Probing Conserved Regions of the Cytoplasmic LOOP1 Segment Linking Transmembrane Segments 2 and 3 of the *Saccharomyces cerevisiae* Plasma Membrane H\(^+\)-ATPase*  

(Received for publication, May 31, 1996, and in revised form, July 19, 1996)

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Genetic probing was used to examine conserved amino acid clusters in the first cytoplasmic loop domain (LOOP1) linking transmembrane segments 2 and 3 of the plasma membrane H\(^+\)-ATPase from *Saccharomyces cerevisiae*. Deletion of the LOOP1 region in *PM1* resulted in a defective enzyme. Scanning alanine mutagenesis of conserved residues produced lethal cell phenotypes in 14 of 26 amino acids, suggesting major enzyme defects. Most viable mutants showed growth characteristics that were comparable to wild type. Two mutations, I183A and D185A, produced reduced growth rates, hygromycin B resistance, and low pH sensitivity, which are phenotypes associated with defects in the H\(^+\)-ATPase. However, both mutant enzymes displayed near-normal kinetics for ATP hydrolysis in *vitro*. Localized random mutagenesis was also performed at sites Glu\(^{195}\), Val\(^{196}\), and Ile\(^{210}\), which all showed lethal phenotypes upon conversion to alanine. Amino acids with polar side groups could substitute for Glu\(^{195}\), while Val\(^{196}\) could not tolerate polar side group moieties. Nine mutations at Ile\(^{210}\) proved lethal, including K, R, E, P, H, N, V, G, and A, while functional enzyme was obtained with S, C, M, and L. Normal rates and extents of pH gradient formation were observed for all mutant enzymes, except I183A and D185A. Detailed analysis of the I183A enzyme indicated that it hydrolyzed ATP like wild type, but it appeared to inefficiently couple ATP hydrolysis to proton transport. In total, these results affirm that conserved amino acids in LOOP1 are important to H\(^+\)-ATPase function, and perturbations in this region can alter the efficiency of energy coupling.

The plasma membrane H\(^+\)-ATPase from *Saccharomyces cerevisiae* is an electrogenic proton pump that couples ATP hydrolysis to proton transport. It plays important roles in both intracellular pH regulation and the maintenance of a large electrochemical gradient necessary for nutrient uptake. The H\(^+\)-ATPase is a member of the P-type family of ion translocating enzymes, and it belongs to the P\(_2\)-subclass (P\(_2\)-ATPase) of enzymes that are involved in the transport of non-electrolytes. Differences between M1 and M2 and the central catalytic domain were obtained from suppressor studies, in which the phenotype produced by a primary site mutation in one sector was overcome by a secondary site mutation in the other (8, 9). These studies suggest that interactions between M1 and M2 and the catalytic region are important for normal ATP-linked electrogenic proton transport.

A likely candidate to mediate these long-range interactions is the cytoplasmic loop domain (LOOP1) linking M2 and M3. The LOOP1 region was originally postulated to act as a transduction domain (14), based largely on the observation that Ca\(^{2+}\) transport appeared uncoupled from ATP hydrolysis in the Ca\(^{2+}\)-ATPase following tryptic hydrolysis of Arg\(^{198}\) (15). In contrast, proteolytic (16–19) and genetic studies (20, 21) of residues in this region suggested that perturbed enzymes are fully coupled. While the role of this region in coupling is uncertain, it has been shown to undergo distinct conformational changes during catalysis that are linked to cation binding in the Na\(^{+}\),K\(^{+}\)-ATPase and Ca\(^{2+}\)-ATPase (6, 22). Finally, mutations S234A and D226N in the LOOP1 region of the yeast H\(^+\)-ATPase were suggested to reduce the normal stoichiometry of protons transported to ATP (23), and a recent report showed that a H285Q mutation caused partial uncoupling (19). These latter results suggest that perturbations in the LOOP1 region of the H\(^+\)-ATPase may affect coupling.
Yeast Strains and Cell Culture—All PMA1 wild type and pma1 mutant strains are derivatives of wild type S. cerevisiae strain Y55 (24). All mutations generated in this work were transformed into yeast strain SH122 (HO ade6-1 trp1-51 leu2-1 lys2-1 ura3-1 pma1:LEU2::PMA1) (8). For expression of pma1 in secretory vesicles, yeast strain NY17 set4-4 (MATa, ura3-52, set4-4) was used (25, 26). All yeast cultures were grown in 1-liter flasks of YPD medium (1% yeast extract, 2% peptone and 2% dextrose, pH 5.7) at 22 °C to mid-log phase ($\Delta A_{600} \approx 5.0$).

Growth Phenotypes of PMA1 Mutants—Overnight cultures (18 h) of pma1 mutants (0.4 ml) were inoculated into 30 ml of YPD medium and grown with shaking at both 24 and 37 °C. Aliquots (0.2 ml) were removed hourly from each culture to determine optical density at 590 nm. A microplate-based assay was used to examine the pH sensitivity and hygromycin B resistance of mutants by evaluating cell growth in YPD media with pH ranging from 2.25 to 5.5 or in YPD media containing 0–200 $\mu$g/ml hygromycin B, as described previously (13).

Site-directed Mutagenesis—Site-directed pma1 mutants were constructed as described previously (9). All mutants were prepared in phagemid vector pGW201, which consists of vector pGEM-3zf subcloned with a 6.1-kilobase HindIII fragment containing PMA1 marked with URA3 at the 3' non-coding end (8). The PMA1 gene was modified by a C → T change in the coding sequence at nucleotide position 1344 (residue Phe444), which did not alter the codon for Phe444 but resulted in loss of an EcoRI site at this position. A second silent amino acid change was made at 1,294 to introduce an unique SpeI site by converting Thr428 to C at codon position 1,292 and G → A at codon position 894. The 4.1-kilobase region from pGW201 was excised and transplanted into yeast strain SH122 by the lithium acetate transformation procedure (27). All vectors containing reconstituted pma1 genes were sequenced prior to transplacement into yeast to confirm the primary site mutation and to eliminate potential secondary mutations in the target region. Isogenic pma1 mutants were prepared as described previously (8). Two basic tetrad growth patterns were observed: 4:0 (4 spores viable) and 2:2 (2 viable:2 nonviable) spore patterns were indicative of a recessive lethal mutation, while a 4:0 pattern suggested either a fully functional gene conversion due to lethality of the mutation in question (28). To test for potential secondary mutations in the target region extending from the putative bilayer/cytoplasmic boundary extending into the third transmembrane segment (29). A 125-base pair slippage mutation produced a silent amino acid change (Ser165, and Arg166). The next residue, Leu167, is equivalent to Leu298 in the deleted region in pGW201 to form vector pMP401. The linker region contained a short polar linker consisting of Glu162, Phe163, Leu164, Ser165, and Arg166. The next residue, Leu167, is equivalent to Leu298 in the wild type sequence. A 5.7-kilobase HindIII fragment from pMP401 was used to replace the wild type PMA1 gene by homologous recombination (8). The cellular phenotype associated with this deletion was assessed by evaluating growth of meiotic segregants on YPD plates, essentially as described by Monk et al. (29). A 125-μl assay mixture contained 10 mM MES-Tris, pH 6.5, 5 mM MgSO₄, 25 mM NH₄Cl, 5 μM ATP, and 0.5–1 $\mu$g membrane protein. Samples were incubated at 30 °C and inorganic phosphate released from ATP was determined by the addition of 125 μl of phosphate developing reagent (29). The absorbance at 600 nm ($\Delta A_{600}$) was determined after a 15-min incubation at 22 °C. All $K_m$ and $V_{max}$ values were obtained by determining ATP hydrolysis as a function of substrate concentration (0–10 mM for both ATP and MgSO₄), and the data were fit to the Michaelis–Menten equation. Vanadate sensitivity was determined by measuring ATP hydrolysis in standard ATPase buffer containing sodium vanadate at 0–100 μM.

Measurement of ATP-induced pH Gradient Formation—Deoxycholate-enriched H⁺-ATPase was reconstituted into asolectin-containing liposomes, as described previously (11). ATP-induced proton transport in the isolated vesicles was monitored by measuring fluorescence-quenching assay (30). Liposomes containing 50 μg of reconstituted enzyme were suspended in 1.0 ml of assay buffer containing 10 mM MES-Tris, pH 7.0, 50 mM KCl and 5 mM ATP in a 2.7-milliliter cuvette. The reaction was initiated by the addition of 5 mM MgSO₄. The quenching of acridine orange fluorescence was monitored with a LLS-S spectrophotometer (Perkin-Elmer Corp.) with excitation and emission wavelengths of 420 and 550 nm, respectively.

Isolation of Mutant H⁺-ATPase-containing Secretory Vesicles—pma1 mutants that yield distinct hygromycin B resistant phenotypes were introduced into yeast strain NY17 set4-4, which produces secretory vesicles at elevated temperature (25, 26), by a one-step gene replacement procedure. A 6.1-kilobase HindIII fragment from pGW201 containing pma1 marked with URA3 was transplanted into yeast, as described previously (9). Transformants growing in the absence of uracil (Ura− colonies) were further selected for growth resistance on YPD plates containing 50–250 μg/ml hygromycin B. Hygromycin B-resistant colonies were isolated, and the PMA1 region was amplified by polymerase chain reaction. DNA sequence analysis was used to confirm that the mutations were integrated into the genome. Secretory vesicles were prepared by a modified version of the procedure described by Rao and Slayman (31). Parental strain NY17 and isogenic pma1 mutant strains were grown in YPD at 22 °C until mid-log phase ($\Delta A_{600} \approx 5$). The temperature was increased to 37 °C for 2 h, and the cells were harvested by centrifugation at 3,000 × g for 20 min. The cell pellet was resuspended in homogenization buffer containing 50 mM Tris-HCl, pH 7.5, 1.4 mM sorbitol, 5 mM EDTA, 1 mM EGTA, 5 mM MgCl₂ bovine serum albumin, 2 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2.5 μg/ml chymostatin. The cells were broken in a French press at 20,000 p.s.i., and the lysate was centrifuged at 10,000 × g for 10 min. Secretory vesicles in the supernatant were centrifuged at 100,000 × g for 20 min. The pellet was resuspended in 5 mM Tris-HCl, pH 7.0, containing 0.8 mM sorbitol (31), and used directly for evaluation ATP-induced pH gradient formation. Proton-transport in secretory vesicles was determined by the same procedure used with reconstituted vesicles. In some experiments, proton-transport and ATP hydrolysis were monitored simultaneously. To monitor ATP hydrolysis under these conditions, a 40-μl aliquot was removed at 30-s intervals from the assay mixture, and mixed with 200 μl of phosphate colorimetric reagent (32) to measure phosphate released. Coupling efficiency was assessed by comparing the initial rate of proton pumping (percent fluorescence quenching per min) with the rate of ATP hydrolyzed per min.

Other Procedures—Protein concentrations were determined by modification of the Lowry method (33). SDS-gel electrophoresis of plasma membrane proteins in 10% precast minigels (Bio-Rad) was performed according to the manufacturer's instructions. DNA sequencing was performed with the Sequenase DNA sequencing kit (U. S. Biochemical Corp.).

RESULTS

Deleting the LOOP1 Region—The LOOP 1 region appears similar in all the P-type ATPases (34). As a first step toward understanding the role of LOOP1 in H⁺-ATPase function, the region extending from the putative bilayer/cytoplasmic boundary of transmembrane segment 2 to the same boundary point in transmembrane segment 3, Glu162-Leu298, was deleted. However, because LOOP1 demarcates the bilayer boundaries for transmembrane segments 2 and 3, it was rationalized that linking the transmembrane segments directly would be too

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1 The abbreviation used is: MES, 4-morpholineethanesulfonic acid.
destabilizing to the enzyme. Therefore, the LOOP1 domain was replaced with a short polar linker region consisting of Glu162, Phe163, Leu164, Ser165, Arg166, and Leu167. (Leu167 corresponds to Leu236 in the wild type enzyme.) The deletion construct was introduced into yeast and found to produce a recessive lethal growth phenotype indicative of a defective H^+-ATPase. The fact that the deletion construct produced a recessive lethal phenotype, rather than a dominant lethal phenotype resulting in gene conversion, may suggest that the mutant enzyme structure was not grossly perturbed. Nonetheless, these results demonstrate that deletion of the LOOP1 region results in a defective enzyme.

Alanine Mutagenesis of Conserved Residues in LOOP1—Several highly conserved stretches of amino acids have been identified in the LOOP1 region among the various P-type ATPases (35). These stretches in yeast PMA1 include Val182–Gln188, Glu195–Ile201, Gly207–Arg213, Ile225–Leu235, and Met258–Gly270. Scanning alanine mutagenesis was used to probe residues, in four of the five conserved regions found in LOOP1, by converting all amino acids to alanine. The Met258–Gly270 region was not examined because this region contains the lowest relative percentage of conserved residues (35), and specific residues have been examined in other studies (8, 36, 37). Conserved amino acid clusters or regions are usually considered to be functionally important, and by probing these regions, it is more likely that interesting mutations would be isolated. Alanine substitution was used because it generally causes fewer structural perturbations, and subtly perturbed mutant enzymes were desirable due to the lethality of more severe pma1 mutations in yeast. (In one case, the naturally occurring alanine at position 229 was converted to serine.) The effects of each mutation on enzyme function, as reflected in cell viability, are shown in Table I. It was found that 54% (14/26) of the residues, when modified to alanine, resulted in sufficient enzyme perturbation to prevent cell growth (lethality). The lethal mutations were widely distributed, although clusters did appear such as Glu195–Pro198 and Thr231–Glu233. Lethal mutations were frequently observed with prominent changes in side group character, such as Glu or Arg to Ala. However, they were also observed with more subtle changes, such as Val or Gly to Ala.

Characterization of Growth and Enzymatic Properties of Viable pma1 Mutants—Viable pma1 mutants from the scanning alanine mutagenesis were characterized for cell growth at 24 and 37 °C. All of the pma1 mutants at 24 °C showed growth properties that were comparable to that of wild type (Table I). In contrast, two mutants, I183A and D185A, showed significantly reduced growth rates at the elevated temperature of 37 °C (Fig. 1). Viable mutants were screened for growth resistance to hygromycin B, which has been correlated with the ability of the H^+-ATPase to sustain a hyperpolarized membrane potential (39). As previously observed, wild type yeast are highly sensitive to hygromycin B (24), while pma1 mutants show a wide range of sensitivities. Differential sensitivity was also observed for the LOOP1 mutants, as indicated by the amount of hygromycin B required to produce 50% growth inhibition in rich medium (IC_{50}) (Table I). A majority of the pma1 mutants were found to retain strong sensitivity to hygromycin B, while five mutants I183A, G207A, V209A, S228A, and I230A were three to five times more resistant to the antibiotic. (This shift in sensitivity correlates exactly with plate assays (24) previously used to score hygromycin B resistance.) Hygromycin B resistance was also observed at 37 °C, and in the case of I183A, was somewhat amplified (Table I). The pma1 mutants were also grown as a function of acidic medium pH, which provides a relative measure of the ability of the H^+-ATPase to regulate intracellular pH. Fig. 2 shows that the growth of wild type cells was relatively insensitive to acidic medium above pH 2.3. In contrast to wild type, pma1 mutants, I183A and D185A, showed prominent growth sensitivities below pH 2.9 at both 24 °C (not shown) and 37 °C. pma1 mutant S228A also showed acid sensitivity but only at elevated temperature. These data indicate that several LOOP1 mutants show typical phenotypic growth abnormalities that are associated with defects in the H^+-ATPase (24).

Purified plasma membranes from the viable pma1 mutants were used to assess the biochemical properties of mutant enzymes. The relative amount of H^+-ATPase in the membrane was assessed by examining the amount of the characteristic molecular mass ~100-kDa polypeptide band by SDS-gel electrophoresis. It was determined from this analysis that the level of assembled H^+-ATPase for all the scanning alanine mutants was within 20% of that of wild type, when evaluated as a percent of the total plasma membrane protein. Most of the mutant enzymes showed K_m values within 25% of wild type, while three mutant enzymes, V196M, V209A, and S228A, showed values about 40% of wild type (Table II). Fifty percent of the mutant enzymes showed V_{max} levels that were 50–60% of wild type, including enzymes with modifications in the final cluster (I225A, S228A, A229S, and I230A). These results were qualitatively similar at 37 °C, although the values for V_{max} were two to three times greater. There was a weak correlation

### Table I

| Mutants | Viabilitya | Doubling timeb | Hygromycin B IC_{50}c |
|---------|------------|----------------|----------------------|
|         | 24 °C | 37 °C | 24 °C | 37 °C |
| Wild type | V | 169 | 114 | 32 | 12 |
| V182A | DN | 93 | 78 | 130 |
| I183A | V | 139 | 358 | 75 | 130 |
| R184A | RL | 180 | 363 | 45 | 24 |
| D185A | V | 180 | 100 | 45 | 27 |
| G186A | V | 180 | 100 | 45 | 27 |
| Q187A | RL | 180 | 100 | 45 | 27 |
| E195A | DN | 166 | 101 | 108 | 68 |
| V196A | DN | 129 | 100 | 32 | 13 |
| V197A | RL | 139 | 99 | 32 | 25 |
| P198A | DN | 147 | 121 | 32 | 22 |
| G207A | V | 166 | 101 | 108 | 68 |
| T208A | V | 129 | 100 | 32 | 13 |
| V209A | V | 133 | 98 | 82 | 48 |
| I210A | RL | 130 | 98 | 30 | 20 |
| P211A | RL | 139 | 99 | 32 | 25 |
| T212A | V | 147 | 121 | 32 | 22 |
| D213A | RL | 165 | 125 | 109 | 85 |
| I225A | V | 158 | 120 | 56 | 42 |
| D226A | RL | 174 | 124 | 102 | 64 |
| Q227A | RL | 174 | 124 | 102 | 64 |
| S228A | V | 166 | 101 | 108 | 68 |
| A229A | V | 129 | 100 | 32 | 13 |
| A230A | V | 139 | 99 | 32 | 25 |
| T231A | DN | 147 | 121 | 32 | 22 |
| G232A | RL | 165 | 125 | 109 | 85 |
| E233A | RL | 158 | 120 | 56 | 42 |

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a Viability was determined from the growth of dissected spores on YPD medium. Two viable spores and two dead spores indicated recessive lethality (RL), while four viable spores indicated either viability of a pma1 mutant (V) or a dominant negative mutation (DN) resulting from gene conversion (8, 38), which was confirmed following DNA sequencing of the chromosomal pma1 gene.

b Average doubling time (min) determined from duplicate growth curves. Standard error of the mean was less than 15% for each growth set.

c Hygromycin B concentration (μg/ml) required to inhibit growth 50% (IC_{50}) in YPD growth medium.

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D. Seto-Young, unpublished results.
between the level of ATP hydrolysis under \( V_{\text{max}} \) conditions and growth phenotypes, as has been observed previously (11, 13). In particular, the I183A and D185A mutants, which show prominent growth phenotypes displayed normal hydrolytic properties. All of the mutant enzymes showed \( K_m \) values for vanadate that ranged from 1.0 to 2.5 \( \mu M \), which are comparable to wild type (not shown). There were no enzymes showing significant vanadate insensitivity (>100-fold), which has been observed with other \( pma1 \) mutant enzymes (40). These results suggest that the mutant enzymes were only mildly perturbed in ATP hydrolysis by the introduction of alanine at the various positions.

Localised Random Mutagenesis of Glu\(^{195} \), Glu\(^{196} \), and Ile\(^{210} \)—Localised random mutagenesis was used to introduce diverse mutations at defined sites. Ile\(^{210} \) is located in a sequence region that is largely tolerant of mutation (Table I). However, the I210A mutation is lethal, and it was of interest to determine whether a more subtle perturbation of this residue might be tolerated. Random mutagenesis resulted in 12 different amino acid substitutions at position 210. Nine mutations were lethal, including K, R, E, P, H, N, V, G, and A. Functional enzyme was obtained with S, C, M, and L substitutions. Cys\(^{210} \) and Leu\(^{210} \) mutant enzymes supported relatively normal rates of growth (Fig. 1) and sensitivities to hygromycin B (Fig. 2). In contrast, the mutants carrying I210M and I210S grew slower (Fig. 1), were resistant to hygromycin B (Table III), and were sensitive to acid loading (Fig. 2). The growth properties of the I210M- and I210S-containing mutants were consistent with a decreased \( V_{\text{max}} \) (<40% of wild type) for these enzymes in vitro, relative to wild type and other mutant enzymes at position 210 (Table II). These results suggest that nonpolar residues of similar hydrated volume, Ile, Leu, and Met or small, relatively polar residues, Ser and Cys, may fill a structural role at this position. Clearly, the conserved nature of Ile\(^{210} \) does not equate with it being essential.

Saturation mutagenesis of amino acid residues Glu\(^{195} \) and Val\(^{196} \) was performed because these residues define the begin-

![Fig. 1. Cell growth profiles for \( pma1 \) mutants.](image)

Assessing Proton Transport by Mutant Enzymes—The proton transport properties of \( pma1 \) mutant enzymes were assessed by reconstituting the enzymes in asolectin liposomes and following ATP-induced pH gradient formation by the acridine orange fluorescence quenching assay. All reconstituted enzyme preparations were compared by examining the rate and extent of ATP-induced pH gradient formation at a fixed level of total activity (ATP hydrolysis units) in the assay. A survey of the mutant enzymes indicated that all but two, I183A and D185A, showed proton transport characteristics that were nearly identical to wild type (Fig. 3A). The mutant enzymes D185A and I183A consistently showed less pH gradient formation relative to wild type. The ATPase activity of reconstituted vesicles was confirmed before and after the proton pumping assay, and SDS-gel electrophoresis was used to confirm that the enzyme remained intact following reconstitution (Fig. 3B).

To rule out the possibility that the diminished proton transport observed for the I183A mutant enzyme was due to alterations occurring during detergent extraction and reconstitution, the I183A mutant was evaluated in naturally occurring Sec vesicles (25). This was accomplished by transplacing the I183A \( pma1 \) mutant gene into strain NY17, which contains the temperature-sensitive Sec6-4 mutation that results in accumulation of Sec vesicles at 37 °C (25). The new strain contained only the \( pma1-1 \)I183A gene and induction of Sec vesicles at 37 °C resulted in the incorporation of the mutant enzyme into the vesicles. It was not possible to introduce the D185A mutation because it did not confer significant hygromycin B resistance (Table I) suitable for selection in NY17 cells. ATP-mediated proton transport in the Sec vesicles was assessed in the same way as for the reconstituted liposomes. Fig. 4A shows the pH gradient profiles for wild type and the I183A mutant enzymes in the presence of increasing amounts of vanadate to partially inhibit enzyme activity. Wild type and mutant enzyme activities were adjusted to produce nearly equivalent rates of ATP hydrolysis in each assay, as illustrated in Fig. 4B. Proton transport was observed to differ between the mutant and wild type enzymes at equivalent rates of ATP hydrolysis, which was consistent with the reconstitution data. The most
significant differences were observed at the highest rates of ATP hydrolysis (Fig. 4B, curves c, d, g, and h). It can be seen that the extent of ATP-induced proton transport by the I183A mutant enzyme in the Sec vesicles was lower than that of wild type. A semiquantitative assessment of proton transport by the two enzymes determined by dividing the initial rate of proton transport (fluorescence quenching) by the rate of ATP hydrolysis under conditions when the enzymes were turning over at approximately 70% of capacity indicated that the mutant enzyme was approximately 45–65% less efficient than the wild type enzyme in ATP-mediated proton pumping. These results, along with the whole-cell growth data, are consistent with the notion that the I183A mutant enzyme couples protons less efficiently than the wild type enzyme.

**DISCUSSION**

Mutagenesis of 26 conserved residues in the LOOP1 domain revealed that conversion to alanine in approximately one-half the residues (14) induced significant enzyme perturbation, resulting in a lethal cell phenotype (Table I). Fig. 5 summarizes the scanning alanine mutagenesis growth data reported in this study, and illustrates that only eight residues could be changed to alanine without a major affect on the H⁺-ATPase. The remaining four viable mutants displayed typical defects associated with a functionally perturbed H⁺-ATPase, including hygromycin B resistance and low pH sensitivity. Many of the lethal mutations occurred in a region predicted to form β-strand structure (41) (Fig. 5), although a cluster of lethal and viable hygromycin B-resistant mutants occurred in a transition region between the predicted α-helical stalk segment and the β-strand sector. It is possible that the lethal mutations generated in this study were each sufficient to cause a structural perturbation that led to either an assembly-defective enzyme or an assembled but catalytically deficient enzyme. In this regard, it has been widely suggested that the LOOP1 region interacts with the large central cytoplasmic domain, LOOP2, and this interaction is important for catalysis. Mutations in the LOOP1 domain are known to influence vanadate sensitivity, phosphate binding, and the rate of phosphorylated intermediate turnover (8, 37, 42, 43). Protein-protein interactions often occur at the interfaces of conserved residues, and the clusters of
mutations in conserved regions producing lethal phenotypes in this study may be consistent with such interacting regions. LOOP1 would be expected to interact with LOOP2 through local interactions of individual amino acid residues between structural domains. Such interactions could help explain why many of the individual amino acid substitutions in portions of the conserved region of LOOP1 failed to significantly inhibit ATPase activity. In addition, these interactions help account for the saturation mutagenesis data obtained at positions Glu195, Val196, and Ile210, in which both viable and lethal amino acid substitutions were identified (Table III). If a specific side group were essential, then all substitutions would be lethal. However, if the targeted residue was part of a local interacting domain, then more diverse side groups within a given molecular size or charge family could be accommodated, as was observed. Overall, the subtle effects of viable LOOP1 mutations in highly conserved regions suggest that these regions play a minimal role in catalysis and are more likely to be of structural importance.

Subtle mutations should have a minimal effect on the structural properties of LOOP1, while more perturbing mutations should induce both local and global shape changes, which could lead to a severe catalytic deficiency or lack of enzyme assembly. If LOOP1 contributes to the active center, then local structural changes could influence catalysis. It is well established that mutations in LOOP1 strongly influence the sensitivity of the yeast H⁺-ATPase to vanadate (8, 37, 44), as well as phosphate interactions (37). These mutations could directly alter the phosphorylation domain or could alter the distribution of conformational intermediates leading to vanadate insensitivity, as previously proposed (8, 16, 36, 44). The effects of these mutations on vanadate sensitivity, the reduced catalytic activity observed for a factor Xa-engineered mutant (16), and some of the I210 mutations in this study (Table II), are consistent with the effects of LOOP1 mutations on the Ca²⁺-ATPase (20, 21). In the Ca²⁺-ATPase, mutations in the LOOP1 β-strand domain are believed to alter the progression of catalysis by blocking the interconversion of catalytic intermediates during the E₁P-E₂P transition (20, 21). Such perturbations in LOOP1 are believed to indirectly affect catalytic turnover. Thus, deleterious mutations in LOOP1 could alter the progression of catalysis by indirectly blocking the interconversion of catalytic intermediates. Definitive structural changes in LOOP1 appear to be an important part of the overall catalytic cycle for the Na⁺,K⁺-ATPase (6). Finally, it is known that formation of E₁P exposes a tryptic cleavage site on LOOP1 in the Ca²⁺-ATPase, whereas the site is largely protected in E₂P (18).

It is not clear whether the structural features contributed by LOOP1 play a direct role in ATP hydrolysis by the enzyme. Nonetheless, perturbations in LOOP1 can indirectly influence catalysis, and this may be relevant to coupling. A survey of proton transport by the viable LOOP1 pma1 mutant enzymes showed that, in nearly all cases, there was no significant difference in the proton transport properties between wild type and mutant enzymes. This finding suggests that perturbations

### TABLE III

| Mutants         | Viability† | Doubling time 24°C | Hygromycin B IC₅₀ 24°C | Hygromycin B IC₅₀ 37°C |
|-----------------|-----------|--------------------|------------------------|------------------------|
| Wild type       | V         | 169                | 32                     | 12                     |
| E195A           | RL        | 161                | 55                     | 72                     |
| E195V           | DN        | 166                | 54                     | 50                     |
| E195D           | V         | 134                | 75                     | 53                     |
| E195C           | V         | 159                | 81                     | 45                     |
| E195G           | V         | 304                | 75                     | 59                     |
| V196A           | RL        | 219                | 74                     | 77                     |
| V196D           | DL        | 131                | 24                     | 30                     |
| V196I           | DN        | 177                | 80                     | 99                     |
| V196R           | DN        | 150                | 46                     | 40                     |
| V196G           | RL        | 219                | 74                     | 77                     |
| V196M           | V         | 169                | 32                     | 12                     |
| E195C, V196A    | V         | 304                | 75                     | 59                     |
| E195W, V196A    | RL        | 219                | 74                     | 77                     |
| E195I, V196A    | V         | 169                | 32                     | 12                     |
| I210A           | RL        | 219                | 74                     | 77                     |
| I210S           | V         | 169                | 32                     | 12                     |
| I210C           | V         | 304                | 75                     | 59                     |
| I210M           | V         | 219                | 74                     | 77                     |
| I210L           | V         | 169                | 32                     | 12                     |
| I210K           | RL        | 219                | 74                     | 77                     |
| I210G           | DN        | 169                | 32                     | 12                     |
| I210R           | RL        | 219                | 74                     | 77                     |
| I210V           | DN        | 169                | 32                     | 12                     |
| I210N           | DN        | 219                | 74                     | 77                     |
| I210E           | DN        | 169                | 32                     | 12                     |
| I210H           | DN        | 219                | 74                     | 77                     |
| I210P           | DN        | 169                | 32                     | 12                     |

† See legend to Table I.
in LOOP1 do not generally alter proton transport. Interestingly, several of the LOOP1 mutations, which failed to elicit significant differences in in vitro proton transport or the kinetics for ATP hydrolysis, displayed prominent growth phenotypes such as temperature sensitive growth, resistance to hygromycin B, and sensitivity to low external pH suggesting potential defects in electrogenic proton transport by the H$_1$-ATPase under stress conditions. It is apparent from the relationship between H$_1$-ATPase function and cellular physiology that subtle perturbations in the enzyme can have pronounced affects on phenotype. This likely reflects the fact that the H$_1$-ATPase in the cell is not operating at maximal capacity due to the influence of numerous constraining factors such as membrane potential, pH gradient, turgor pressure, and other regulatory phenomena. Thus, small perturbations in enzyme function may be amplified at the cellular level. In contrast, mutant pma1 enzymes analyzed in vitro do not have the same physiological constraints placed upon them, and an analysis of kinetic capacity under maximum turnover conditions ($V_{max}$) may not reveal significant differences.

However, two mutations, I183A and D185A, were notable because they induced in vitro and in vivo properties suggestive of coupling defects. Both mutations induced temperature sensitive growth properties at 37 °C that resulted in diminished rates of cell growth (Table I, Fig. 1), sensitivity to low external pH (Fig. 2), and resistance to hygromycin B (Table I). When reconstituted in liposomes, both mutant enzymes appeared to pump protons less efficiently than wild type (Fig. 3A). This deficiency was not a reconstitution artifact because a more detailed analysis of proton transport by the I183A mutant enzyme in naturally occurring Sec vesicles confirmed the reconstitution study (Fig. 4). These mutations are predicted to lie near the interface between where the α-helical stalk region ends and the β-strand region begins. It remains to be determined whether this region directly or indirectly impacts the coupling process. The three-dimensional organization of transmembrane segments could bring M2 within close proximity to M4 and M5, which have been implicated in ion binding (1, 7). Our findings are fully consistent with the previous assertion of Serrano and Portillo, who suggested that mutations at D226, E233, and S234 in LOOP1 diminish the efficiency of coupling in the H$^+$-ATPase (23, 43). It should be noted that D226A and
E234A was found to be lethal in this study (Table I). In addition, a recent report by Wach et al. (19) that a H285Q mutation induces partial uncoupling is also relevant. This mutation is predicted to reside on the α-helical stalk segment emerging from transmembrane segment 3, but it could be in close proximity to I183A, which lies in the α-helical stalk segment emerging from transmembrane segment 2. The likely close proximity of these two helical elements (6) and the partial uncoupling effects of local mutations suggests a role for local structure of the stalk region in coupling. Yet, it cannot be ascertained whether the partial uncoupling observed is a consequence of disrupting the physical mechanism of coupling or whether it reflects an alteration in the transition between $E_1$P and $E_2$P intermediate states, as has been previously proposed (19). The fact that mutations in both the Ile$^{170}$ and His$^{285}$ region induce vanadate insensitivity (8, 16) may be more consistent with the latter proposal. Although, none of the mutations in this study altered vanadate sensitivity.

Collectively, the data in this study suggest that changes in LOOP1 can influence energy coupling. Unfortunately, it cannot yet be determined whether this involves a direct or indirect interaction with the central catalytic loop domain, LOOP2, which is involved in nucleotide binding and the phosphorylation-dephosphorylation of the critical aspartate residue.

Acknowledgments—G. W. acknowledges the helpful advice and support of Drs. Carolyn Slayman and Neville Kallenbach.

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