Genetic Probing of the First and Second Transmembrane Helices of the Plasma Membrane H\(^+\)-ATPase from Saccharomyces cerevisiae*

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Structural features of the putative helical hairpin region comprising transmembrane segments 1 (TM1) and 2 (TM2) of the yeast plasma membrane H\(^+\)-ATPase were probed by site-directed mutagenesis. The importance of phenylalanine residues Phe-116, Phe-119, Phe-120, Phe-126, Phe-144, Phe-159, and Phe-163 was explored by alanine replacement mutagenesis. It was found that substitutions at all positions, except Phe-120 and Phe-144, produced viable enzymes, although a range of cellular growth phenotypes were observed like hygromycin B resistance and low pH sensitivity, which are linked to in vivo activity of the H\(^+\)-ATPase. Lethal positions Phe-120 and Phe-144 could be replaced with tryptophan to produce viable enzyme, although the F144W mutant was highly perturbed. ATP hydrolysis measurements showed that \(K_m\) was not significantly altered for most mutant enzymes, whereas \(V_{max}\) was moderately reduced with two mutants, F144W and F163A, showing less than 50% of the normal activity. Double Phe→Ala mutations in TM1 and TM2 were constructed to examine whether such substitutions would result in a higher degree of enzyme destabilization. Mutant F116A/F119A was viable and gave a normal phenotype, while F159A/F163A was not viable. Other double mutants, F116A/F159A and F119A/F159A, which are predicted to lie juxtaposed on TM1 and TM2, produced non-functional enzymes. However, a viable F119V/F159A mutant was isolated and showed hygromycin B resistance. These results suggest that double mutations eliminating 2 phenylalanine residues strongly destabilize the enzyme. A putative proline kink at Gly-122/Pro-123 in TM1 is not essential for enzyme action since these residues could be variously substituted (G122A or G122N; P123A, P123G, or P123F) producing viable enzymes with moderate effects on in vitro ATP hydrolysis or proton transport. However, several substitutions produced prominent growth phenotypes, suggesting that local perturbations were occurring. The location of Pro-123 is important because Gly-122 and Pro-123 could not be exchanged. In addition, a double Pro-Pro created by a G122P mutation was lethal, suggesting that maintenance of an \(\alpha\)-helical structure is important. Other mutations in the hairpin, including modification of a buried charged residue, E129A, were not critical for enzyme action. These data are consistent with the view that the helical hairpin comprising TM1 and TM2 has important structural determinants that contribute to its overall stability and flexibility.

The yeast plasma membrane H\(^+\)-ATPase is a typical P-type ion translocation ATPase that is related to the family of enzymes, which includes the mammalian Na\(^+\),K\(^+\)-ATPase, Ca\(^{2+}\)-ATPase, and H\(^+\),K\(^+\)-ATPase; the plant H\(^+\)-ATPase; and the bacterial K\(^+\)-ATPase, Mg\(^{2+}\)-ATPase, and Cu\(^{2+}\)-ATPases (1–3). These enzymes couple ATP hydrolysis to ion transport and cycle between two principal conformational states during catalysis. They do not form a single conformational state during catalysis and are sensitive to inhibition by vanadate (4). The plasma membrane H\(^+\)-ATPase from yeast is essential for growth (5), where it plays a critical role in the maintenance of electrochemical proton gradients and the regulation of intracellular pH (6). Significant sequence similarity exists between the various family members, with the greatest degree of sequence homology found within the cytoplasmic domain catalyzing ATP hydrolysis (7, 8). The topology of these enzymes is similar, with the N and C termini residing in the cytosol (9, 10), and most recent data are consistent with the presence of 10 transmembrane segments (11, 12). There is general agreement on the identity and orientation of the first four transmembrane segments, with discrepancies occurring in the remaining C-terminal transmembrane elements.

The mechanistic nature of how the H\(^+\)-ATPase couples energy from ATP binding and hydrolysis within the cytosolic domain to transport of ions within the membrane sector is not understood. Diverse studies involving drug interactions and immunological probing of higher eukaryotic enzymes (13–18), as well as genetic modifications of the yeast H\(^+\)-ATPase (19–21), support the involvement of long-range conformational interactions. There is growing evidence that transmembrane segments 1 (TM1) and 2 (TM2) are conformationally linked to the catalytic ATP hydrolysis domain (19, 20, 22).

Recently, we proposed a detailed structural model for TM1 and TM2 and used molecular dynamic simulations to assess potential conformational determinants in this region that help account for its functional role (23). TM1 and TM2 are predicted to form a helical hairpin structure that has a number of prominent structural features including a short 4–6-amino acid turn region linking TM1 and TM2, a tightly packed head region, an \(\alpha\)-helical structure, and a flexible region consisting of Gly-122/Pro-123. These features are critical for enzyme function and are conserved in the various family members, with the greatest degree of sequence homology found within the cytoplasmic domain. Recent studies involving drug interactions and immunological probing of higher eukaryotic enzymes (13–18), as well as genetic modifications of the yeast H\(^+\)-ATPase (19–21), support the involvement of long-range conformational interactions. There is growing evidence that transmembrane segments 1 (TM1) and 2 (TM2) are conformationally linked to the catalytic ATP hydrolysis domain (19, 20, 22).

Recently, we proposed a detailed structural model for TM1 and TM2 and used molecular dynamic simulations to assess potential conformational determinants in this region that help account for its functional role (23). TM1 and TM2 are predicted to form a helical hairpin structure that has a number of prominent structural features including a short 4–6-amino acid turn region linking the \(\alpha\)-helices, a tightly packed head region, an N-cap structure stabilizing the turn region, a cluster of phenylalanine residues near the cytoplasmic face of the hairpin structure, and a flexible region consisting of Gly-122/Pro-123 that may kink TM1. The hairpin structure is hydrophobic, and only one charged amino acid, Glu-129, is present in TM1. The short turn region linking TM1 and TM2 was extensively probed by mutagenesis and found to be highly conformationally active with perturbations being manifested as alterations in catalytic function (21). In this report, we examined the effects of amino acid substitutions on the 7 phenylalanines, the flexible

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‡ The abbreviations used are: TM1, transmembrane segment 1; TM2, transmembrane segment 2; kb, kilobase pair(s); Mes, 4-morpholineethanesulfonic acid.
proline kink region, and other putatively important residues
within the hairpin region of TM1 and TM2. We provide evi-
dence that the hairpin region is conformationally sensitive
since viable mutations in this region yield hygromycin B-resistant
and low pH-sensitive cellular phenotypes, and many of the
mutant enzymes show altered catalytic properties. We further
provide evidence that the cluster of phenylalanine residues
near the cytoplasmic face of the bilayer may be important for
structural stability.

MATERIALS AND METHODS

Yeast Strains and Cultures—All yeast strains utilized in this study
are isogenic derivatives of Y55 (HO gal3 MAL1 SAC1) (24). Wild type
cell strain GW201 (HO ade6–1 trp5–1 leu2–1 lys1–1 ura3–1
PMA1::URA3) was constructed by transplacing a 6.1-kb HindIII frag-
ment containing intact PMA1 linked 3’ to URA3 into SN122 (HO ade6–1 trp5–1 leu2–1 lys1–1 ura3–1 pma1::LEU2 PMA1), as de-
scribed by Harris et al. (19). Wild type strain SN236 (HO ade6–1 trp5–1
leu2–1 lys1–1 ura3–1 PMA1) is a derivative of SN236 (20) in which the
URA 3 marker has been lost. All yeast cultures were grown to early
log-phase in YEPD medium (1% yeast extract, 2% peptone, and 2%
glucose). Wild type and isogenic mutants were prepared in phagemid
vector pGW201, which consists of an 11.3-kb HindIII fragment from
SN236 and a 1.5-kb HindIII fragment from SN267. pGW201 was
linearized by S1 nuclease and used to transform the wild type strain
SN236 (HO ade6–1 trp5–1 leu2–1 lys1–1 ura3–1). Transformants were
selected on YEPD plates containing 50 μg/ml hygromycin B and 50 μg/ml
ampicillin. The transformants were grown to mid-log phase in YEPD
medium at 30°C. The cells were harvested by centrifugation, washed
with water, and resuspended in 10 mM Hepes-KOH (pH 7.0). The cell
pellet was resuspended in 400 μl of dialysis buffer containing 100 μM
Hesper-KOH (pH 7.0), 100 μM KCl, and 1 mM dithiothreitol. The reconstituted
vesicles were recovered by centrifugation at 250,000 × g for 1 h. The
pellet was resuspended in 400 μl of the dialysis buffer. Proton transport
measurements were made according to the method of Perlin et al. (28).
A fluorescence quenching reaction volume consisted of 1 ml of 10 mM
hygromycin B (pH 7.0), 50 mM KCl, 5 mM ATP, 1.5 μM acridine orange, and
50 μg of reconstituted vesicles. The reaction was initiated by the addition of 5 μg
MgCl2. Fluorescence intensity was monitored on a Perkin-Elmer LS-5 spectrophotometer.

H+-ATPase Abundance Measurements—SDS-polyacrylamide gel
electrophoresis and semidensitometry of plasma membrane proteins
were performed, as described previously (26). Western blot analysis
was performed with a polyclonal anti-H+-ATPase antibody, de-
scribed by Sato-Young et al. (21). Western blots were scanned with a
UMAX color scanner (UMAX Data Systems, Inc.), and Adobe Photoshop
and NIH Image software were used to quantitate the level of the intact
H+-ATPase (molecular mass ~ 100 kDa). Standard default settings
were used for all measurements, and all mutant enzymes were com-
pared to an internal wild type control on the same gel.

Other Procedures—Protein was determined by a modified Lowry
method (28). Yeast transformants were prepared by the lithium acetate
treatment method, as described in the alkaline cation kit (Bio 101, Inc.).
DNA sequencing of plasmid DNA was performed either with Sequenase
(20, 2), United States Biochemical Corp.) or by polymerase chain
reaction amplification of the target region and sequencing with the
sequencing system (Promega). Transmembrane helices 1 and 2 of the
yeast H+-ATPase were constructed with the molecular modeling pro-
gram Insight II (version 2.2.1; Biosym Technologies) on a Silicon Graph-
ics IRIS computer (model 4D/70GT).
assessed by SDS-polyacrylamide gel electrophoresis and Western blot analysis; it was found to exceed 70% of the wild type level (Table II). ATP hydrolysis measurements (Table II) showed that the $K_m$ for ATP was not significantly altered for most mutant enzymes; only enzymes from mutants F159A and F144W showed slightly lower $K_m$ values. In contrast, $V_{\text{max}}$ (adjusted for enzyme abundance) was significantly reduced in mutants F144W and F163A to 39 and 49%, respectively, of the wild type level of activity, while other mutants were reduced from 57 to 74%. The F144W enzyme produced an enzyme with lower activity (39%) than the F120W mutation (68%).

The vanadate inhibition profiles ($I_{50}$) for most of the Phe-pma1 mutant enzymes were comparable to the wild type (Table II). The pH dependence of ATP hydrolysis was assessed at pH 5.5, 6.5, and 7.5, and the hydrolysis activities at pH 5.5 and 7.5 were expressed as a function of activity at the normal pH optimum pH 6.5. All the Phe-pma1 mutant enzymes showed a near wild type-like activity ratio, which at most, was 20% less than wild type (data not show). The $H^+$ transport properties of mutants showing hygromycin B-resistant phenotypes were examined in a reconstituted vesicle system. The mutations had no significant effect on ATP-mediated proton transport when equivalent amounts of ATP hydrolysis units were assayed. The F144W enzyme showed a somewhat lower initial rate of pumping, although the steady state pH gradient reached the same level as wild type (Table II).

Structural stability was assessed by measuring ATPase activity in sucrose gradient-purified plasma membranes at increasing temperature (30, 35, 40, 50, and 55°C). Mutant enzymes showed the same relative heat inactivation profile as wild type (data not show). However, when mutant and wild type enzymes were heated for 15 min at 45°C in the presence of increasing concentrations of urea (0–2.67 M), mutant enzymes F163A and F144W showed enhanced heat inactivation (Fig. 2). Mutant enzymes F144A, F126A, and F159A were comparable to wild type (data not shown).

Double Phe Mutants in the Hairpin Region—Double Phe mutants were constructed to examine whether two Phe residues could be substituted, which would result in a higher degree of enzyme destabilization. Double mutant F116A/F119A, lying nearby on TM1, was viable and gave normal growth phenotypes and enzymatic properties (Table II). This was somewhat curious result because the individual mutant, F119A, showed abnormal growth and enzymatic properties (Tables I and II). It appears that the F116A mutation relieved the stress created by the F119A mutation. In contrast, double mutant F159A/F163A, lying nearby on TM2, was not viable. Other double mutants, F116A/F159A and F119A/F159A, which are expected to be juxtaposed on TM1 and TM2, also produced non-functional enzymes. The results suggest that a double substitution with Ala on TM1 and TM2 is not tolerated. However, a viable double mutant, F119V/F159A, was isolated. This double mutant with Val on TM1 at position 119 showed hygromycin B-resistant and low pH-sensitive growth phenotypes, indicative of a perturbed enzyme. However, the mutant enzyme
showed rates of ATP hydrolysis (Table II) and proton transport
that were wild type in behavior, indicating that the in vivo perturbation was relieved upon plasma membrane purification.

Overall, these results suggest that eliminating two Phe from the base of each transmembrane segment is highly destabilizing. The ability of F119V to stabilize a second mutation at F159A may result from the side chain partially filling a cavity created by the loss of the aromatic residues from each helix. This would imply that, at the very least, a space-filling role is indicated for the phenylalanines.

Mutagenesis of Proline Kink Region—TM1 contains a Gly-122/Pro-123 combination that may form a highly flexible and helix distorting region. Pro-123 is conserved among all fungal ATPases (8, 23). Mutagenesis was used to investigate whether a potential Pro kink involving Pro-123 might play an important role in the function of the $H^+$-ATPase. A P123A mutation produced viable cells that showed growth phenotypes with hygromycin B resistance and low pH sensitivity (Table I). An additional substitution with a small Gly residue or a bulker Phe residue resulted in hygromycin B-resistant phenotypes (Table I). The P123G and P123A mutations showed 62% and 71%, respectively, of the normal abundance of enzyme in the membrane, although they showed only a moderate reduction in ATP hydrolysis rates (Table II). Gly-122, which precedes Pro-123, was substituted with Asn, Ala, and Pro. The G122N and G122A mutations showed growth resistance to hygromycin B, but only G122N showed low pH sensitivity (Table I). However, the G122N mutation showed normal biochemical properties, while the G122A mutation was significantly reduced in activity. A G122P mutation, which produced double Pro-Pro residues at positions 122 and 123, was not permissible. Doublets of Pro-Pro are rarely, if ever, found in $\alpha$-helices (33). A double mutant, G122P/P123G, was constructed to shift the position of the Pro down the helix, but it resulted in a nonfunctional enzyme (Table I). A Pro was also introduced into TM2 by substituting Pro for Ala at position 155. The mutant was highly perturbed, showing mutant growth phenotypes (Table I) and ATP hydrolysis rates that were 20% of the wild type level (Table II). This result suggests that a Pro in TM2, potentially kinking or altering the helical structure, was not favorable.

Enzymes from mutants G122N, G122A, and G122A mutations showed growth resistance to hygromycin B, but only G122N showed low pH sensitivity (Table I). However, the G122N mutation showed normal biochemical properties, while the G122A mutation was significantly reduced in activity. A G122P mutation, which produced double Pro-Pro residues at positions 122 and 123, was not permissible. Doublets of Pro-Pro are rarely, if ever, found in $\alpha$-helices (33). A double mutant, G122P/P123G, was constructed to shift the position of the Pro down the helix, but it resulted in a nonfunctional enzyme (Table I). A Pro was also introduced into TM2 by substituting Pro for Ala at position 155. The mutant was highly perturbed, showing mutant growth phenotypes (Table I) and ATP hydrolysis rates that were 20% of the wild type level (Table II). This result suggests that a Pro in TM2, potentially kinking or altering the helical structure, was not favorable.

Enzymes from mutants G122A, G122N, and P123A showed normal biochemical properties, but only G122N showed low pH sensitivity (Table I). However, the G122N mutation showed normal biochemical properties, while the G122A mutation was significantly reduced in activity. A G122P mutation, which produced double Pro-Pro residues at positions 122 and 123, was not permissible. Doublets of Pro-Pro are rarely, if ever, found in $\alpha$-helices (33). A double mutant, G122P/P123G, was constructed to shift the position of the Pro down the helix, but it resulted in a nonfunctional enzyme (Table I). A Pro was also introduced into TM2 by substituting Pro for Ala at position 155. The mutant was highly perturbed, showing mutant growth phenotypes (Table I) and ATP hydrolysis rates that were 20% of the wild type level (Table II). This result suggests that a Pro in TM2, potentially kinking or altering the helical structure, was not favorable.

Enzymes from mutants G122A, G122N, and P123A showed normal biochemical properties, but only G122N showed low pH sensitivity (Table I). However, the G122N mutation showed normal biochemical properties, while the G122A mutation was significantly reduced in activity. A G122P mutation, which produced double Pro-Pro residues at positions 122 and 123, was not permissible. Doublets of Pro-Pro are rarely, if ever, found in $\alpha$-helices (33). A double mutant, G122P/P123G, was constructed to shift the position of the Pro down the helix, but it resulted in a nonfunctional enzyme (Table I). A Pro was also introduced into TM2 by substituting Pro for Ala at position 155. The mutant was highly perturbed, showing mutant growth phenotypes (Table I) and ATP hydrolysis rates that were 20% of the wild type level (Table II). This result suggests that a Pro in TM2, potentially kinking or altering the helical structure, was not favorable.

Enzymes from mutants G122A, G122N, and P123A showed normal biochemical properties, but only G122N showed low pH sensitivity (Table I). However, the G122N mutation showed normal biochemical properties, while the G122A mutation was significantly reduced in activity. A G122P mutation, which produced double Pro-Pro residues at positions 122 and 123, was not permissible. Doublets of Pro-Pro are rarely, if ever, found in $\alpha$-helices (33). A double mutant, G122P/P123G, was constructed to shift the position of the Pro down the helix, but it resulted in a nonfunctional enzyme (Table I). A Pro was also introduced into TM2 by substituting Pro for Ala at position 155. The mutant was highly perturbed, showing mutant growth phenotypes (Table I) and ATP hydrolysis rates that were 20% of the wild type level (Table II). This result suggests that a Pro in TM2, potentially kinking or altering the helical structure, was not favorable.

Enzymes from mutants G122A, G122N, and P123A showed normal biochemical properties, but only G122N showed low pH sensitivity (Table I). However, the G122N mutation showed normal biochemical properties, while the G122A mutation was significantly reduced in activity. A G122P mutation, which produced double Pro-Pro residues at positions 122 and 123, was not permissible. Doublets of Pro-Pro are rarely, if ever, found in $\alpha$-helices (33). A double mutant, G122P/P123G, was constructed to shift the position of the Pro down the helix, but it resulted in a nonfunctional enzyme (Table I). A Pro was also introduced into TM2 by substituting Pro for Ala at position 155. The mutant was highly perturbed, showing mutant growth phenotypes (Table I) and ATP hydrolysis rates that were 20% of the wild type level (Table II). This result suggests that a Pro in TM2, potentially kinking or altering the helical structure, was not favorable.

Enzymes from mutants G122A, G122N, and P123A showed normal biochemical properties, but only G122N showed low pH sensitivity (Table I). However, the G122N mutation showed normal biochemical properties, while the G122A mutation was significantly reduced in activity. A G122P mutation, which produced double Pro-Pro residues at positions 122 and 123, was not permissible. Doublets of Pro-Pro are rarely, if ever, found in $\alpha$-helices (33). A double mutant, G122P/P123G, was constructed to shift the position of the Pro down the helix, but it resulted in a nonfunctional enzyme (Table I). A Pro was also introduced into TM2 by substituting Pro for Ala at position 155. The mutant was highly perturbed, showing mutant growth phenotypes (Table I) and ATP hydrolysis rates that were 20% of the wild type level (Table II). This result suggests that a Pro in TM2, potentially kinking or altering the helical structure, was not favorable.

Enzymes from mutants G122A, G122N, and P123A showed normal biochemical properties, but only G122N showed low pH sensitivity (Table I). However, the G122N mutation showed normal biochemical properties, while the G122A mutation was significantly reduced in activity. A G122P mutation, which produced double Pro-Pro residues at positions 122 and 123, was not permissible. Doublets of Pro-Pro are rarely, if ever, found in $\alpha$-helices (33). A double mutant, G122P/P123G, was constructed to shift the position of the Pro down the helix, but it resulted in a nonfunctional enzyme (Table I). A Pro was also introduced into TM2 by substituting Pro for Ala at position 155. The mutant was highly perturbed, showing mutant growth phenotypes (Table I) and ATP hydrolysis rates that were 20% of the wild type level (Table II). This result suggests that a Pro in TM2, potentially kinking or altering the helical structure, was not favorable.

Enzymes from mutants G122A, G122N, and P123A showed normal biochemical properties, but only G122N showed low pH sensitivity (Table I). However, the G122N mutation showed normal biochemical properties, while the G122A mutation was significantly reduced in activity. A G122P mutation, which produced double Pro-Pro residues at positions 122 and 123, was not permissible. Doublets of Pro-Pro are rarely, if ever, found in $\alpha$-helices (33). A double mutant, G122P/P123G, was constructed to shift the position of the Pro down the helix, but it resulted in a nonfunctional enzyme (Table I). A Pro was also introduced into TM2 by substituting Pro for Ala at position 155. The mutant was highly perturbed, showing mutant growth phenotypes (Table I) and ATP hydrolysis rates that were 20% of the wild type level (Table II). This result suggests that a Pro in TM2, potentially kinking or altering the helical structure, was not favorable.

Enzymes from mutants G122A, G122N, and P123A showed normal biochemical properties, but only G122N showed low pH sensitivity (Table I). However, the G122N mutation showed normal biochemical properties, while the G122A mutation was significantly reduced in activity. A G122P mutation, which produced double Pro-Pro residues at positions 122 and 123, was not permissible. Doublets of Pro-Pro are rarely, if ever, found in $\alpha$-helices (33). A double mutant, G122P/P123G, was constructed to shift the position of the Pro down the helix, but it resulted in a nonfunctional enzyme (Table I). A Pro was also introduced into TM2 by substituting Pro for Ala at position 155. The mutant was highly perturbed, showing mutant growth phenotypes (Table I) and ATP hydrolysis rates that were 20% of the wild type level (Table II). This result suggests that a Pro in TM2, potentially kinking or altering the helical structure, was not favorable.
disulfide linkage could be established between the 2 residues. A previous study indicated that E129Q or E129L mutations had little effect on enzyme activity. Unfortunately, more subtle growth effects could not be examined in the expression system utilized (35). We explored whether Glu-129 plays a role in function by substituting a small Ala at this position. The E129A mutant had a weak effect on phenotype, producing only mild hygromycin B resistance (Table I). As previously proposed (35), this result suggests that the bilayer-buried charged moiety Glu-129 is not important for catalytic function. The mutation has some effect on the initial rate of the H⁺ transport, but steady state pH gradient formation was comparable to the wild type (Table I). It is puzzling that a membrane-embedded charged moiety would be so highly conserved among the fungal enzymes (8), if it plays no apparent role in function.

According to the molecular structure model, Met-128 on helix 1 is predicted to lie within close proximity to Cys-148 on helix 2. An M128C mutation was created to examine whether a disulfide linkage could be established between the 2 residues. The M128C mutant yielded hygromycin B-resistant and low pH-sensitive phenotypes, and the mutant enzyme was significantly reduced in ATP hydrolysis and initial rate of H⁺ transport (Table I). However, there was no effect of sulfhydryl reagents on enzyme activity, which precluded an affirmation of a disulfide linkage. In addition, Ala and Ser substitutions at Met-128 were not viable, which further complicates the analysis, since it is not possible to distinguish between perturbations created by the mutation in helix 1 and a potential cross-linking of the hairpin structure.

**DISCUSSION**

A Role for TM1 and TM2—It has been suggested for P-type ATPases that the primary transported ion binds to a cytoplasmically-exposed site on the membrane surface of the enzyme, which is a considerable distance (50-60 Å) (36, 37) away from the catalytic center engaged in ATP hydrolysis. Thus, long range structural interactions are necessary for coupling to occur. Emerging evidence suggests that local interactions in TM1 and TM2 provide important clues to the nature of such long distant energy coupling. In the yeast H⁺-ATPase, the notion that the hairpin region encompassing TM1 and TM2 is conformationally linked to the catalytic ATP hydrolysis domain has arisen from genetic studies of the extracellular turn region (21), and from studies identifying second site suppressor mutations, which either partially or fully complement phenotypes produced by a primary site mutation in PMA1. For example, it was shown that the phenotype induced by a primary site mutation, S368F, near the site of phosphorylation (Asp-378), could be suppressed by second-site mutations in TM1 and TM2 (19). Conversely, the phenotypes induced by a mutation, A135V, near the extracytoplasmic face of TM1, could be suppressed by secondary site mutations within the catalytic core of the ATP binding domain (20). Additional evidence linking the TM1 and TM2 to the catalytic ATP hydrolysis domain in the yeast enzyme was provided by showing that modification of Cys-148 in TM2 with omeprazole was closely correlated with enzyme inactivation (38). In addition, a G158D mutation in TM2 was found to produce a partially uncoupled enzyme when assayed in vitro (28). Diverse studies on higher eukaryotic enzymes also support a potential linkage between TM1, TM2 and the catalytic ATP hydrolysis domain. Genetic modification of residues in TM1 and TM2, which alter ouabain sensitivity, was also observed to alter catalysis by the Na⁺,K⁺-ATPase (39). A monoclonal antibody that recognizes an epitope in the extracellular turn region between TM1 and TM2 of the Na⁺,K⁺-ATPase inhibited catalysis (16). Finally, the H⁺,K⁺-ATPase antagonist SCH-28080, which blocks ATP hydrolysis, appears to bind within the loop region linking TM1 and TM2 (13).

TM1 and TM2 are predicted to form a helical hairpin structure (13, 23). We have used molecular dynamic simulations to predict how perturbations in the hairpin head region could be propagated throughout the structure (23). In addition, we used a detailed genetic analysis to explore limited conformational flexibility and tight packing in the head region (21), as predicted from the model studies. In this study, we systematically investigated residues comprising putatively important features of this structural region.

Importance of Aromatics—Interacting aromatic residues in proteins are believed to be important for structural stability and assembly of proteins (29, 40, 41). In addition, clustered aromatics may be important for translocation across the bilayer as has been recently proposed for sugar transport through the porin channel (42). The TM1/TM2 hairpin region contains seven Phe residues, with five residues predicted to form a clustered grouping at the cytoplasmic interface. These residues are predicted to be important for structural stability through the involvement of potential π-π interactions (23). In fact, the sequence arrangement of Phe-116 and Phe-120 on TM1 and Phe-159 and Phe-163 on TM2 should place these residues on the same face of their respective α-helical segments. A similar cluster of aromatics is found in the ω-subunit of the Na⁺,K⁺-ATPase and in the Ca²⁺-ATPase (43, 44). We substituted each of the seven Phe residues with Ala and found only two positions, Phe-120 and Phe-144 (described previously; Ref. 21), which could not support the loss of side chain mass (Table I). A Trp substitution at these positions produced viable enzymes, suggesting that bulky aromatic character was required. Of the remaining Phe residues that could be substituted with Ala (Phe-116, Phe-119, Phe-126, Phe-159, and Phe-163), the viable mutants showed varying growth phenotypes ranging from wild type to strongly hygromycin B-resistant and low pH-sensitive (Table I). Two mutants, F163A and F144W, showed enhanced thermal/chaotropic denaturation (Fig. 2) indicative of a stability defect. More severe effects were observed with double mutants constructed to remove a single Phe residue each from TM1 and TM2. Double mutants F163A/F159A and F119A/F159A were recessive lethal, as was a double mutant F159A/F163A on TM2 (Table I). However, an F116A/F119A on TM1 produced a viable enzyme that appeared wild type in behavior. Unless aromatic residues on TM2 are interacting with Phe-120 on TM1, it appears that potential interactions between the helical segments are not as important as the presence of aromatic character on TM2. Of course, interactions with other elements or lipid cannot be ruled out. The bulkiness of the Phe side group is likely to be important. Deletion of these two residues from TM1 and TM2 could create a cavity that could be highly destabilizing, as has been observed with T4 lysozyme (45). The fact that a F119V/F159A mutation was viable (Table I) suggests that Val may partially substitute for the bulky Phe in this position. Overall, these data suggest that aromatic residues are important for the stability and/or folding of the TM1, TM2 hairpin structure.

In many cases, reduced enzyme activity is correlated with growth phenotype, as previously observed (19, 20, 28). However, in some cases (e.g. F119V/F159A), cells showing prominent mutant growth phenotypes produced mutant enzymes displaying normal enzymatic properties when examined in vitro (Table I). One possible explanation may be that altering phenylalanines in TM1, TM2 influences the assembly efficiency of this region, which could result in an apparent growth irregularity. However, once the enzyme is assembled, it behaves normally. This suggestion is intriguing in view of the recently
proposed role of TM1/TM2 as a catalyst in the membrane assembly of the H\textsuperscript{+}-ATPase in *Neurospora* (46). Alternatively, since the in vivo enzyme is displaced from equilibrium and experiences numerous potential constraints on catalytic activity such as pH gradients, membrane voltage, turgor pressure, and regulation due to phosphorylation, it may be that small perturbations are amplified and show more pronounced effects on cell physiology. In contrast, the in vitro enzyme operating at \(V_{\text{max}}\) capacity would not be subject to these constraints, and would not be expected to show significant differences from the wild type enzyme under the same conditions. This latter explanation would be pertinent to all subtly perturbing mutant enzymes which show differential in vivo and in vitro properties.

Is a Proline Kink Important in TM1?—The helix in TM1 was predicted to kink toward TM2 about one third into the bilayer due to the presence of a flexible Gly-122 and a helix-breaking Pro-123 (23). Pro is an unusual amino acid in which its side chain is cyclized back on the backbone amide position and backbone conformation is restricted leading to a kinked structure (47). From a structural point of view, a Pro kink provides a reasonable description of the TM1/TM2 region of the H\textsuperscript{+}-ATPase. They provide additional evidence that perturbations within the helical hairpin, which are most significantly manifested as changes in cellular growth phenotypes, alter the efficiency of enzyme action.

**REFERENCES**

1. Serrano, R. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 61–94
2. Snavely, M. D., Miller, C. G., and Maquire, M. E. (1991) J. Biol. Chem. 266, 815–823
3. Odermatt, A., Suter, H., Kräpf, R., and Soliz, M. (1993) J. Biol. Chem. 268, 12775–12779
4. Bielman, B. J., and Slayman, C. W. (1979) J. Biol. Chem. 254, 2928–2934
5. Serrano, R., Kielland-Brandt, M. C., and Fink, G. R. (1986) Nature 319, 689–693
6. Goffeau, A., and Slayman, C. W. (1981) Biochim. Biophys. Acta 639, 197–223
7. Taylor, W. R., and Green, N. M. (1989) Eur. J. Biochem. 179, 241–248
8. Wach, A., Schlesser, A., and Goffeau, A. (1992) J. Bioenerg. Biomembr. 24, 309–317
9. Hennessy, J. P., Jr., and Scarborough, G. A. (1990) J. Biol. Chem. 265, 532–537
10. Mandala, S. M., and Slayman, C. W. (1989) J. Biol. Chem. 264, 16276–16282
11. Singh, D. L., Tao, T., and Maguire, M. E. (1993) J. Biol. Chem. 268, 22469–22479
12. Lin, J., and Addison, R. (1995) J. Biol. Chem. 270, 6942–6948
13. Munson, K. B., Gutierrez, C., Balaji, V. N., Ramnarayan, K., and Sachs, G. (1993) J. Biol. Chem. 268, 18976–18988
14. J ewell-Motz, E. A., and Linged, J. B. (1993) Biochemistry 32, 13523–13530
15. Schultheis, P. J., and Linged, J. B. (1993) Biochemistry 32, 544–550
16. A rystarkhova, E., Gasparian, M., Modyanov, N. N., and Seward, K. J. (1992) J. Biol. Chem. 267, 13694–13701
17. Munson, K. B., and Sachs, G. (1988) Biochemistry 27, 5932–5938
18. Price, E. M., Rice, D. A., and Linged, J. B. (1989) J. Biol. Chem. 264, 21902–21908
19. Harris, S. L., Perlin, D. S., Seto-Young, D., and Haber, J. E. (1991) J. Biol. Chem. 266, 24439–24445
20. Na, S. Perlin, D. S., Sato-Young, D., Wang, G. A., and Haber, J. E. (1993) J. Biol. Chem. 268, 11792–11797
21. Seto-Young, D., Na, S., Monk, B. C., Haber, J. E., and Perlin, D. S. (1994) J. Biol. Chem. 269, 23998–24005
22. Perlin, D. S., and Haber, J. E. (1995) Adv. Mol. Cell Biol., in press
23. Monk, B. C., Feng, W. C., Marshall, C. J., Seto-Young, D., Na, S., Haber, J. E., and Perlin, D. S. (1994) J. Bioenerg. Biomembr. 26, 101–115
24. McCusker, J. H., Perlin, D. S., and Haber, J. E. (1987) Mol. Cell. Biol. 7, 4082–4088
25. Seto-Young, D., Monk, B. C., and Perlin, D. S. (1992) Biochim. Biophys. Acta 1102, 213–219
26. Monk, B. C., Kurtz, M. B., Marrinan, and Perlin, D. S. (1991) J. Biocem. 173, 6826–6836
27. Perlin, D. S., and Brown, C. L. (1987) J. Biol. Chem. 262, 6788–6794
28. Perlin, D. S., Harris, S. L., Seto-Young, D., and Haber, J. E. (1989) J. Biol. Chem. 264, 21857–21864
29. Harzer, C. A., Singh, J., and Thornton, J. M. (1991) J. Mol. Biol. 216, 1–10
30. Deber, C. M., Brandi, C. J., Deber, R. B., Hsia, C. L., and Young, K. (1986) Arch. Biochem. Biophys. 251, 68–76
31. Luthy, R., McLaughlin, A. D., and Eisenberg, D. (1991) Proteins Struct. Funct. 10, 229–239
32. Perlin, D. S., Brown, C. L., and Haber, J. E. (1988) J. Biol. Chem. 263, 18118–18122
33. Pietta, L., Nemethy, G., and Schera, H. A. (1987) Biopolymers 26, 1587–1600
34. Sussman, M. R., Strickler, J. E., Hager, K. M., and Slayman, C. W. (1987) J. Biol. Chem. 262, 4569–4573
35. Portillo, F., and Serrano, R. (1989) EMBO J. 7, 1793–1798
36. Martinosi, A. N., Jona, I., Molnar, E., Szeider, N. W., Buchet, R., and Varga, S. (1990) FEBS Lett. 268, 365–370
37. Inoue, G., and Kirtley, M. (1992) J. Bioenerg. Biomembr. 24, 271–283
38. Monk, B. C., Mason, B., Abramchikin, G., Haber, J. E., Seto-Young, D., and Perlin, D. S. (1995) Biochim. Biophys. Acta 1239, 81–90
39. Price, E. M., Rice, D. A., and Linged, J. B. (1990) J. Biol. Chem. 265, 6538–6641
40. Kanel-Ishii, C., Sarai, A., Sawazaki, T., Nakagoshi, H., He, D.-N., Ogata, K.,
41. Nishimura, Y., and Ishii, S. (1990) J. Biol. Chem. 265, 19990–19995
42. von Heijne, G., and Gavel, Y. (1988) Eur. J. Biochem. 174, 671–678
43. Hofnung, M. (1995) Science 267, 473–474
44. Shull, G. E., Schwartz, A., and Lingrel, J. B. (1985) Nature 316, 691–695
45. MacLennan, D. H., Brandl, C. J., Korczak, B., and Green, N. M. (1985) Nature 316, 696–700
46. Matthews, B. W. (1993) Annu. Rev. Biochem. 62, 139–160
47. Lin, J., and Addison, R. (1995) J. Biol. Chem. 270, 6935–6941
48. Williamson, M. P. (1994) Biochem. J. 297, 249–260
49. Woolfson, D. N., and Williams, D. H. (1990) FEBS Lett. 277, 185–188
50. Henderson, R., Baldwin, J. M., Ceska, T. A., Zemlin, F., and Downing, K. H. (1990) J. Mol. Biol. 213, 899–929
51. Brandl, C. J., and Deber, C. M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 917–921
Genetic Probing of the First and Second Transmembrane Helices of the Plasma Membrane H-ATPase from *Saccharomyces cerevisiae*
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Additions and Corrections

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Genetic probing of the first and second transmembrane helices of the plasma membrane H^+-ATPase from Saccharomyces cerevisiae.

Donna Seto-Young, Michael J. Hall, Songqing Na, James E. Haber, and David S. Perlin

Page 583, Fig. 1: This figure did not reproduce adequately. An improved version is shown below.

Vol. 270 (1995) 20051-20058

Feasibility of a mitochondrial pyruvate malate shuttle in pancreatic islets. Further implication of cytosolic NADPH in insulin secretion.

Michael J. MacDonald

Page 20052, “Experimental Procedures,” first paragraph: Two numbers were transposed in line 6. The correct sentence should read: Briefly, when mitochondria were isolated from islets, about 2,000 islets were homogenized in 0.4 ml of 230 mM mannitol, 70 mM sucrose, and 5 mM potassium Hepes buffer pH 7.5 (MSH solution) and centrifuged at 600 × g for 10 min.

Vol. 271 (1996) 478-485

Inositol 1,4,5-trisphosphate receptor is located to the inner nuclear membrane vindicating regulation of nuclear calcium signaling by inositol 1,4,5-trisphosphate. Discrete distribution of inositol phosphate receptors to inner and outer nuclear membranes.

Jean-Paul Humbert, Nathalie Matter, Jean-Claude Artault, Pascal Köppler, and Anant N. Malviya

Page 485, line 17: 1000 nmol of calcium uptake/mg of protein was equivalent to 2.4 μM, and not 2.4 mM as printed.

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