like that of His-701) remains to be determined.

An additional residue that deserves mention is Asp-143 in M2. Mutations at this position reduced but did not totally block biogenesis, and, consistent with this fact, D143N and D143V...
were relatively resistant to trypsin. They were barely able to hydrolyze ATP, however, and proton transport (if any) fell below the limit of detection by the acridine orange assay. Furthermore, Seto-Young et al. (56) found that D143A, D143N, and even D143E acted as lethal mutations when expressed in intact cells. Thus, like His-701, Asp-739, and Arg-811, where mutations could not be examined for possible effects on proton transport owing to defects in protein folding and biogenesis, Asp-143 remains on the list of residues that may conceivably contribute to the proton translocation pathway.

Conclusions—The data presented in this study serve to strengthen the idea that M5 and M8 play a direct role in cation transport by the PMA1 H⁺-ATPase, presumably bundling together with other membrane-spanning helices (such as M4 and M6) to form the actual transport pathway. If, as suggested above, the H⁺-ATPase pumps hydronium ions rather than protons, then the fundamental mechanism of transport may be the same as in mammalian Na⁺, K⁺-, and Ca²⁺-ATPases, where multiple polar residues from M4, M5, M6, and M8 are thought to ligand the transported cations as they move across the membrane. From the behavior of certain H⁺-ATPase mutants (E703Q, E703L, and E703D in M5, E803Q and E803N in M8), it appears that residues lining the transport pathway may dictate the stoichiometry as well as the velocity and specificity of transport; further work will be needed to examine this possibility.

Acknowledgment—We are grateful to Philippe Male for help with illustrations.

REFERENCES

1. Serrano, R., Kielland-Brandt, M. C., and Fink, G. R. (1986) Nature 319, 689–693
2. Fagan, M. J., and Saier, M. H. (1994) J. Mol. Evol. 38, 57–99
3. Lutsenko, S., and Kaplan, J. H. (1995) Biochemistry 34, 15607–1561328
4. Palmgren, M. G., and Axelsson, K. B. (1998) Biochim. Biophys. Acta 1365, 37–45
5. Warneke, J., and Slayman, C. L. (1980) Biochim. Biophys. Acta 591, 224–233
6. Slayman, C. L., and Zackier, G. R. (1989) Ann. N. Y. Acad. Sci. 574, 233–245
7. Perlin, D. S., San Francisco, M. J. D., Slayman, C. W., and Rosen, B. P. (1986) Arch. Biochem. Biophys. 248, 53–61
8. Post, R. L., and Jolly, P. C. (1957) Biochim. Biophys. Acta 25, 118–121
9. Glynis, E. M., and Karlis, S. J. D. (1986) Annu. Rev. Biochem. 59, 171–205
10. Auer, M., Scarborough, G. A., and Kuhlbbrandt, W. (1998) Nature 392, 840–843
11. Zhang, P., Toyoshima, C., Yonekura, K., Green, N. M., and Stokes, D. (1998) Nature 392, 835–839
12. Green, N. M. (1992) Ann. N. Y. Acad. Sci. 671, 104–112
13. Nakamoto, R. K., Rao, R., and Slayman, C. W. (1991) J. Biol. Chem. 266, 7940–7947
14. Sarkar, G., and Sommer, S. S. (1990) BioTechniques 8, 404–407
15. Cid, A., Perona, R., and Serrano, R. (1987) Curr. Genet. 12, 105–110
16. Schekman, R., and Novick, P. (1982) in The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression (Strathern, J. N., Jones, E. W., and Broach, J. R., eds) pp. 361–388, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY