Mutations in the plasma membrane H+-ATPase gene (PMA1) of Saccharomyces cerevisiae that confer growth resistance to hygromycin B have been shown recently to cause a marked depolarization of whole cell membrane potential (Perlin, D. S., Brown, C. L., and Haber, J. E. (1988) J. Biol. Chem. 263, 18118–18122). In this report, the biochemical and genetic properties of H+-ATPases from four prominent hygromycin B-resistant pma1 mutants, pma1-105, pma1-114, pma1-147, and pma1-155, are described. Single base pair changes were identified in pma1-105, pma1-114, and pma1-147 that resulted in amino acid substitutions of Ser-368 → Phe, Gly-158 → Asp, Pro-640 → Leu, respectively. An A → G transition mutation at −39 in the 5'-untranslated region of the mRNA of pma1-155 was also found. This mutation creates an out-of-Frame upstream AUG initiation codon that apparently reduces normal translation of PMA1. DNA sequence analysis of PMA1 from strain Y55 identified 9 base pair substitutions that resulted in 6 amino acid changes in nonconserved regions when compared to the published sequence for strain S288C. Plasma membranes of three of the four pma1 mutants contained normal amounts of H+-ATPase; membranes from pma1-155 contained enzyme at 62% of the wild-type level. The kinetics of ATP hydrolysis were most strongly altered for enzymes from pma1-105 and pma1-147 which showed changes in both Kₐ and Vₘₐₓ.

A striking pH dependence for these parameters was found for enzyme from pma1-105 which resulted in a precipitous decline in Kₐ and Vₘₐₓ below pH 6.5. ATP hydrolysis by enzymes from pma1-105 and pma1-147 was insensitive to inhibition by vanadate. These enzymes, in contrast to wild-type and vanadate-sensitive mutant enzymes, were poorly protected from trypsin-induced inactivation by MgATP and vanadate or P₁ alone. These results are pertinent to the mechanism of vanadate-induced enzyme inhibition and suggest that Ser-368 and Pro-640 influence the affinity of the phosphate-binding site for P₁. All mutant enzymes catalyzed ATP-induced pH gradient formation following purification and reconstitution into liposomes. Finally, these results further demonstrate the usefulness of hygromycin B as a generalized screening tool for isolating diverse plasma membrane ATPase mutants.

The fungal plasma membrane contains an H+-ATPase that maintains the electrochemical proton gradient necessary for ion and nutrient transport and also plays an important role in intracellular pH regulation (1, 2). The H+-ATPase has been extensively characterized from the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe, as well as from the ascomycete fungus Neurospora crassa (1, 2). It consists of a single subunit of about Mᵣ = 100,000 (3–5), forms a phosphorylated intermediate (6–8), is sensitive to inhibition by vanadate (9), and cycles between at least two distinct conformational states during its reaction cycle (10, 11). The primary amino acid sequence of the H+-ATPase has been deduced from the DNA sequence of its structural gene (PMA1) and shown to share regions of direct homology with the Ca⁺⁺-ATPase, Na⁺,K⁺-ATPase and H⁺-K⁺-ATPase of animal cells (12–14). Stains of yeast carrying mutations in PMA1 are of considerable importance because they may provide valuable information about specific amino acid residues and/or protein structure domains participating in ATP hydrolysis, proton transport, and energy coupling. The cloning of PMA1 (12), along with the development of a suitable gene expression system (15), has now made it possible to apply site-directed mutagenesis techniques to produce targeted amino acid substitutions. With this approach, Serrano and colleagues (16) have started to map different functional domains of the H+-ATPase.

In contrast to a targeted mutagenesis approach, our approach has been to isolate random mutants with the prospect that mutations will arise in different regions of PMA1 and alter separate functional properties of the enzyme. Random mutagenesis, unlike site-directed mutagenesis, requires no assumptions to be made about essential residues. Recently, we described the isolation of a large number of UV-induced pma1 mutations from S. cerevisiae by selecting for resistance to the aminoglycoside antibiotic hygromycin B (17). These mutants exhibited a variety of phenotypes including growth sensitivity to weak acids, low pH medium (pH < 3.5) and NH₃. However, one of the most interesting properties of these mutants was a generalized depolarization of cellular membrane potential which was postulated to account for the cellular resistance of pma1 mutants to hygromycin B (18). Since the H+-ATPase is primarily responsible for the highly hyperpolarized membrane potential state in fungi (19, 20), it is of considerable interest to understand how specific genetic alterations influence catalysis and hence, enzyme function. Preliminary evidence indicated that the kinetic properties of the H+-ATPase were altered (17).

In this paper, we present a detailed biochemical study of the H+-ATPases associated with four of the most prominent hygromycin B-resistant pma1 mutants, pma1-105, pma1-114,
EXPERIMENTAL PROCEDURES

Materials—All culture media supplies were from Difco. Trypsin, trypsin inhibitor, acrylamide (99.9%), bisacyramide, MEGA-8, and octyl glucoside detergents were from Boehringer Mannheim. [35S] Protein A (50 μCi/mg) was from Amersham Corp. Crude phosphodiesterase was obtained from Sigma. Acetone/ether-washed aseolin was from Avanti Polar Lipids. Deoxycholate was purchased from Kodak. All restriction endonucleases were purchased from New England Biolabs. Oligonucleotides used for sequencing were made on a Cyleone DNA Synthesizer (Milligen/Biosearch).

Yeast Strains and Cell Culture—All pma1 mutant strains of yeast used in this study were derived from parental wild-type strain Y55 (HO gal1 MALI SUC1) as described by McCusker et al. (17). Cells were grown in 10-liter batches of yeast extract peptone dextrose at 30°C until mid-log phase and harvested by centrifugation, as described previously (11).

Cloning Mutant Alleles of PMA1—Mutant alleles of pma1-105, pma1-141, pma1-114, pma1-147, and pma1-155, and the wild-type PMA1 of S. cerevisiae strain Y55 were cloned by gel recovery (21). Plasmid YCP60-PMA1 containing a 5-kilobase pair HindIII fragment including the PMA1 gene (12) was obtained from G. Fink (Whitehead Institute for Biomedical Research, Cambridge, MA). The coding region was recovered from PMA1 by partial digestion with KpnI and complete digestion with XbaI. The ends of the 8.4-kilobase pair fragment were made blunt with Klenow fragment and deoxyxylulose triphosphates, and XbaI linkers were added before ligation to create plasmid pSH10. When pSH10 was linearized by restriction digestion with XbaI and transformed into ura3-1, pma1 strains, the free ends of PMA1 flanking region DNA invaded homologous chromosomal DNA, served as primers for repair synthesis to repair the gap and produced an autonomously replicating repaired plasmid containing a copy of the mutant allele.

Mapping the Site of Mutation within PMA1—Restriction enzyme digestion of pma1-105 and pma1-141 indicated that the clones contained a new EcoRI restriction site at the same position. The novel EcoRI site thus mapped the mutations in these genes very precisely. The region surrounding the new site was sequenced by the manufacturer (United States Biochemical Corp.). Mutations in the remaining alleles were mapped using a modification of the single step gene replacement technique (23). Mutant strains were transformed with DNA fragments containing portions of the wild-type PMA1 gene, and the ability of these fragments to rescue the mutant phenotypes was measured. URA3 present in the 3' nontranslated portion of PMA1 enabled chromosomal integration of these fragments to be selected (Fig. 1). Plasmid pSH11 was digested with the restriction endonuclease HindIII (which yields a 6-kilobase pair URA3-containing fragment) or with HindIII and complete digestion with XbaI. This produced a set of DNA fragments containing progressively smaller portions of PMA1. By assessing which fragments rescued the mutants, the search for the position of the mutations was narrowed sufficiently so that only a small portion of each gene had to be sequenced.

Plasma Membrane Isolation—Plasma membranes were isolated by passing 75 g of cells resuspended in 300 ml of homogenization buffer (50 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 5 mM Na2EDTA, 1 mM EGTA, 5 mg/ml bovine serum albumin, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2.5 μg/ml chymostatin) through a French pressure cell at 20,000 p.s.i. The lysate was adjusted to pH 7.5 with 1 M Tris and then centrifuged at 3500 × g for 5 min. The supernatant was removed and centrifuged at 14,000 × g for 20 min. The supernatant was again removed and then centrifuged at 300,000 × g for 1 h. The pellet from this high speed spin was resuspended in 100 ml of membrane wash buffer (10 mM Tris-HCl, pH 7.0, 1 mM EGTA, and 10% glycerol (v/v) plus 0.1 mM phenylmethylsulfonyl fluoride, homogenized vigorously with a glass homogenizer and tight-fitting teflon plunger and centrifuged at 300,000 × g for 1 h. The pellet was resuspended in membrane wash buffer at 10 mg/ml and frozen at −80°C. When being prepared for enzyme purification, plasma membranes were washed, as above, in membrane wash buffer containing 1 mM phenylmethylsulfonyl fluoride and 2 mM DTT. Plasma membranes were then resuspended in buffer containing 10 mM HEPES-KOH, pH 7.0, 1 mM EGTA, 25% (v/v) glycerol, and 2 mM DTT. All preparative operations were performed at 4°C. The final pellet was highly enriched with plasma membranes and contained less than 1% mitochondrial (azide-sensitive) ATPase contamination.

Purification and Reconstitution of H+-ATPase—A modification of the enzyme purification procedure of Koland and Hammes (24) was used to isolate normal and mutant forms of the H+-ATPase. This procedure was developed to address problems of enzyme instability and excessively proton leaky reconstituted vesicles. Yeast plasma membranes were resuspended at 2 mg/ml in a solubilization buffer consisting of 10 mM HEPES-KOH, pH 7.2, 0.1 M KCl, 45% glycerol, 0.2 mM EDTA, 1 mM DTT. Crude phosphatidylserine and acetone/ether washed aseolin were added to this suspension at a final concentration of 1 mg/ml. The suspension was stirred slowly at 4°C for 1h and deoxycholate was added slowly to a final concentration of 0.5% in order to make claimed suspensions centrifuged at 300,000 × g for 75 min. The upper translucent layer of the pelleted membranes was resuspended at 2 mg/ml in a solubilization buffer containing 0.3 M KCl, and then re-extracted with 0.4% (w/v) deoxycholate, as before. The suspension was centrifuged at 300,000 × g for 75 min. The upper translucent portion of the pellet was again removed and resuspended in solubilization buffer containing 10 mM KCl. The suspension was centrifuged as above for 90 min. The KCl-washed pellet was resuspended at 1 mg/ml in solubilization buffer containing 2 mg/ml phosphatidylserine and aseolin and then extracted with 1.5% MEGA-8 detergent. The suspension was centrifuged at 198,000 × g for 90 min. The pellet was resuspended at 1 mg/ml in solubilization buffer containing 3 mg/ml phosphatidylserine and L5 mg/ml aseolin and used immediately for reconstitution.

Reconstitution was performed by a modification of the method described by Newman and Wilson (25). Octyl glucoside was added to the resuspended MEGA-8 washed pellet, at, a final concentration of 0.8% (w/v), and the suspension was mixed by gentle vortexing. The suspension was centrifuged at 198,000 × g for 60 min, and the supernatant was removed. 1 ml of this fraction was added to 1 ml of solubilization buffer, 0.57 ml of 50 mg/ml sonicated aseolin, and 0.06 ml of 50 mg/ml phosphatidylserine. (For mutant enzymes, 1.5 ml of octyl glucoside-extracted supernatant and 0.5 ml of solubilization buffer were used.) The suspension was adjusted to 0.8% (w/v) octyl glucoside, placed on ice for 5 min, and then diluted to 25 ml with buffer containing 10 mM HEPES-KOH, pH 6.8, 300 mM KCl, and 1 mM DTT. The proteoliposomes formed spontaneously and were centrifuged at 300,000 × g for 60 min. The lower translucent portion of reconstituted liposomes was washed by resuspension with dilution buffer and then centrifuged at 303,000 × g for 60 min. The final pellet was resuspended in 150 μl of the dilution buffer. SDS gel electrophoresis was used to verify that the reconstituted enzymes represented >80% or more of the total reconstituted protein and remained intact following incorporation into liposomes.

Proton Transport Measurements—Proton transport measurements of reconstituted H+-ATPases were made using the acridine orange fluorescent quenching assay (26). Relative fluorescence was monitored with a Perkin-Elmer LS-5B Luminescence Spectrophotometer using excitation and emission wavelengths of 419 and 535 nm, respectively. Reconstituted proteoliposomes (15 to 75 μg) were incubated at 25°C in a 2-ml quenching buffer containing 10 mM HEPES pH 6.8, 50 mM KCl to 300 mM KCl, 5 mM ATP, and 2 μM acridine orange. The reaction was initiated by adding 5 mM MgCl2, Valinomycin (1 μM), NH4Cl (10 mM), and vanadate (10 μM) were added as indicated in text.

Abundance of H+-ATPase in Plasma Membranes—The appearance of intact H+-ATPase in plasma membrane fractions was evaluated by SDS gel electrophoresis and Western blot techniques as described previously (11). The relative abundance of intact H+-ATPase was evaluated by scanning Coomassie Blue-stained SDS gels with a video densitometer (Bio-Rad LS-5B). The digitized data was analyzed with an IBM PC-AT using a one-dimensional analysis program (Bio-Rad Corp.). The relative abundance of H+-ATPase was calculated by comparing total area for the intact, M, to 100,000, protein with total area represented by all membrane protein.

Protection from Trypsin-induced Inactivation—Protection studies...
Sequence of Wild-type PMA1 Allele from Strain Y55—The wild-type allele of PMA1 from strain Y55 was cloned by gap repair and sequenced in its entirety. We expected that sequence differences would exist between the published sequence from strain S288C (12) and the wild-type allele from Y55, the strain background in which all our hygromycin B-resistant mutants were isolated (17). Nine single nucleotide substitutions were found which resulted in 6 amino acid changes: Pro-74 → Leu, Val-209 → Ile, Lys-444 → Met, Ser-479 → Phe, Ala-480 → Val, and Ala-836 → Ser. A number of these amino acid changes are apparently nonconservative, but generally occur in positions not strongly conserved within the family of ATPases having phosphorylated intermediates (12).

Sequence of Mutant pma1 Alleles—Mutant alleles pma1-105, pma1-114, pma1-141, pma1-147, and pma1-155 were cloned and sequenced. Single base pair changes were found in pma1-105, pma1-114, and pma1-147 that resulted in amino acid substitutions of Ser-368 → Phe, Gly-158 → Asp, and Pro-640 → Leu, respectively. Mutant allele pma1-155 has an A → G transition at -39 in the 5' untranslated region (Fig. 1). Mutant allele pma1-141 was found to have the same mutation as pma1-105 (Ser-368 → Phe). The genetic sequence of pma1-105 and pma1-141 was confirmed by the nearly identical biochemical properties of their respective H+-ATPases; hence, only pma1-105 is discussed below.

Appearance of H+-ATPase in pma1 Mutant Membranes—Plasma membranes derived from log-phase pma1 mutant cells were evaluated by SDS gel electrophoresis (Fig. 2A). Analysis of the protein profiles by video densitometry indicated that the H+-ATPase, as indicated by the appearance of the M, = 100,000 band, represented 9.1% of the total plasma membrane protein in wild-type, pma1-105, and pma1-147 membranes, 8.3% in pma1-114 membranes, and 5.6% in pma1-155 membranes. These relative proportions of enzyme have now been observed in more than nine different membrane preparations of each pma1 mutant. A Western blot analysis (Fig. 2B) was performed with a specific anti-H+-ATPase antibody capable of detecting 20 or more proteolytic cleavage products (11). To enhance the detection of proteolysis products, the portion of the blot containing mutant membranes was exposed nearly twice as long as the wild-type portion. The results confirm that the mutant enzymes are intact and, despite the presence of a single prominent breakdown product, show no signs of generalized proteolysis. The breakdown peptide apparent in pma1-105, pma1-114, and pma1-147 mutant membranes has also been observed in different preparations of wild-type membranes and is believed to be a product of the normal turnover pathway. These results suggest that proteolysis is not likely to account for the reduced amount of H+-ATPase in pma1-155 membranes.

Kinetic Properties of pma1 Mutant Enzymes—The kinetic properties of the pma1 mutant enzymes are summarized in Fig. 3, A and B. Of the four mutants, only pma1-105 and pma1-147 mutant enzymes showed prominent decreases in $K_m$ and $V_{max}$. The kinetic behavior of enzymes from pma1-114 and pma1-155 appeared most defective in $V_{max}$, but these differences were diminished following normalization of these values for the amount of enzyme in the membrane relative to wild type. The same generalized kinetic properties found for these mutants were also observed in kinetic characterizations of nine other hygromycin B-resistant pma1 mutant enzymes (data not shown).

The effect of pH on kinetic parameters $K_m$ and $V_{max}$ was evaluated for enzymes from pma1-105, which is unable to grow at low pH, and pma1-155 which shows no growth inhibi—

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1. D. S. Perlin, unpublished data.
2. D. S. Perlin, unpublished data.
Faulty H⁺-ATPase

of Yeast

FIG. 2. Appearance of H⁺-ATPase in mutant membranes.
Plasma membranes derived from pmal mutant strains were analyzed by SDS gel electrophoresis (A). Duplicate samples were analyzed by Western blot technique (B) using anti-H⁺-ATPase antibody as described under "Experimental Procedures." The portion of the blot containing mutant membranes was exposed 1.8 X as long as wild type to allow detection of proteolytic breakdown products. The arrows indicate the position of intact H⁺-ATPase (Mᵣ = 100,000).

FIG. 3. Mutant enzyme kinetics. Kinetic parameters $Kₘ$ and $V_{max}$ were determined for mutant enzymes at pH 6.5, as described under "Experimental Procedures." The cross-hatch areas in B represent corrected $V_{max}$ values obtained after multiplying the experimental $V_{max}$ (solid bars) by the ratio of intact H⁺-ATPase protein found in wild type to that of mutant membranes (Fig. 1).

FIG. 4. Effect of pH on mutant enzyme kinetics. Kinetic parameters $Kₘ$ and $V_{max}$ were determined over the pH range 5.5-8.0, as described under "Experimental Procedures," for membrane-bound enzymes from PMA1⁺ (■■), pmal-105 (○○), and pmal-155 (□□).
pmal-114 and pmal-155 were strongly inhibited by vanadate, whereas enzyme from pmal-147 was only weakly sensitive to vanadate over a concentration range that produced 95% inhibition of wild-type enzyme. This result suggests that for pmal-105 and pmal-147 enzymes, vanadate is unable to form a transition state complex which locks the enzyme in an $E_2 (E_2$-$P$ or $E_2$-$P$) conformational state. To examine this possibility, the ability of vanadate to induce a stable $E_2$ conformational state that would confer protection from trypsin-induced inactivation was examined for the mutant enzymes. It was observed previously that trypsin treatment of wild-type H+-ATPase caused a rapid loss of ATP hydrolysis and this effect was strongly protected by the formation of a vanadate-induced $E_2$ conformational state (11). Fig. 6 indicates that wild-type enzyme and vanadate-sensitive mutant enzyme pmal-114 were strongly protected from trypsin-induced inactivation, whereas vanadate-insensitive enzymes, pmal-105 and pmal-147, were poorly protected from trypsin treatment. The level of protection paralleled the degree of vanadate-insensitive ATP hydrolysis (Fig. 5) and supports the suggestion that vanadate insensitivity correlates with an inability to attain a stabilized $E_2$ ($E_2$-$P$ or $E_2$-$P$) intermediate species.

Effects on Phosphate Binding—If vanadate acts via an interaction at a phosphate binding site, then it is possible that vanadate insensitivity results from a change in the affinity of this site for Pi. ($K_r = 177 \text{ mM}$ (10)) precludes such a measurement. Alternatively, phosphate binding can be evaluated by an indirect assay utilizing phosphate-induced protection from trypsin proteolysis (11). The results in Fig. 7 show that 150 mM P, was able to induce protection from trypsin-induced inactivation more strongly in vanadate-sensitive enzymes, wild-type and pmal-155, than in vanadate-insensitive enzymes, pmal-105 and pmal-147. This result suggests that a change in phosphate binding has occurred in the vanadate-insensitive enzymes.

Proton Transport by Mutant Enzymes—It was reported previously from measurements of whole cells that hygromycin B-resistant pmal mutants are depolarized in cellular membrane potential but maintain near normal levels of proton transport (18). Since the evaluation of proton transport in whole cells relied on measurements of glucose-induced medium acidification, which reflects the combined action of both the H+-ATPase and organic acid transport, it was important to independently verify that pmal mutant enzymes actively catalyze proton transport. To accomplish this objective, wild-type and mutant enzymes were purified and reconstituted in liposomes. The specific ATPase activities for reconstituted mutant enzymes pmal-105, pmal-147, and pmal-114 were 8.8, 11.7, and 10.0 $\mu$mol P, mg$^{-1}$ min$^{-1}$; wild-type specific activities were in the range 27.7–32.8 $\mu$mol P, mg$^{-1}$ min$^{-1}$.

The relative purity of reconstituted enzymes, as determined from SDS gel electrophoresis, was greater than 80% for wild-type and mutant enzymes. Each mutant enzyme was reconstituted at least two times from separate membrane preparations and the results, as illustrated below, were always consistent.

Mutant enzymes pmal-105, pmal-147, and pmal-114 readily formed MgATP-dependent pH gradients in reconstituted vesicles, as measured by fluorescence quenching of the pH gradient probe acridine orange (Fig. 8, A–C). In this assay, reconstituted vesicles were suspended in buffer containing ATP and the fluorescent probe acridine orange. Enzyme-mediated proton transport was initiated by the addition of Mg$^{2+}$ and assessed by a continuous measurement of relative fluorescence. The formation of interior acid pH gradients, as indicated by the quenching of acridine orange fluorescence, reached a steady state after several minutes. The rate and extent of pH gradient formation was always lower with the mutant enzymes relative to wild type and this was expected from their decreased rates of ATP hydrolysis (see above). However, it was observed in three separate reconstitution experiments that pmal-114 enzyme always formed smaller pH gradients (Fig. 8C) relative to the other mutant enzymes, despite having a similar rate of ATP hydrolysis (10 $\mu$mol P, mg$^{-1}$ min$^{-1}$). The possibility that this enzyme is partially uncoupled from ATP hydrolysis is currently being investigated by a detailed analysis of H$^+$/ATP stoichiometry. The addition of 10 $\mu$M vanadate during steady-state pH gradient formation had no effect on vanadate-insensitive enzymes, pmal-105 and pmal-147 (Fig. 8, A and B), but caused a rapid reversal of the pH gradient in vanadate-sensitive enzymes pmal-114 and wild type (Fig. 8C). In each case, the pH gradient was completely collapsed by NH$_4$Cl.

The H$^+$-ATPase catalyzes electrogenic proton transport and it was found that pH gradient formation was optimal in the presence of valinomycin which eliminated interior positive membrane potential formation by allowing compensating change movement from the K$^+$-loaded vesicles. When the mutant enzymes were allowed to form transient membrane potentials by initiating proton transport in the absence of valinomycin, as illustrated for pmal-105 (Fig. 8A), there was a marked decline in their apparent rate of proton transport.

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1. D. Seto-Young and D. S. Perlin, unpublished data.
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Fig. 6. Protection from trypsin-induced inactivation. Plasma membranes from pma1 mutant strains, as indicated, were incubated with trypsin (0.25 mg/mg membrane protein) for 1-30 min in the presence (■) or absence (□) of 5 mM MgATP plus 100 μM vanadate. Treated membranes were then analyzed for ATP hydrolysis as described under "Experimental Procedures." ATP hydrolysis rates at time 0 were taken to represent the control value. WT, wild type.

Fig. 7. Pi-mediated protection from trypsin-induced inactivation. Plasma membranes from pma1 mutant strains, as indicated, were incubated with trypsin (0.25 mg/mg membrane protein) for 10 min in the absence or presence of 150 mM Pi. Membranes were washed free of Pi by centrifugation and then assayed for ATP hydrolysis. Values for control level ATP hydrolysis were determined from membranes pretreated with a 20-fold excess of trypsin inhibitor. Relative to wild type. A subsequent addition of valinomycin restored pH gradient formation to its optimal level. This behavior raises the possibility that the pma1 mutant enzymes may have an altered sensitivity to membrane voltage.

Discussion

Membrane Potential-altering Mutations—The results in this study demonstrate that mutations in PMA1 which cause depolarization of cellular membrane potential (18) are widely distributed throughout the gene (Fig. 1). The mutant enzymes were present in near wild-type quantities in the membrane except for pma1-155 which showed 62% of the wild-type level. An A → G mutation at −98 base pairs within the promoter region of pma1-155 creates an AUG sequence at position −41 in the PMA1 mRNA that should be the site of translational initiation. However, only a tripeptide should be produced since an UAA codon is found in frame at position −32. Previous experiments in yeast (30) have shown that only the first AUG is used to initiate translation and that there is little or no reinitiation at downstream AUG sites, even when the first AUG is followed by a termination signal. Our results suggest either that the first AUG is poorly recognized or that reinitiation does occur, as the level of PMA1 protein is only reduced by about 40%. The failure to detect significant pro-
teolytic breakdown products in the membrane (Fig. 2B) suggests that decreased translational efficiency of PMAI is likely to account for the reduced amount of enzyme detected.

The kinetic properties of pmal-105, pmal-114, pmal-147, and pmal-155 mutant enzymes are diverse (Figs. 3 and 4). However, a reduction in membrane-associated H⁺-ATPase activity (except for pmal-114 enzyme) appears to be a common feature and has been observed in kinetic analyses of nine other enzymes from hygromycin B-resistant pmal mutants. A decrease in total membrane-associated H⁺-ATPase activity, resulting from either altered translational efficiency (pmal-155) or from direct protein structure modifications (pmal-105 and pmal-147), appears sufficient to alter the cellular membrane potential and hence confer growth resistance to hygromycin B. Capieaux et al. (31) reached a similar conclusion from a study where mutations within upstream UAS<sub>HRG</sub> sequences, which control transcription of PMAI, decreased the level of H⁺-ATPase in the membrane and conferred hygromycin B resistance to cells.

A close correlation between membrane potential and membrane-associated H⁺-ATPase activity assumes that ATP hydrolysis and proton transport are strictly coupled and that decreased proton transport leads to depolarization of the cellular membrane potential. This assumption is supported by studies of Dio-9-resistant pmal mutants (32, 33). However, we recently reported for pmal-105 and pmal-147 that proton transport, as deduced from whole cell medium acidification, was not significantly different from wild type (18) despite the fact that these mutants show reduced levels of membrane-associated ATPase activity (Fig. 3). Thus, predictions of net proton transport and steady-state membrane potential from measurements of membrane-associated H⁺-ATPase activities can be misleading. It would be best to measure the coupling efficiency between ATP hydrolysis and proton transport for each mutant enzyme and recognize that membrane potential defects may reflect inherent changes in the enzyme-mediated charge-transfer mechanism (18). In this respect, recent patch-clamp studies of isolated membrane patches from pmal-105 have demonstrated an ATP-dependent activation of voltage-gated K⁺ conductance that may reflect a direct participation of the H⁺-ATPase in K⁺ transport (34). A detailed analysis of coupling (H⁺/ATP) stoichiometry, membrane voltage effects, and ion specificity by the mutant enzymes is currently in progress.

Vanadate-insensitive Enzymes—The inhibition of ATP hydrolysis by vanadate in P-type ATPases is generally considered a major characteristic feature of this class of enzyme. Vanadate is believed to bind at the site from which phosphate is released and enzyme inhibition results from the formation of a pentacoordinate complex at the site of phosphorylation (35–37). The importance of vanadate as a mechanistic probe suggests that mutants with altered vanadate sensitivity should provide important information about enzyme mechanism. In this study, two mutant H⁺-ATPases with amino acid substitutions of Ser-386 → Phe (pmal-105) and Pro-640 → Leu (pmal-147) were found to be strongly resistant to inhibition by vanadate (Fig. 5). Both amino acids are contained within conserved regions of the large hydrophilic domain which is believed to form all or part of the catalytic site (38). Vanadate-insensitive H⁺-ATPases have also been identified in Dio-9-resistant pmal mutants from S. cerevisiae and S. pombe (32). The S. pombe mutant has been identified as a Gly-268 → Asp substitution (33) within a highly conserved region near the end of the second putative hydrophilic domain (Fig. 1). This domain is believed to be separated from the large hydrophilic domain by two membrane-spanning helices (38, 39).

On the basis of site-directed mutations, Glu-233 → Gln and Asp-200 → Asn, which influence the level of phosphorylated intermediate at Asp-378, Serrano (16, 38) speculated that the two hydrophilic regions encompassing vanadate-insensitive loci Gly-268 and Ser-386 interact to form a "phosphatase" domain that would function in the hydrolysis of the aspartyl phosphate intermediate. However, the conferment of vanadate insensitivity is not localized to this domain since Pro-640, which also confers vanadate-insensitive enzyme behavior, lies within the putative ATP binding site (16). In addition, the relative position of an amino acid within a specific protein structure domain cannot account for vanadate insensitivity, since Asp-638 → Asn and Asp-378 → Asn, Glu, and Thr mutations which reduce ATP hydrolysis and lie close to vanadate-insensitive loci have no effect on vanadate sensitivity (16). Although the importance of Gly-268, Ser-386, and Pro-640 in vanadate-insensitive enzyme behavior cannot yet be defined precisely, it appears likely that they may play an essential role, either directly or indirectly, in phosphate binding.

In these pmal mutants, vanadate interactions at the phosphate-binding site may have been altered by a structural modification in the site or from a decrease in the steady-state level of E<sub>0</sub> conformational intermediate necessary for P<sub>i</sub> binding (33, 36, 39). Such behavior was supported by a decrease in phosphate-induced protection from trypsin-induced inactivation for vanadate-insensitive enzymes, pmal-105 and pmal-147 (Fig. 7).

pH Dependence of Enzyme Activity—As shown in Fig. 4, the kinetic properties of wild-type enzyme and pmal-105 mutant enzyme vary greatly with pH. Wild-type enzyme shows a relatively constant K<sub>i</sub> and changing V<sub>max</sub> over the pH range 5.5–8.0. In contrast, pmal-105, which is unable to grow at low pH (17), shows a significant decline in K<sub>i</sub> and V<sub>max</sub> over this same range (Fig. 4, A and B). This kinetic behavior may be explained by proposing that a Ser-386 → Phe mutation in pmal-105 results in exposure of a normally buried residue with an acidic pK<sub>a</sub>, such as glutamate or aspartate. Ionization of this residue at acidic pH values would inhibit the enzyme by altering the steady-state distribution of E<sub>i</sub> and E<sub>c</sub> catalytic intermediates. Acidic pH (below pH 6.0) has been proposed to stabilize a low affinity ATP conformation of the Ca<sup>2+</sup>-ATPase (40) and Na<sup>+</sup>,K<sup>+</sup>-ATPase (41) and such an effect may be mimicked in the pmal-105 enzyme.

Conclusion—The detailed genetic and biochemical characterizations of pmal mutant enzymes, pmal-105, pmal-114, pmal-147, and pmal-155, in this study confirm that hygromycin B is a valuable selection tool for isolating random and diverse pmal mutants. Unlike more popular site-directed mutagenesis approaches, random mutagenesis requires no assumptions to be made about essential residues. The major potential advantage of this approach is that numerous and diverse pmal mutants can be isolated which affect different partial catalytic reactions. Finally, through a detailed examination of proton transport by pmal mutant enzymes and a comprehensive revertant analysis of vanadate-insensitive pmal mutants, we hope to better define electrogenic proton transport and ATP hydrolysis by the H⁺-ATPase.

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