Amino Acid Replacements at Seven Different Histidines in the Yeast Plasma Membrane H^+−ATPase Reveal Critical Positions at His285 and His701†

Achim Wach,‡,§ Philip Supply,‡ Jean-Pierre Dufour,‡ and André Goffeau*,‡

Unité de Biochimie Physiologique, Université Catholique de Louvain, Place Croix du Sud 2-20, B-1348 Louvain-La-Neuve, Belgium

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ABSTRACT: The plasma membrane H^+−ATPase (Pma1) from *Saccharomyces cerevisiae* contains 14 histidine residues. The seven most highly conserved of these were selected as targets for oligonucleotide-directed, site-specific mutagenesis. Substitutions at H240, H488, H614, H686, and H914 with a variety of amino acid residues had no effect either on cell viability or on temperature and pH growth sensitivity. In contrast, substitutions at H701, located in the putative fifth membrane-spanning region, with Asp, Gln, or Arg were dominant lethal, indicating that H701 is essential for H^+−ATPase activity. The mutations H285Q and H285R, but not H285E, located in the hydrophilic β-stranded domain, were tolerated in normal growth conditions. Growth of H285Q mutants was sensitive to acid pH, indicating impaired *in vivo* ATPase activity. The H285Q and H285R mutants showed increased *in vitro* ATPase-specific activity, increased vanadate resistance, increased proton competition of vanadate sensitivity, accelerated ATP hydrolysis rates at a substrate concentration much lower than the K_m, and slightly uncoupled proton pumping. The most reasonable hypothesis which would take into account these observations is that H285 would not be involved in the H^+ transport process but rather in the E_2 to E_1 transition step of the ATP hydrolysis catalytic cycle.

The plasma membrane H^+−ATPase (Pma1) from *Saccharomyces cerevisiae* is a P-type ATPase (Pedersen & Carafoli, 1987) that generates a transmembrane proton gradient and is essential for growth [see reviews by Goffeau and Slayman (1981), Serrano (1989), Nakamoto and Slayman (1989), Scarborough (1992), and Rao *et al*. (1993)]. It is generally accepted that, during turnover, the enzyme passes through two major conformational states, the E_1-conformation, capable of binding ATP, and the E_2-conformation, incapable of binding ATP but able to be phosphorylated by P_i (Amory *et al*., 1982). Kinetic studies have shown that yeast H^+−ATPase is reversibly inhibited when the pH is raised from 6.0 to 8.0, the apparent p*K* being 6.8 (de Meis *et al*., 1987) to 6.9 (Blanpain *et al*., 1992) for *Saccharomyces pombe* and 7.4 for *S. cerevisiae* (Wach & Gräber, 1991). These p*K* values may reflect protonation/deprotonation of a histidine residue. However, none of the kinetic approaches used has been able to provide information about the nature, number, location, and function of the histidine residues involved. A more direct approach was used by Morjana and Scarborough (1989), who reported that the histidine reagent diethyl pyrocarbonate irreversibly inactivates the *Neurospora crassa* plasma membrane H^+−ATPase with a p*K* of 7.5. These data indicate that at least one unidentified histidine residue is essential for Pma1-mediated catalysis of ATP hydrolysis. Pma1 from *S. cerevisiae* contains 14 histidines distributed throughout the protein. To our knowledge, none has yet been studied by site-directed mutagenesis and no systematic studies of the role of these residues have been performed on any P-type ATPase. In this article, we present data obtained using an approach combining systematic site-directed mutagenesis and kinetic analysis to identify those histidine residues that are important for activity of the H^+−ATPase in *S. cerevisiae* plasma membranes.

MATERIALS AND METHODS

Materials. Na_2−ATP, pyruvate kinase (rabbit muscle, glycerol solution), phospho(enol)pyruvate (monoammonium salt), and the ATP bioluminescence kit CLS were obtained from Boehringer Mannheim, 5-fluoroorotic acid (5-FOA) from PCR Inc. (Gainesville, FL), and sodium orthovanadate from BDH Chemicals Ltd. (Poole, U.K.). All other chemicals were of high-quality reagent grade.

Strains and Media. A list of strains used is given in Table 1. Yeast was grown on completely synthetic media containing the following (per liter): 7 g of yeast nitrogen base (Difco Laboratories), 1.1 g of drop out mix supplemented with all amino acids (Treco, 1989) except those used for selection, and 2% glucose (MGlu), 3% glycerol/2% ethanol (MGlyEt) or 3% glycerol/2% ethanol/2% galactose (MGlyEtGal). Solid media contained 2% agar (Difco Laboratories). For selection of Ura3$^-$ strains, 1 g of 5-fluoroorotic acid was added to 1 L of medium (Treco, 1989). For biochemical analysis, the cells were grown in 1 L of 2% yeast extract containing 2% glucose (YD) at 25 °C until mid-log phase. For phenotypic analysis, mutant cells were plated on MGlu, adjusted to pH 3.0 with 1 N HCl (MGlu, pH 3.0), MGlu

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* Corresponding author. Tel: +32-10-473614. FAX: +32-10-473872. E-mail: GOFFEAU@FYSA.UCL.AC.BE.

‡ Université Catholique de Louvain.

§ Present address: Institut für Angewandte Mikrobiologie, Biozentrum, Universität Basel, Klingelbergstr. 70, CH-4056 Basel, Switzerland.

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Table 1: Yeast Strains and Plasmids[a]

name	relevant characteristics	source
	Saccharomyces cerevisiae	
YPS229C	*MAT*α, *pmaI-*Δ*::HIS3, pma2-*Δ*::TRP1, lys2-801, ade2-101* [P_{PMA1}*::PMA2, URA3, lue2-d,* 2μ*m*]	P. Supply
YPS12-1	*MAT*α, *pma1-*Δ*::HIS3, pma2-*Δ*::TRP1, lys2-801, ade2-101, ura3-52* [P_{PMA1}*::PMA2, LEU2, CEN6, ARSH4*]	P. Supply
YAWP$_G$PMA1	*MAT*α, *pma1-*Δ*::HIS3, pma2-*Δ*::TRP1, lys2-801, ade2-101, ura3-52* [P_{GAL1}*::PMA1, LEU2, CEN6, ARSH4*]	this study
YAW2P$_G$PMA1	*MAT*α, *pma1-*Δ*::HIS3, pma2-*Δ*::TRP1, lys2-801, ade2-101, ura3-52* [P_{PMA1}*::PMA2, URA3, lue2-d,* 2μ*m*] [P_{GAL1}*::PMA1, LEU2, CEN6, ARSH4*]	this study
YAWP$_{Gpma1*}$	*MAT*α, *pma1-*Δ*::HIS3, pma2-*Δ*::TRP1, lys2-801, ade2-101* [P_{PMA1}*::PMA2, URA3, leu2-d,* 2μ*m*] [P_{GAL1}*::pma1*, LEU2, CEN6, ARSH4*]	this study
YAWP$_{ppma1*}$	*MAT*α, *pma1-*Δ*::HIS3, pma2-*Δ*::TRP1, lys2-801, ade2-101, ura3-52* [P_{PMA1}*::pma1*, LEU2, CEN6, ARSH4]0*	this study
	Plasmids	
pSel-PMA1	pSelect-1 (Promega) containing the 4650 bp *Hin*dIII−*Xba*I fragment of *PMA1*	this study
pSel-pmal	pSel-PMA1 mutated by oligonucleotide-directed site-specific mutagenesis	this study
pGal1PMA1v	pRS315, P*GAL1-10::PMA1C* (promotor GAL1-10 from pBM150 and PMA1C, *Cla*I−*Eco*RV fragment from pPSPMA1C)	(Figure 1)

[a] Strains and plasmids constructed in this study are described in more detail in Materials and Methods. pma1* is the substitution nomenclature for the different *pma1* mutant alleles that were constructed by site-directed mutagenenis. Promotor sequences are indicated by a P followed by the gene name from which it is derived (e.g., P_{GAL1}). A "::" indicates the fusion of two DNA fragments, and a Δ following a gene name means that the corresponding gene has been deleted from the genome. Gene names in square brackets denote that these genes are present on a plasmid. *CEN/ARS* sequences promote 1−2 plasmid copies per cell, whereas a 2 μm origin of replication or the *leu2-d* allele produces 50−150 plasmid copies per cell.

containing 200 mM sodium acetate, adjusted to pH 5.0 with 1 N HCl (MGlu, pH 5.0 Ac), or MGlu containing 50 mM Tris, adjusted to pH 7.5 with 1 N HCl (MGlu, pH 7.5).

Plasmid Constructions. The plasmids used are listed in Table 1. For site-specific mutagenesis, the 4650 bp *Hin*dIII−*Xba*I fragment of *PMA1* (Serrano *et al*., 1986) was subcloned into pSelect-1 (Promega Corp., Madison, WI), giving plasmid pSel-PMA1. Plasmids obtained from the different mutagenesis reactions were named pSel-pma1*, the asterisk indicating the presence of a mutant allele. pPGAL1PMA1v was constructed by ligation of the blunted *Eco*RI−*Bam*HI fragment from pBM150, containing the *GAL1-10* promoter (Johnston & Davis, 1984), into the *Cla*I site, blunted by *T4* DNA polymerase, from a pRS315 (Sikorski & Hieter, 1989) construct already containing the *Cla*I−*Eco*RV fragment from pPSPMA1C (Supply *et al*., 1993). This ligation placed the first 130 bp of the *PMA1* structural gene, including its start codon, downstream of the *GAL1* promoter.

Oligonucleotide-Directed, Site-Specific Mutagenesis. Site-specific mutations were directed using synthetic oligodeoxynucleotide primers complementary to the sense strand of the *PMA1* gene. Primer length was 19−22 nucleotides and 26−30 nucleotides when on mismatch or two/three mismatches, repectively, were introduced. The sequences of the 12 primers used are as follows (the nucleotide changes introduced in the mutagenic oligonucleotides are underlined): 5′-ttggtcaccgta**gt**a**ct**tgtcgacagcc-3′ for the mutation H240Y, 5′-aacttcagtgaa**ttc**accttgaccacc-3′ for the mutation H825E, 5′-cttcagtgaa**t**tgaccttgacc-3′ for the mutation H825Q, 5′-caaaacttcagtgaa**tct**accttgaccacc-3′ for the mutation H285R, 5′-ccggttttg**t**tggaacacaag-3′ for the mutation D378N, 5′-tctgggattggg**at**atcttcttcgac-3′ for the mutation H488I, 5′-ctgtatttat**c**ttgtgggaa-3′ for the mutation H614D, 5′-agagtacattc**aga**aaaaatttgtctg-3′ for the mutation H686S, 5′-tttccaaat**c**tagagacaa-3′ for the mutation H701D, 5′-gatttccaa**t**tgtagagac-3′ for the mutation H701Q, 5′-gaagatttccaa**tct**tagagacaaagc-3′ for the mutation H701R, and 5′-ccttttcgtt**t**tgagtagag-3′ for the mutation H914N. All mutations were made as described in the technical manual accompanying the Promega (Madison, WI) mutagenesis kit, using *T4* DNA polymerase to synthesize the mutant strand *in vitro*. Successful mutagenesis was first screened, where possible, by restriction site analysis or by dideoxynucleotide sequencing. For each mutagenesis reaction, four independent clones were screened and one positive clone was selected for further experiments. At least two of the four screened reaction products per mutagenesis reaction carried the introduced point mutation. A final check was made by sequencing the complete mutated *pma1* genes as described below.

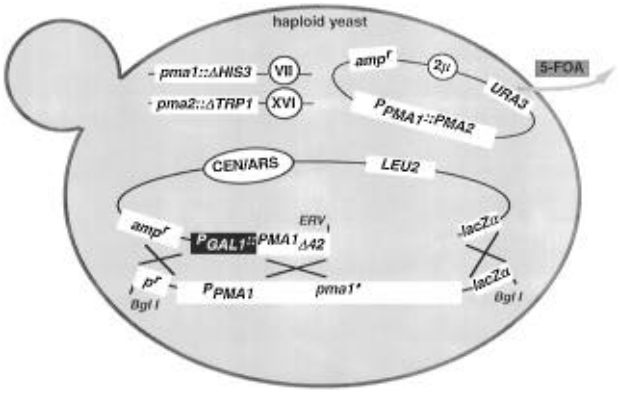

FIGURE 1: Schematic description of the homologous recombination between two DNA fragments cotransformed into yeast. Regions of homology on both fragments are indicated by an ×. The restriction sites *Eco*RV (ERV) and *Bgl*I allowing identification of the recombinogenic event carried out *in vivo* are shown in black. See Materials and Methods and Table 1 for detailed descriptions of the DNA fragments used. The labels *pma2::*Δ*His3* and *pma1::*Δ*TRP1* mean that the PMA2 and PMA1, located respectively on chromosomes XVI and VII, have been deleted and substituted with the HIS3 and TRP1 markers, respectively.

Recombination in Yeast. Yeast recombinants, expressing a plasmid harboring a mutant allele of *PMA1*, were obtained by homologous recombination of two linear DNA fragments (Ma *et al*., 1987). A schematic illustration of the process is presented in Figure 1. The homologous DNA regions,

located at the extremities of both linear fragments, were provided on one side by the *lacZ′* gene fragment and on the other side either by the fragment −4 to +125 of the *PMA1* structural gene or by the 3′-terminal half of the *amp*^{r} gene. Recombination in the *PMA1* structural gene yields a repaired plasmid carrying a mutant allele, whose expression is controlled by the inducible *GAL1* promoter (P*GAL1::pma1**). Recombination in the *amp*^{r} gene gives a paired plasmid containing a constitutively expressed mutant allele, controlled by the constitutive *PMA1* promoter (P*PMA1::pma1**). Independently of the recombination event, all the plasmids produced contain the yeast selectable marker *LEU2* and the *CEN6/ARSH4* cassette. The plasmid to be repaired (pPGAL1PMA1v) was linearized by *Pst*I/*Sac*I digestion, and the insert, containing the *pma1** mutant allele, was extracted by *Bgl*I. The restriction fragments, separated by agarose gel electrophoresis (Heery *et al*., 1990), were then introduced into yeast cells by cotransformation using the lithium acetate method (Ito *et al*., 1983). The *PMA1* wild type allele from pSel-PMA1 was used as control for successful recombination.

Analysis of the in Vivo Growth Characteristics of the PMA1 Mutant Alleles. For each mutant allele, four yeast recombinants were analyzed for growth on different media (see legend to Figure 3 for detailed description). From those recombinant strains showing significant growth differences as compared to the wild type control, one clone was selected for mutant allele sequencing and biochemical analysis.

Mutant Allele Sequencing. The total DNA of the yeast strain was transformed into *Escherichia coli*, and *PMA1*-containing plasmids were extracted from *amp*^{r} colonies. The *PMA1* allele was then entirely sequenced by double-strand dideoxynucleotide sequencing, using primer walking to check the presence of the introduced mutation and to exclude any inadvertent second-site change that might have been introduced during *in vitro* mutagenesis or *in vivo* recombination.

Isolation of Plasma Membranes. For each strain, three different preparations of plasma membranes were made from independent cultures as described by Dufour *et al*. (1988) with the following modifications. When glucose incubation (Serrano, 1983) was used, yeast cells were harvested, washed three times in cold double-distilled water, and then incubated for 30 min at 30 °C in 0.1 M MES/TRIS,[1] pH 6.5. After addition of 1 M glucose (1:10 v:v) (activated cells) or 1 M sorbitol (1:10 v:v) (deactivated cells) to the cell suspension, incubation was continued for another 15 min prior to isolation of plasma membranes. Cells were then disrupted with glass beads in 50 mM TRIS/CH_3COOH, pH 7.5, 1 mM $MgCl_2$, 1 mM PMSF, and either 250 mM glucose (activated cells) or 250 mM sorbitol (deactivated cells) as described elsewhere (Dufour *et al*., 1988). The membranes were aliquoted and stored at −80 °C at a protein concentration of 5−10 mg/mL in 0.01 M TRIS/CH_3COOH, pH 7.5, 1 mM EDTA, 1 mM ATP.

Preparation of Sealed Plasma Membranes. Liposomes were made by sonicating a 2 mL aliquot of a suspension of purified asolectin (50 mg/mL) in buffer A containing 25 mM K_2SO_4, 5.2% (w/v) glycerol, 50 mM MES/KOH, pH 6.0, at room temperature in a Laboratory Supplies Co. bath sonicator as previously described (Dufour *et al*., 1982). As described by Venema *et al*. (1993), 1.5 mL of liposome was mixed with 450 μL of 10% w/v deoxycholate in buffer A, 1.05 mL of buffer A was then added, and the resulting solution was stored on ice. A total of 5 mg of protein of plasma membranes was diluted with buffer A to a concentration of 2.5 mg of protein/mL and kept on ice. A 3 mL amount of the liposome−deoxycholate dispersion was added to 2 mL of the plasma membrane suspension at 0 °C, and the resulting mixture was manually shaken every 30 s for 10 min. The mixture was centrifuged at 100 000*g* (38 000 rpm) for 1 h in a Beckman Ti50 rotor at 4 °C. The supernatant was discarded, and the pellet was resuspended in 2.2 mL of buffer A and kept on ice until use.

Measurement of ATP-Dependent Proton Transport. ATP-dependent proton translocation was measured by recording the fluorescence quenching of ACMA. Fluorescence measurements were carried out with a Bio-Logic Co. (Zirst, Meylan, France) fluorimeter. The excitation wavelength was set at 410 nm, and emission was detected after passage through a Balzers K 50 broad-band filter (500 nm) and a Corning 3-72 cutoff filter to reduce light scattering. The assay medium at 30 °C contained 2.45 mL of buffer A, 450 μL of sealed plasma membrane suspension (approximately 400 μg of protein), and 5 μL of ACMA (0.5 mM stock solution in ethanol). All assay ingredients except MgATP were preincubated for 7 min at 30 °C. The fluorescence intensity obtained, after correcting for light scattering by the vesicles, by full-scale reading on the recorder was taken as 100%. A 30 μL aliquot of MgATP solution (150 mM $MgSO_4$, 150 mM ATP, 10 mM MES/KOH, pH 6.0) was added to start the reaction.

Kinetic Studies of Plasma Membrane H^+−ATPase. All data were obtained in duplicate from three different membrane preparations isolated from independent cultures. Standard ATPase activity was measured at 35 °C in 1.0 mL of reaction mixture, containing 1 mg of protein/mL, 50 mM MES, 50 mM MOPS, 50 mM TRIS, adjusted with HCl or KOH to the pH indicated, 6 mM $(MgATP)^{2-}$, and 10 mM NaN_3. Other conditions were as described by Dufour *et al*. (1988). The concentration of free Mg^{2+} was 1 mM. The amounts of $MgSO_4$ and Na_2−ATP to be added to the reaction mixture to produce the required final concentrations of free Mg^{2+} and $(MgATP)^{2-}$ were calculated as described by Wach *et al*. (1990). Measurements of apparent K_m and specific activity were performed in 50 mM MES/KOH, pH 6.0, 1 mg of protein/mL, and the concentration of $(MgATP)^{2-}$ was varied between 0.1 and 6.0 mM, as indicated. An ATP regenerating system [100 μg of pyruvate kinase and 5 mM phospho(enol)pyruvate] was added to the reaction mixture. ATPase activity at low substrate concentrations (about 30 nM MgATP final concentration) was measured by monitoring the concentration of free ATP remaining in the reaction mixture with the luciferine/luciferase bioluminescence assay (Wach & Gräber, 1991), using an integrating photometer (SAI Technology Co., San Diego, CA). Prior to these measurements, ATP present in the plasma membranes suspension buffer was removed by washing 150 μL of plasma membranes 4 times in 8 mL of 10 mM TRIS/CH_3-COOH, pH 7.5, 1 mM EDTA. The reaction mix was 3 mL of 50 mM MES, 50 mM MOPS/KOH, pH 6.0, 1 mM $MgSO_4$, 10 mM NaN_3, and 35 μg of protein. The reaction was started by adding 500 μL of 200 nM ATP. At the indicated times, 300 μL of the reaction mixture was mixed with 300 μL of ATP bioluminescence CLS reagent. The luminescence signal was calibrated by the addition of a

[1] Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; K_i, inhibitor constant.

Table 2: Kinetic Parameters of the Purified Plasma Membrane ATPase from Pma1, Pma1-H285Q, and Pma1-H285R in Sorbitol-Incubated or Glucose-Activated Cells[a]

	ATPase activity				K_i (μM vanadate)			
	K_m (mM)		V_{max} (μmol min^{-1} mg^{-1})		vanadate/ATP		vanadate/H^+	
strains	glucose	sorbitol	glucose	sorbitol	glucose	sorbitol	glucose	sorbitol
H285	0.73 ± 0.12	1.70 ± 0.30	11.4 ± 0.4	7.1 ± 0.5	9.5 ± 1.2	18 ± 5.2	2.4 ± 1.0	4.2 ± 0.8
H285Q	0.73 ± 0.37	1.24 ± 0.25	17.9 ± 0.3	11.9 ± 2.8	184 ± 34	416 ± 137	64 ± 34	209 ± 97
H285R	0.81 ± 0.23	2.27 ± 0.66	19.2 ± 0.8	9.6 ± 1.7	131 ± 23	493 ± 226	56 ± 17	277 ± 62

[a] ATPase activity was assayed in purified plasma membranes as described under Materials and Methods. Results are mean values ±SD (n = 3). V_{max} and K_m values were calculated as illustrated in Figure 3. Inhibition constants were obtained from Dixon plot analysis. The noncompetitive inhibition between vanadate and ATP was measured at pH 6.0 and varying MgATP concentrations. The competition between vanadate and H^+ was measured at pH 6.0 and 6.4 at 6 mM MgATP.

known amount of ATP to the assay cuvette. Protein concentration was determined by the method of Lowry *et al*. (1951) using BSA as a standard.

RESULTS

Construction and Verification of the pma1 Mutants. More than 100 genes coding for members of the P-type ATPase family from different organisms have been sequenced, and 17 H^+−ATPases from plants and fungi have been aligned (Wach *et al*., 1992). On the basis of this alignment, the seven most highly conserved histidines from *S. cerevisiae* Pma1, H240, H285, H488, H614, H686, H701, and H914, were selected for oligonucleotide-directed, site-specific mutagenesis. In addition to the histidine mutants, the negative mutation D378N, which prevents the formation of the aspartyl phosphate catalytic intermediate, was used as a control. Recombinant strains were obtained in the host strain YPS299C grown on glucose medium by co-transformation with linearized plasmid and DNA fragments bearing different *PMA1* mutant alleles (see Table 1 for list of strains). YPS299C is the haploid progeny of the diploid strain YPH501 (Sikorski & Hieter, 1989), whose genomic copies of *PMA1* and isogene *PMA2* are both deleted. PMA1 is an essential gene encoding the H^+−ATPase gene. PMA2 is a poorly expressed isogene of PMA1 (Schlesser *et al*., 1988) which can sustain cell growth in the absence of the PMA1 gene when put under the control of a strong promoter such as that of PMA1 (Supply *et al*., 1993). The YPS299c host strain carries a 2 μm plasmid with *URA3* and *leu2-d* markers and the *PMA2* structural gene, under the control of the *PMA1* promotor to ensure viability in the absence of Pma1 protein (Supply *et al*., 1993). Our recombination system allowed for recombination events producing mutant alleles fused either to the *GAL1* promotor or to the *PMA1* promotor. In this situation, recombinants carrying toxic negative mutant alleles grow only on glucose when the *GAL1* promotor is repressed and do not grow when put under *PMA1* promotor control. Successful recombinations were checked by restriction site analysis of recombinant plasmids extracted from *E. coli* transformed with DNA preparations from individual Leu^+ recombinant yeast strains grown in glucose. This analysis showed that all histidine substitutions listed in Table 2, except H285 and H701, were found in fusions with the *PMA1* promotor, whereas the expression of the negative mutation D378N and the mutations H285E and H701D were found in fusions with the *GAL1* promotor only when tested on glucose. This was the first indication that yeast could grow only when expression of the alleles H285E, D378N, and H701D was repressed.

To exclude any accidental mutation during *in vitro* mutagenesis or recombination, all mutants were checked for the presence of newly-created restriction sites, introduced by the site-directed mutations. In addition, mutant alleles giving a detectable phenotype (H285E, H285Q, H285R, D378N, and H701R) were entirely sequenced. The presence of the site-directed mutation was confirmed in all cases. In addition, comparison with the published sequence of *PMA1* from yeast strain S288C (Serrano *et al*., 1986) showed that seven nucleotide differences were found in all sequenced mutant alleles. Six gave silent triplet changes (C1380G, A1562G, T2055C, C2241A, C2465A, G2474A, numbering relative to the initiating ATG codon), one of which (T2055C) has been reported to occur in the *PMA1* sequence of strain Σ1278b (van Dyck *et al*., 1990). Only one difference, G2152A, changed the coding triplet in such a way that another amino acid was generated (D718N). Since these differences were observed in all the mutant alleles, they must correspond to strain polymorphism. This was confirmed independently in the same parental strain for mutants C1380G (P. Supply, personal communication) and G2152A (A. de Kerchove d'Exaerde, personal communication). All the phenotypic and biochemical effects observed therefore result only from the introduced site-directed mutation.

Viability of the pma1 Mutants. The viability of the different yeast recombinants was first tested in MGlu (Figure 2A, upper panel). On MGlyEtGal containing 5-fluoroorotic acid (Figure 2A, middle panel) the *URA3*, *leu2-d* multicopy (2 μm) plasmid with the P*PMA1::PMA2* construct is counterselected in the strain YPS229C. Under these conditions, all constructed mutant alleles are transcribed, but growth is only seen in the case of mutant alleles able to complement the disrupted essential *PMA1* gene. The substitutions D378N (control), H285E, and H701D were not tolerated by the cells, suggesting an important role for these residues in plasma membrane H^+−ATPase function. Analysis of the plasmids extracted from all viable 5-FOA-resistant yeast cells confirmed the presence of the introduced mutation, the *PMA1* promotor-controlled mutant alleles and the loss of the *URA3* multicopy plasmid. Moreover, on galactose where the mutant is expressed as well as the wild type (MGAl, Figure 2A, lower panel), a dominant negative effect was observed for mutations *pma1-D378N* (control) and *pma1-H701D*. Both mutants did not grow on galactose, although a functional *PMA2* allele was expressed.

Other amino acids were substituted for the histidine residues H285 and H701, which were found to be lethal in the first screen. Glutamine and arginine were used, since they are similar in bulkiness to histidine and also make it

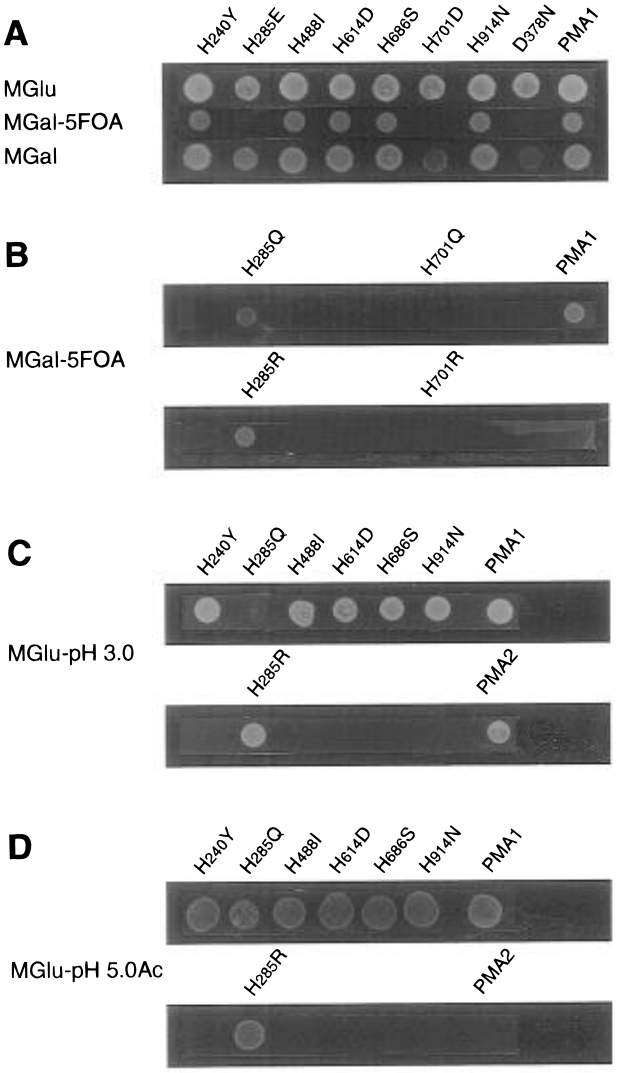

FIGURE 2: Drop test for growth of the constructed yeast strains on selective media. Tests were made by depositing 5 μL of preculture, grown in MGlyEt-L to a density of 3×10^7 cells/mL, on selective plates. Strains are recombinants carrying the different *pma1* mutant alleles (particular amino acid replacement are indicated for each strain) or the *PMA1* wild type allele (PMA1) on a plasmid. As an additional control we have used the strain YPS12-1 in which genomic alleles of *PMA1* and *PMA2* are deleted and which carries the P*PMA1::PMA2* fusion on a centromeric plasmid with the *LEU2* selectable marker (PMA2). Plates were photographed after 36 h of growth at 30 °C. (A) Upper panel: MGlu-L (control). Middel panel: MGlyEtGal containing 1 g of 5-fluoroorotic acid/L. Lower panel: MGlyEtGal-L. (B) As in A, middle panel, but for recombinant strains from the second screen. (C) MGlu-L plates adjusted to pH 3.0 with 1 N HCl. (D) MGlu-L plates containing 200 mM sodium acetate adjusted to pH 5.0 with 1 N HCl.

possible to test whether a positive charge is a prerequisite. Both amino acids have been statistically scored as being preferentially accepted as spontaneous histidine substitutes in many protein families (Dayhoff, 1978; Bordo & Argos, 1991). The results of this screen (Figure 2B) demonstrated that the *PMA1* deletion was complemented by *PMA1-H285Q* or *PMA1-H285R* but not by either *PMA1-H701Q* or *PMA1-H701R*. Restriction site analysis of the individual mutant alleles showed that *PMA1-H285Q* and *PMA1-H285R* were fused to the *PMA1* promotor, whereas all *PMA1* mutant H701 alleles were fused to the *GAL1* promotor and were thus only expressed in galactose. All tolerated *PMA1* mutations were then checked for temperature sensitivity (18 or 37 °C) and pH sensitivity (pH 3.0 or 5.0 in the presence of acetate or

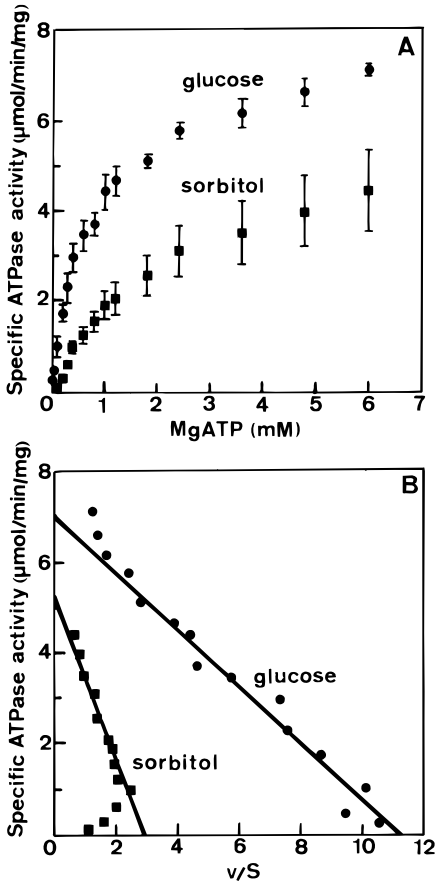

FIGURE 3: Substrate dependence of ATP hydrolysis (A) and determination of K_m by Eadie−Hofstee plots (B) for plasma membrane ATPase from Pma1 sorbitol-incubated (squares) or glucose-activated (circles) cells. ATP hydrolysis measurements were done as described under Materials and Methods using purified plasma membranes (error bars represent the standard deviation; $n = 3$).

pH 7.5). As an additional control, we used strain YPS12-1, which is isogenic with YPS229C, except that it carries the *PMA1* promotor fused to the *PMA2* structural gene on a centromeric, *LEU2*-containing plasmid instead of the 2 μm plasmid (Supply *et al*., 1993). As shown in Figure 2C,D, this strain grows slowly at pH 3.0 and does not grow in acetate-containing medium at pH 5.0. These figures also show that a significant difference in growth between mutant and control strains was seen only with *PMA1-H285Q*, which was sensitive to pH 3.0 and grew slowly on acetate-containing medium at pH 5.0. It is concluded that H701 is an essential residue, whereas H285, while not strictly essential, is important for growth under acid conditions. No other histidine replacement tested was found to be important under any growth condition used.

Kinetic Analysis of pma1-H285 Mutants. The ATPase activity of plasma membranes isolated from the H285 strain containing only the wild type ATPase was tested at different MgATP concentrations in the presence of a constant concentration of 1 mM free magnesium. Immunoblot analysis reveals that equivalent amounts of the major 100 kDa subunit was found in the three tested strains, H285, H285R, and H285Q) (data not shown). Figure 3A shows that typical Michaelis−Menten kinetics was not obtained, since the activity from glucose activated cells did not plateau at concentrations of MgATP up to 6 mM. This trend was even more accentuated in membranes from sorbitol-treated cells. The complexity of the kinetics is illustrated by the

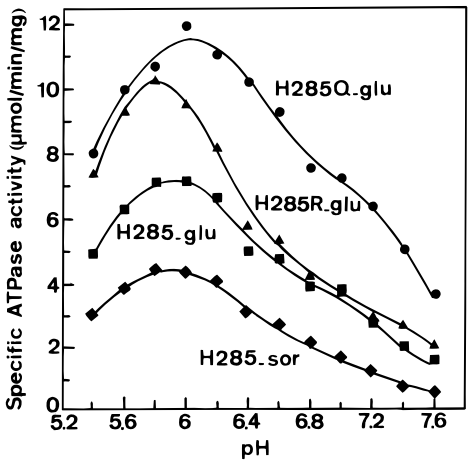

FIGURE 4: pH dependence of ATP hydrolysis and stimulation of plasma membrane ATPase activity by glucose. pH dependence of ATP hydrolysis for plasma membranes from glucose- or sorbitol-activated cells of H285, H285Q, and H285R, ATP hydrolysis was measured as described under Materials and Methods with purified plasma membranes (error bars represent the standard deviation; $n = 3$).

curved Eadie−Hofstee plots, shown in Figure 3B. Attempts were made to analyze these data by considering either two independent or two interactive substrate binding sites. Even though some best-fit data were obtained giving two distinct K_m and V_{max} values, their physiological and biochemical relevance was doubtful, as some K_m values were not realistic, e.g., as high as 50−100 mM MgATP. Therefore only the specific activities at pH 6.0 and 6 mM MgATP were considered, as was the apparent K_m, which was determined by the substrate concentration at which half of the specific ATPase activity at pH 6.0 and 6 mM MgATP was reached. Table 2 shows the mean data from three different membrane preparations from each strain, incubated in either glucose or sorbitol. It has been shown indeed that a 15 min incubation of yeast cells with glucose (but not with sorbitol) remarkedly stimulated the ATPase activity which was measured subsequently in the isolated plasma membranes (Serrano, 1983). ATPase activity was markedly enhanced in plasma membranes from mutants H285Q and H285R, but glucose activation of ATPase activity was not modified by mutation. These data indicate that H285 participates in the ATPase catalytic cycle but not in its regulation by glucose.

One way of testing whether it is the H^+ acceptor−donor property of H285 that is relevant to ATP hydrolysis is to compare the proton concentration dependency of the ATPase activity of wild type and mutant proteins. If ATPase activity was directly influenced by the positive charge of the histidine side chain, its pK of 6.9 would be expected to determine the pH sensitivity of the enzymatic activity within the range where acid/base transitions occur. If the ATPase activity was strictly dependent on the protonation state of the histidine residue, its replacement by an uncharged glutamine residue would not be permitted and it would be expected that replacement by arginine (pK = 12.5) would displace the pH optimum of ATPase activity toward basic values. The pH curve for ATPase activity is far from symmetrical, with a peak at pH 5.8 to 6.2 and a broad shoulder from pH 6.4 to 7.6. Figure 4 shows that in our strain, and in contrast to other observations (Serrano, 1983), the pH profile is not influenced by glucose activation. Replacement of wild type H285 by nonprotonatable Q285 had no effect on the biphasic pH profile, even though the activity of the mutant was 1.7-fold higher at all pH values tested. In contrast, replacement of H285 by arginine enhanced the peak activity over the pH range 5.4−6.2 but had no effect at higher pH values.

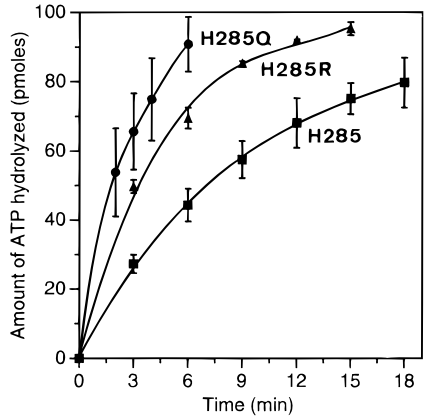

FIGURE 5: ATP hydrolysis at very low ATP concentration. Experiments were done as described under Materials and Methods using purified plasma membranes (35 μg of protein) of glucose-incubated cells from H285 (squares), H285Q (circles), and H285R (triangles) (error bars represent the standard deviation; $n = 3$).

In the next step, we checked whether H285 was important for vanadate inhibition of the yeast plasma membrane H^+−ATPase (Willsky, 1979). If the positive charge of H285 contributed, either directly or indirectly, to vanadate binding, one would expect to see differences in the pH profile of vanadate inhibition in the mutants. The data obtained from wild type ATPase activity measurements at different proton concentrations and increasing vanadate concentrations were found to be competitive. Inhibition by vanadate was noncompetitive with MgATP (Dufour *et al*., 1980), but protons irrespective of whether the enzyme was prepared from glucose activated cells or from cells incubated with sorbitol showed competitive interaction with vanadate in membranes from H285 as well as from the mutants H285Q and H285R. The derived inhibition constants are shown in Table 2. In all strains, glucose activation decreased both the apparent K_i for vanadate inhibition, measured at pH 6.0, and the K_i for proton/vanadate competition by a factor of 2−5. It can be concluded once more that glucose activation of vanadate sensitivity is not modified by mutation of H285. In contrast, in the mutants, vanadate sensitivity decreased about 20-fold and proton/vanadate competition was decreased 30−50-fold. These drastic changes in vanadate inhibition might be due to alteration of the E_2−E_1 equilibrium in the enzyme, but neither [E_1] nor [E_2] can be directly determined experimentally. However, some conclusions about [E_1] can be drawn from ATP hydrolysis rate measurements at very low substrate concentrations. When [ATP] is much lower than K_m, simple derivation of the Michaelis−Menten equation shows that v equals k_2/K_m[E][ATP] or that [E_1] equals vK_m/k_2[ATP], where k_2 is the turnover rate and E_1 is the free enzyme conformation that binds ATP.

Thus, the initial reaction velocity (v) at very low [ATP] is proportional to [E1]. The data in Figure 5 show higher initial velocities (v) with Pma1-H285Q than with Pma1-H285R or Pma1. We thus infer that [E_1] in the mutant H285R and H285Q is, respectively, 2- and 3-fold higher than in wild type H285.

In order to check whether H285 is directly involved in H^+ pumping, sealed plasma membrane vesicles were prepared from H285 and the mutant H285Q. ACMA quenching as well as ATPase activity was measured under the exact

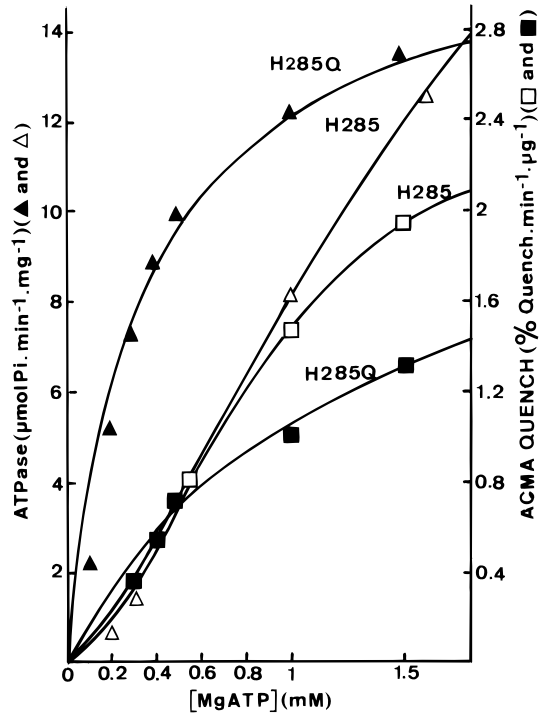

FIGURE 6: ACMA quench and ATP hydrolysis rates at different MgATP concentrations in wild type (H285) and mutant H285Q sealed plasma membranes. The initial rates of ATP-induced quench in H285 (□) and H285Q (■) were measured in sealed plasma membranes as described in Materials and Methods. ATPase activity in H285 (△) and H285Q (▲) was measured for 8 min under the exact same conditions as the ACMA quench assay described in Materials and Methods with the exception that ACMA was not added.

same conditions at different concentrations of MgATP ranging from 0.1 to 1.5 mM. Figure 6 shows that, although the ATPase activity is higher in H285Q than in H285 (especially at the lowest MgATP concentrations), the H^+ pumping activity is lower in the mutant H285Q than in H285 at the concentration of 1 mM MgATP or above. Even though detailed kinetic comparison of the relation between H^+ pumping and ATP hydrolysis is obviously complex, it is clear that increased ATPase activity in the mutant is not accompanied by increased H^+ pumping. In other words, under the conditions tested, the mutant H285Q H^+ pumping is partially uncoupled from hydrolysis.

DISCUSSION

The repair of deleterious mutations, introduced into essential genes such as *PMA1* (Harris *et al*., 1991), can lead to misinterpretation due to recombinations between *PMA1* and *PMA2* [for mutation D378N, see Portillo and Serrano (1988), Serrano *et al*. (1992), and Harris *et al*. (1993)]. The expression system used in this work was designed to decrease this risk by means of the following features: (1) The expression plasmids containing the mutant alleles were made *in vivo* by homologous recombination. On glucose, two alternative recombination products can form, one (P*GAL1::pma1**) under the control of the promoter P*GAL1* conditionally expressing the mutant allele, the other (P*PMA1*::*pma1**) under the control of the promoter P*PMA1* constitutively expressing the mutant allele. (2) When the mutation is toxic, the P*GAL1::pma1** recombination product is *autoselected* on glucose, since this is the only construct where expression is repressed on glucose. Under these conditions, selection pressure for repair by *PMA2* in cells harboring a toxic *pma1* allele is removed. (3) When desired, expression of the toxic allele can be transiently induced by galactose. (4) Placement of the mutant allele on a plasmid facilitates its extraction and verification by restriction mapping or dideoxynucleotide sequencing. (5) The wild type *PMA2* gene, placed on a *URA3* multicopy plasmid under the control of the promoter *PMA1* is selectively removed by plasmid shuffling in case of complementation of the deleted *PMA1* allele by the expressed *pma1** mutant allele. This stabilizes the mutation, since the strain is then devoid of the genomic *PMA1* and *PMA2* genes with which the *PMA1* mutant allele could recombine (Harris *et al*., 1993).

Using simple growth tests on recombinant strains obtained after site-directed mutagenesis of the seven most highly conserved histidine residues of *S. cerevisiae* Pma1 ATPase, three groups of histidine residues were distinguished. The five residues, H240, H488, H614, H686, and H914, are neither essential nor catalytically important, whereas residue H285 is not essential but is catalytically important and residue H701 is essential. H285 and H701 are conserved in all fungal proton ATPases sequenced. In addition, H285 is also conserved in all known plant H^+−ATPase genes (Wach *et al*., 1992).

Two classes of lethal H^+−ATPase mutants, identified in this work, can be distinguished. Some, like H285E, are recessive and do not grow when expressed in the absence of the wild type gene but do grow when wild type and mutant genes are coexpressed. Others, like H701D, H701Q, H701R and D378N, are dominant and do not grow even in the presence of a functional wild type Pma1.

Besides the essential H701, the residue H285 was found to play an important role in H^+−ATPase function. At this position, a positive charge is not required, since charge neutralization of H285Q is tolerated even though the mutant strain grows poorly, especially under conditions where the need for a well-functioning ATPase is more pronounced. The charge inversion mutation H285E is, however, not allowed. *In vitro*, the mutants H285Q and H285R show differences in specific activity and vanadate sensitivity, as well as modification of the pH profile of ATPase activity. None of the observed kinetic differences is significantly modified by glucose activation of yeast cells, excluding any specific participation of H285 in this regulatory process. Direct participation of H285 in proton transport is also excluded, since the enzyme is functional in the absence of the charged His residue, e.g., as in H285Q and since H^+ pumping activity measured in the mutant H285Q proceeds at a significant (although reduced) rate. However, the pH profile of H285R ATPase activity is modified over the pH range 5.8−6.4, indicating that the protonation state of the substituted Arg residue indirectly influences ATPase activity. The rate of ATP hydrolysis of the plasma membrane H^+−ATPase at very low substrate concentrations ($[ATP] \ll K_m$) is increased in both H285Q and H285R. Since, under these conditions, velocity is a function of E_1 concentration, the most plausible interpretation of these data is that the H285R and H285Q substitutions modify the E_2-E_1 equilibrium in favor of increased concentration of the E_1 conformation and decreased concentration of E_2. This would lead to a partly futile use of ATP not completely linked to H^+ transport which in acid conditions might be responsible for growth retardation of the mutants. This conclusion is in agreement with the partial uncoupling of the H^+−ATPase pumping observed in H285Q

as well as with the increased specific activity observed with both mutants H285Q and H285R, since the E_2-E_1 transition is believed to be the rate-limiting step in the ATPase catalytic cycle (Amory *et al*., 1982). An alternative method of studying the E_2-E_1 equilibrium is to compare the pattern of vanadate inhibition of wild type and mutant enzymes. It is believed that vanadate locks the Na^+/K^+−ATPase (Cantley *et al*., 1978) and the Ca^{2+}−ATPase (Pick, 1982) in a transition state equivalent to an inactive E_2·Pi or E_2-P conformation. High pH leads to an increased concentration of E_2 (Amory *et al*., 1982) and to an increased vanadate sensitivity (Blanpain *et al*., 1992). Thus, the increased vanadate resistance in the H285 mutants can be interpreted as a direct consequence of the modification of the E_2-E_1 equilibrium and might serve as an indicator of lower levels of E_2 conformers during turnover. The competition between proton and vanadate indicates that they both act on the same E_2 species. The decreased inhibitory effect of H^+ on vanadate sensitivity in the mutants is thus in agreement with a decreased level of E_2 in these strains. Such a conclusion would not be incompatible with data on vanadate resistance located in the same region of the β-stranded domain of the ATPase domain, such as T231G (Portillo & Serrano, 1989), K250T (Ghislain *et al*., 1992), G270D (Ulaszewski *et al*., 1987), and V289F (Harris *et al*., 1991).

In conclusion, H701 is an essential residue of the yeast plasma membrane ATPase as all amino acid replacements tested at this position produced a dominant negative phenotype. H285, although not essential, is important for ATPase function. If one regards an increased vanadate resistance and an increased rate of ATP binding as indicators of a shift in the E_1-E_2 equilibrium toward an increased E_1 concentration, a crucial role in E_1-E_2 transition of Pma1 can be assigned to H285.

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