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The yeast plasma membrane proton pumping ATPase is a viable antifungal target. I. Effects of the cysteine-modifying reagent omeprazole

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Abstract

The yeast plasma membrane proton pumping ATPase (H^+-ATPase) was investigated as a potential molecular target for antifungal drug therapy by examining the inhibitory effects of the sulfhydryl-reactive reagent omeprazole on cell growth, glucose-induced medium acidification and H^+-ATPase activity. Omeprazole inhibits the growth of *Saccharomyces cerevisiae* and the human pathogenic yeast *Candida albicans* in a pH dependent manner. Omeprazole action is closely correlated with inhibition of the H^+-ATPase and is fungicidal. Glucose-dependent medium acidification is correspondingly blocked by omeprazole and appears to require the H^+-ATPase to proceed through its reaction cycle. A strong correlation is observed between inhibition of medium acidification and H^+-ATPase activity in plasma membranes isolated from treated cells. The inhibitory properties of omeprazole are blocked by pre-treatment of activated drug with β-mercaptoethanol, which is consistent with the expected formation of a sulfhydryl-reactive sulfenamide derivative. Mutagenesis of the three putative membrane sector cysteine residues (C148S, C312S, C867A) in the *S. cerevisiae* H^+-ATPase suggests that covalent modification of the conserved C148 residue may be important for inhibition of ATPase activity and cell growth. Other mutations (M128C and G158D/G156C) mapping near C148 support the importance of this region by modulating omeprazole inhibition of the H^+-ATPase. These findings suggest that the plasma membrane H^+-ATPase may serve as an important molecular target for antifungal intervention.

Keywords: ATPase, H^+-; Plasma membrane; Drug targeting; Omeprazole; (Yeast)

1. Introduction

Fungal disease primarily occurs in humans when natural barriers to infection, such as the skin, mucosal surfaces, the immune system or competition with the endogenous microflora are compromised [1]. While superficial infections of mucosal surfaces are readily treated, disseminated infections are frequently life-threatening because the patient is usually debilitated and because of the limited efficacy and toxic side-effects of existing antifungals. Novel drug targets are required so that new classes of antifungal drugs can be developed. The plasma membrane H^+-ATPase of yeast is an essential enzyme that may represent an important new target [2]. It is a P-type ion translocating ATPase, a diverse class of ion pumps found in higher and lower eukaryotes, and many prokaryotes. Many of these enzymes perform a critical physiological function by maintaining electrochemical ion gradients across the plasma membrane of their respective cells. The binding of ATP within a cytoplasmic domain results in the formation of an aspartyl-phosphate intermediate that initiates a series of conformational intermediates leading to the stoichiometric movement of an ion(s) across the membrane. This fundamental role and the fact that these membrane-spanning enzymes have exposed extracellular do-

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mains have been key factors in the development of clinically important therapeutics which target these enzymes.

The Na^+,K^+-ATPase, which is inhibited by ouabain and the cardiac glycosides [3], and the gastric H^+,K^+-ATPase, which is inhibited by omeprazole [4] and a range of K^+-competitive compounds including SCH28080 [5], are currently the two most important pharmacological targets among the P-type ATPases. The interaction of therapeutic agents with these targets is known to occur from outside the cell but a detailed understanding of their mechanism of inhibitory action is just emerging [3–6]. These inhibitors block the cytoplasmic catalytic activity and interact with the enzyme most favorably during the E_2 phase of the reaction cycle [3,5]. Several cell surface amino acid residues have been implicated in the interaction between ouabain and the α_1-subunit of the Na^+,K^+-ATPase. They include two carboxyl groups at the edges of the turn between transmembrane segments H1 and H2 [7–9], a proline in the middle of the H1-H2 turn [8], membrane embedded cysteine and tyrosine residues in H1 [10–12], aromatic groups in the extracytoplasmic turn between transmembrane segments 3 and 4 [3,13] and, most recently, an arginine residue located in the extracytoplasmic turn between transmembrane segments H7 and H8 [11]. Although many of these residues or regions affect the overall affinity of the enzyme for ouabain, or were identified on the basis of photoaffinity labeling studies, their precise role(s) in the interaction between ouabain and the Na^+,K^+-ATPase has yet to be defined.

Covalent binding of the photoaffinity analog MeDA-ZIP$^+$ to the gastric H^+,K^+-ATPase implicated F124, near the C-terminal end of transmembrane segment H1 and D136, near the N-terminal end of transmembrane segment H2, in the binding of SCH28080 to the enzyme [14,15]. This led to a molecular model of the SCH28080 H1-H2 complex, in which the inhibitor interacts with the extracellular face of the transmembrane loop structure [14,15]. The highly effective anti-ulcer drug omeprazole is known to rapidly and selectively covalently react with cysteine residues following acid activation to a sulfenamide; however, labeling of the H^+,K^+-ATPase by omeprazole has been problematic. Morii et al. [16] first reported that the sulfenamide interacted with an externally located cysteine in the N-terminal half of the enzyme. More recently, Besancon et al. [4] reported that omeprazole labeled C813 and C822 in the external turn between transmembrane segments H5 and H6 and possibly C892 at the end of transmembrane segment H7. Shin et al. [6] used pantoprazole, a more stable chemical congener of omeprazole, to covalently modify only C813 and C822, suggesting that C892 may not be essential for omeprazole inhibition of the ATPase. If C813 and C822 are indeed the critical residues modified by omeprazole, it remains to be seen how this modification leads to enzyme inactivation.

Genetic and biochemical studies of the yeast plasma membrane H^+-ATPase have shown that the region linking transmembrane segments 1 and 2 is highly conformationally active [17–20]. The equivalent region in the gastric H^+,K^+-ATPase is also believed to undergo a conformational change during phosphorylation [21]. Mutations in this region of the yeast H^+-ATPase are known to alter the kinetic properties of the enzyme including the V_{max} and K_m for ATP hydrolysis [17,18]. The effects of such mutations are therefore communicated to the cytoplasmic active site, either by conformational interaction or by altering the kinetic states of intermediates required for the normal reaction cycle of the enzyme. Many of these mutations also confer growth resistance to hygromycin B [17–20,22–24], which is thought to be associated with a defect in the ability of the H^+-ATPase to generate an hyperpolarized membrane potential [23,25]. In addition, several mutations in the region are either strongly selected against in heterozygous diploid cells or give recessive lethal mutations in haploid progeny, indicating that the modified residues may be essential for enzyme structure/function and cell growth/viability [19,20]. Taken together, such observations suggest that the first transmembrane loop of the yeast plasma membrane ATPase has the attributes of a useful inhibitory target site [2]. Given the urgent need to develop new classes of antifungal agents and the fact that the fungal plasma membrane ATPase is an essential enzyme which can control the rate of cell growth [26,27], our research has focused on the properties of the extracytoplasmic and transmembrane regions of the yeast ATPase.

The development of the plasma membrane H^+-ATPase as a molecular target for antifungal drug therapy requires the demonstration that inhibition of enzyme activity correlates with cessation of cell growth. Furthermore, drugs which target extracytoplasmic regions of the H^+-ATPase are highly desirable since they do not need to enter the cell, thus avoiding most cellular pump-based resistance mechanisms. We therefore chose omeprazole, an acid-activated sulfhydryl-scavenging reagent [28] which can be effectively localized to the extracellular portion of the cell, to modify and inhibit the yeast plasma membrane ATPase in vivo. This approach was also based on the presence of two conserved cysteine residues near the cell surface of the ATPase [2] as well as the ability of yeast cells growing in glucose medium to maintain an acidic extracellular milieu. We have shown that omeprazole inhibits the growth of *Saccharomyces cerevisiae* and the human pathogenic yeast *Candida albicans* in a pH-dependent manner that is consistent with the acid-activation profile of the drug [29]. Omeprazole action is fungicidal and inhibition of cell growth corresponds well with inhibition of both proton pumping, determined in whole cells, and H^+-ATPase activity, measured in isolated plasma membranes derived from treated cells. Genetic probing suggests that covalent modification of C148 in transmembrane segment 2 is important for inhibition of enzyme function and cell growth. The potential of the H^+-ATPase as a molecular antifungal target is discussed.

2. Materials and methods

2.1. Yeast strains and cell culture

The yeast strains used in this study, along with their phenotypic characteristics are described in Table 1. All strains are isogenic derivatives of *Saccharomyces cerevisiae* wild type strain Y55 (*HO gal3 MAL1 SUC1*). The control strain SN236 (*HO ade6-1 trp-5-1 leu2-1 lys1-1 ura3-1 PMA1::URA3*) [17] was made by selecting a *leu URA* spore from an SH122 derivative in which the *LEU2*-disrupted *pma1* gene was replaced with *PMA1:URA3*. Strain C148S was obtained from the C148S S368F double mutant by transforming this mutant with a 4.2 kb *Pvu*II-*Hin*dIII fragment of *PMA1:URA3*, thereby reverting to wild type the S368F mutation [18]. Strain G158D/G156C was obtained as a pH resistant revertant of G158D [30]. The *Candida albicans* strains used in this study was ATCC10261, ATCC23187 and B311. Cells were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) at pH 5.5 or in medium adjusted to the indicated pH with concentrated HCl.

2.2. Site-directed mutagenesis

Site-directed *pma1* mutants were constructed in vectors pSN54/57, essentially as described by Na et al. [17]. To obtain isogenic *pma1* mutants a 6.1 kb linear *Hin*dIII DNA fragment, containing *URA3* inserted into the *Bgl*II site in the 3'-nontranslated portion of *PMA1*, was excised from each reconstituted pSN57 vector and was used to transform yeast strain SH122 (*HO ade6-1 trp5-1 leu2-1 lys1-1 ura3-1 pma1Δ::EU2/PMA1*) [18]. *Ura leu* transformants were sporulated and dissected, and the growth phenotypes of each dissected *URA* colony determined. All *pma1* mutants (Table 1) were assayed for growth on YPD medium at pH 5.0 containing 0.57 mM hygromycin B and on YPD medium adjusted to pH 2.5 with 1 M HCl [24].

2.3. Drug challenge growth assays

Cells were grown to mid-log phase ($A_{595nm} \sim 5$) on YPD medium and diluted to an $A_{595nm} \sim 0.2$ in YPD medium at the pH used for subsequent growth. Omeprazole (145 mM in 90% DMSO) was either added directly to YPD medium at the required pH or acid-activated for 1 h in the presence of 0.1 M HCl prior to addition. Growth assays were conducted in sterile flat-bottomed 96 well microtitre plates containing 200 μl of YPD per well. The cells were agitated on a gyratory shaker at 150 rpm for 20–24 h at 30°C and the growth of the cultures was measured using either a SLT Elisa reader (SLT Labinstruments, Research Triangle Park) or an EAR 340 microtitre plate reader (SLT Instruments, Austria). Absorbance measurements were made at either 590 or 620 nm, with about a 10-fold difference between absorbance 'unit' readings measured on the Elisa readers and the true absorbance values determined by a spectrophotometer. Blanks containing omeprazole but no cells were included to take into account the background contribution (A_{590nm} or $A_{620nm} < 0.05$) of acid-activated omeprazole. All readings were in duplicate or triplicate, giving absorbance readings within 5% of each other.

Table 1
Phenotypes of wild type and *pma1* mutant strains

Strain	Phenotype [a]	
	HygB	low pH
SN236 (wt)	S	R
C148S	R	R
C312S	S	R
C867A	S	R
M128C	R	S
M128A	Recessive lethal	
G156C, G158D [b]	R	R

[a] Growth resistance (R) or sensitivity (S) to YEPD solid media at pH 5.0 containing 0.57 mM hygromycin B (HygB) or media adjusted to pH 2.5.
[b] Phenotypic revertant of G158D (hygB^r, low pH^r) [31].

2.4. Glucose-induced medium acidification

Cells were grown to late log phase ($A_{595nm} \sim 5$) in 30 ml of YPD medium and harvested by centrifugation at 3500 × g for 10 min. The culture was washed once with an equal volume of distilled water and once with an equal volume of 50 mM KCl, pH 4.5. The cells were fully carbon starved by incubation overnight at 4°C in 15 ml of 50 mM KCl, pH 4.5, recovered by centrifugation and concentrated about 20 × in 50 mM KCl, pH 4.5. The concentrated cells were diluted to $A_{595nm} = 8.3$ in 1.8 ml of reaction medium (3 ml stirred cuvette) containing 50 mM KCl, pH 4.5, the test reagents added and the mix adjusted to pH 4.5. After a 10 min preincubation, medium acidification was initiated by adding 0.2 ml of 20% glucose. The pH of the medium was determined using a rapidly responding pH electrode (Microelectrodes). Omeprazole is essentially fully active after 20 min exposure to 0.1 M HCl in DMSO and the drug is about 50% inactivated after about 8 h under these conditions. All experiments on proton pumping were conducted within 2 h following 1 h of acid-activation.

For experiments requiring comparative measurements between relative proton pumping and the ATPase activities associated with plasma membranes, the volume of the assay was scaled up 50-fold. After a 30 min incubation with drug, the cells were pelleted by centrifugation at 4°C, and washed twice with buffer consisting of 50 mM Tris pH 7.0, 0.5 mM EDTA, 2% glucose, 0.5 mM reduced glutathione and 1 mM PMSF. The cells were lysed in a French pressure cell, the pH adjusted to 7.0 with KOH and the plasma membranes purified by sucrose gradient centrifugation, as described by Monk et al. [31].

2.5. ATPase and other biochemical assays

Microscale plasma membrane ATPase assays were conducted as described by Monk et al. [31]. The treatment of H^+-ATPase in vitro with omeprazole was performed in a stabilization buffer (100 mM sodium citrate, pH 3.5, 40% glycerol and 100 mM KCl) which minimized pH-dependent enzyme-inactivation during a 5 min pretreatment with the drug. All in vitro experiments were performed with deoxycholate-treated plasma membranes [25] since native plasma membranes rapidly precipitated under these conditions.

Protein was estimated using the Bio-Rad microassay using bovine γ-globulin as standard [32].

3. Results

3.1. Inhibition of fungal cell growth by omeprazole

Omeprazole is an acid-activated reagent that forms a reactive sulfenamide derivative capable of modifying free sulfhydryl groups [28,29]. The growth of wild type strains of *Saccharomyces cerevisiae* (SN236) and *Candida albicans* (ATCC10261) was significantly inhibited by omeprazole in a dose- and pH-dependent manner. Fig. 1 shows that *Saccharomyces* was more sensitive to growth inhibition by omeprazole than its *Candida* counterpart. At pH 3.0, 0.43 mM omeprazole was required to fully inhibit growth of SN236 whereas approximately twice as much omeprazole was required for ATCC10261 (Fig. 1A,B.) The growth of SN236 was maximally inhibited by 0.22 mM omeprazole at pH 2.5 and 3.0. Between pH 3.0 and pH 4.5, omeprazole inhibition declined by about 75% for each unit increase in pH. The growth of *C. albicans* strain ATCC10261 was generally 2–3-times less sensitive than *S. cerevisiae* strain SN236 at pH 3.5 and below, and was essentially unaffected by the drug at pH 4.0 and above (Fig. 1B). Similar behavior was observed for other *Candida* wild type strains (ATCC23187 and B311). The pH-dependence for omeprazole-mediated growth inhibition of both *S. cerevisiae* and *C. albicans* is consistent with the acid-activated properties of omeprazole which result in the formation of a highly reactive sulfenamide.

The formation of this specific sulfenamide species from acid-treated omeprazole can be monitored at a wavelength of 355 nm [29]. The conversion is relatively rapid with a $T_{1/2}$ for omeprazole activation of ~7 min (data not shown). Acid-activated omeprazole and untreated omeprazole were similarly effective at inhibiting growth of SN236 below pH 3.5, while inhibition of growth at pH 4.0 and 4.5 was enhanced by acid activation (Fig. 2A and Fig. 1A). Acid-activated omeprazole did not inhibit growth at pH 5.0. The sulfenamide is unstable at pH > 4.0, rapidly generating several inactive derivatives [29]. Pre-treatment of acid-activated omeprazole with a 70% excess of either

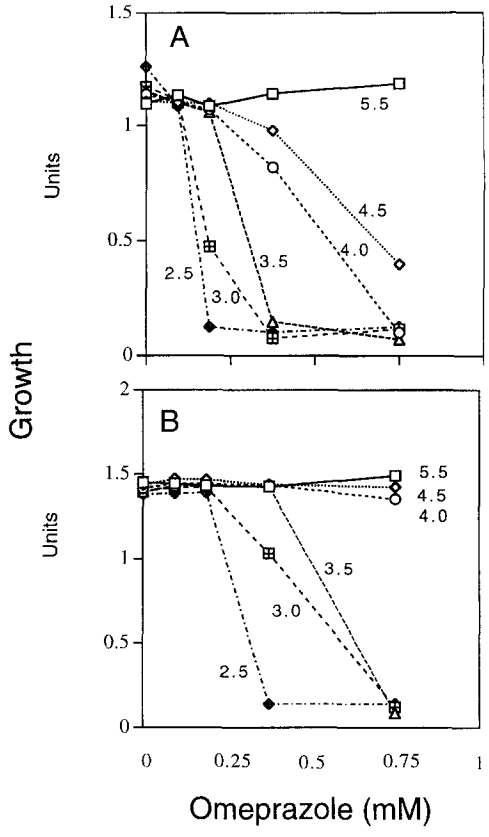

Fig. 1. The pH-dependence for inhibition of yeast growth by omeprazole. The pH-dependence for omeprazole-induced inhibition of growth by (A) *S. cerevisiae* (SN236) or (B) *C. albicans* (ATCC10261) was determined in 96 well microtitre plates, as described in Section 2. Growth (A_{620nm}) was determined after 24 h at pH 2.5 to 5.5, as indicated.

2-mercaptoethanol or reduced glutathione blocked the action of the drug, consistent with the sulfenamide acting as the inhibitor of growth (Fig. 2B). The sulfhydryl reagents (1.5 mM) and the carrier DMSO (0.8%) had no effect on growth. Preincubation of omeprazole with a slight excess of the two sulfhydryl reagents in YPD medium at pH 3.5 also substantially blocked (> 80%) omeprazole-dependent inhibition of growth (data not shown). The growth inhibitory effect of the sulfenamide was blocked by pretreatment of acid-activated omeprazole in DMSO with increasing amounts of 2-mercaptoethanol in a stoichiometric manner (Fig. 2C).

3.2. Omeprazole action is fungicidal

Omeprazole was fungicidal for cells under conditions that inhibited cell growth (pH 3.5 medium and reagent concentrations > 0.43 mM) (Fig. 3). Omeprazole at 0.80 mM killed greater than 95% of all cells after 24 h. Cell viability was unaffected by 1 h of exposure but was substantially reduced by 6 h exposure to 0.43–0.80 mM omeprazole (data not shown). At lower omeprazole concentrations (0.22 mM) growth rates were reduced but cell viability was only modestly impaired. Since the sulfen-

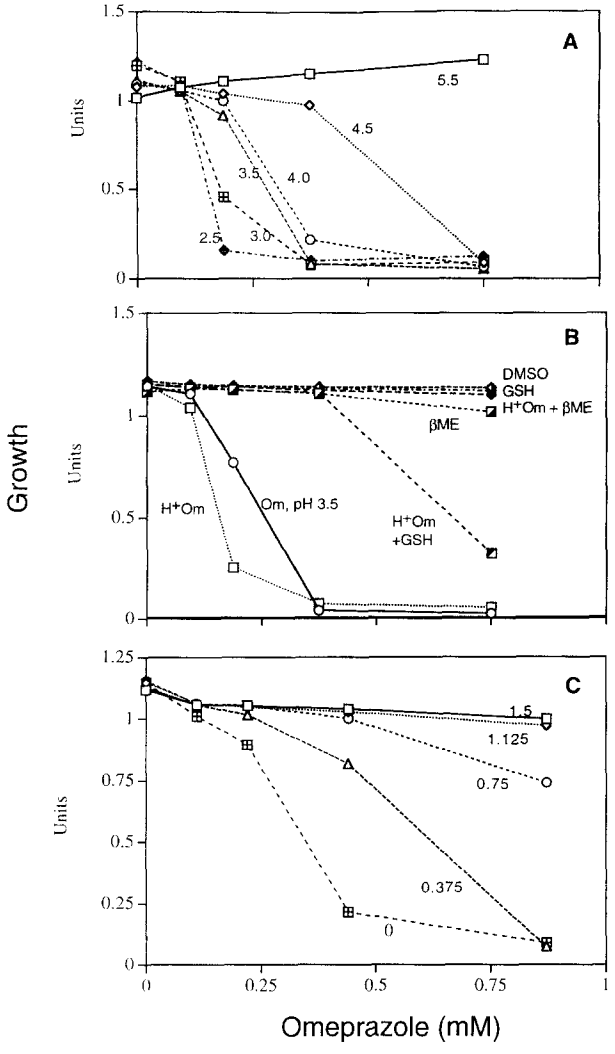

Fig. 2. The sulfenamide of omeprazole affects growth of *S. cerevisiae*. (A) The pH-dependence for inhibition of *S. cerevisiae* growth by acid-activated omeprazole. Assays were conducted in 96-well microtitre plates, as described in Section 2 with strain SN236. Growth was determined after 24 h. Values for medium pH are indicated on the figure. (B) Treatment of acid-activated omeprazole with sulfhydryl reagents. Omeprazole (Om) (50 mg/ml, 0.145 mM in 90% DMSO) was acid activated for 1 h with 100 mM HCl. The sulfhydryl reagents, glutathione (GSH) and β-mercaptoethanol (βME), were added directly to the omeprazole at 0.25 M. After 20 min, 20 μl of the treated omeprazole was diluted into 1.25 ml YPD medium at pH 3.5 and the standard 24 h growth inhibition assay was performed. (C) Effects of β-mercaptoethanol on the growth inhibitory effects of acid-activated omeprazole. Aliquots (5 μl) of acid-activated omeprazole were incubated with either 0, 1.25, 2.5, 3.75 or 5 μl of 0.25 M β-mercaptoethanol in a total volume of 10 μl. After 20 min, the treated omeprazole was diluted into 0.675 ml YPD medium at pH 3.5 and the standard growth inhibition assay was conducted. The final concentrations of β-mercaptoethanol in wells containing 0.87 mM acid-activated omeprazole were 0, 0.375, 0.75, 1.125, and 1.5 mM, as indicated.

amide of omeprazole is only moderately stable and can interact with non-essential accessible cysteine residues (such as those in the cell wall), populations of cells rendered partially viable recovered as the effective concentration of omeprazole declined with time. These data suggest that a majority of target(s) must be modified by omeprazole action before cell viability is significantly impaired.

3.3. Correlating inhibition of in vivo proton pumping with H^+-ATPase activity

Glucose-dependent medium acidification provides a relative measure of proton pumping by the plasma membrane H^+-ATPase [23,26,33]. Fig. 4 shows the effect of acid-activated omeprazole on glucose-dependent proton pumping by SN236. Carbon-starved cells were incubated with the indicated amount of acid-activated omeprazole at pH 4.5 for 10 min prior to the addition of glucose. Glucose-dependent proton pumping was initiated at normal or near normal rates at all levels of omeprazole treatment, but the initial rate response gave way to a dose-dependent inhibition of proton pumping. Acid-activated omeprazole at < 0.22 mM progressively inactivated proton pumping until a new steady state external pH was reached. At higher omeprazole concentrations, > 0.36 mM, proton pumping was progressively inhibited and a new low pH steady-state was not attained as the cells appeared to resorb protons from the medium. The inhibition profiles suggest that a specific catalytic intermediate may be required for interaction with omeprazole since the initial rate of pumping was essentially unaffected by the inhibitor treatments. Full inhibition of enzyme activity would likely be dependent on the rate of catalytic turnover, the half-time for the reactive intermediate, and the concentration of inhibitor. As expected, inhibition of proton pumping was extensively (~ 80%) blocked by pre-treatment of acid-activated omeprazole with a slight excess of 2-mercaptoethanol, while DMSO and 2-mercaptoethanol added at equivalent concentrations had no effect on pumping (not shown).

Glucose-dependent proton pumping in *S. cerevisiae* is a complex process which requires glucose uptake, glucose

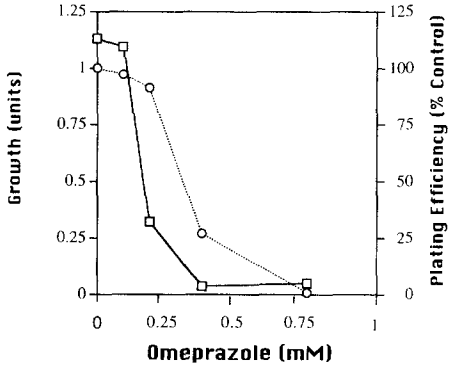

Fig. 3. The fungicidal activity of omeprazole at pH 3.5. Cells incubated with omeprazole in a standard growth assay for 20 h at pH 3.5 were diluted in YPD medium and plated on solid YPD medium in duplicate. The percentage cell viability was estimated as 100×(percent plating efficiency of treated cells)/(percent plating efficiency of control cells not treated with omeprazole). Cell growth (□) and viability (○) are indicated.

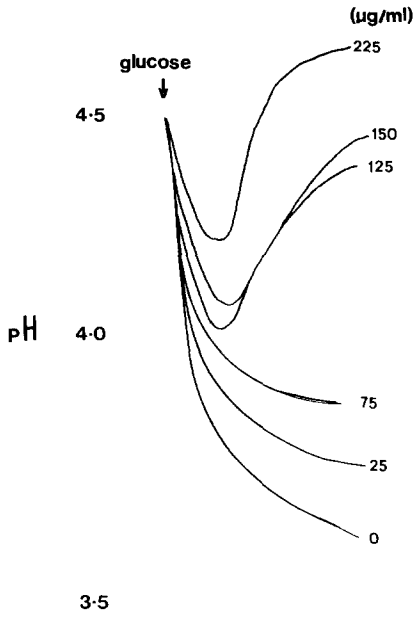

Fig. 4. Omeprazole-induced inhibition of glucose-dependent proton pumping by *S. cerevisiae*. Effect of acid-activated omeprazole on the inhibition of glucose-dependent medium acidification. Acid-activation of omeprazole and glucose-induced medium acidification by strain SN236 were carried out as described in Section 2. Acid-activated omeprazole was added in a maximum volume of 20 μl. Cells were preincubated with acid-activated omeprazole at the indicated concentrations for 10 min prior to initiation of medium acidification with glucose.

metabolism, and activation of the proton pump [34,35]. To establish whether inhibition of proton pumping by acid-activated omeprazole was due to a corresponding inhibition of the H^+-ATPase, identical batches of cells were treated with acid-activated omeprazole at varying concentrations and assayed for glucose-induced medium acidification. Plasma membranes were isolated from the cells and the kinetic properties of the vanadate sensitive H^+-ATPase assayed in vitro. Fig. 5 shows the strong correlation between the extent of in vivo proton pumping after treatment with omeprazole and the residual activity of the plasma membrane ATPase recovered in the highly purified plasma membrane fraction. The loss of enzyme activity following in vivo omeprazole treatment is consistent with irreversible covalent modification of the ATPase. A partially-inhibited, omeprazole-treated enzyme showed a $K_{0.5}$ for ATP ~ 0.7 mM and a vanadate K_i ~ 0.5 μM that were nearly identical to non-inhibited control enzyme. The inhibition of H^+-ATPase in omeprazole-treated cells was not due to glucose-depletion, which is known to inactivate the enzyme but with different kinetic effects [36]. Collectively, these data are consistent with the H^+-ATPase serving as a principal target for omeprazole-induced inhibition of cell growth and proton pumping.

3.4. Site of action for omeprazole inhibition

The reactive sulfenamide of omeprazole is not expected to cross the bilayer and therefore accessible cell surface and/or membrane sector sulfhydryl groups of the H^+-ATPase are expected to be the principal site(s) modified. This notion was tested by analyzing the growth of wild type and *pma1* mutant cells at important cysteine residues. Three cysteine residues, C148, C312 and C867, are predicted to lie either within or near the extracellular surface of transmembrane segments 2, 3 and 10, respectively (these assignments are based on a topology model for the yeast H^+-ATPase which incorporates ten transmembrane segments [2]). In YPD medium at pH 3.5, similar levels of omeprazole were required to inhibit the growth of wild type and *pma1* mutant strains carrying the C312S and C867A mutations (Fig. 6A). However, the C148S mutant was 2–3-times less sensitive than the wild type to omeprazole. This result suggests that C148 in transmembrane segment 2 may be an important initial site of interaction with omeprazole.

Transmembrane segment 2 is believed to be part of a helical hairpin structure that is formed with transmembrane segment 1 and a short linking turn region [19]. To examine whether this region significantly affects omeprazole interaction, additional *pma1* mutants were analyzed in which cysteine residues were introduced into different regions of the hairpin structure. It was found that the introduction of a cysteine as M128C, a position on helix 1 predicted to be adjacent to C148 on helix 2, gave a 3-fold enhancement of growth sensitivity to omeprazole (Fig. 6). Interestingly, the introduction of a cysteine, G156C (isolated as a suppressor of G158D [30]) near the C-terminus of helix 2, resulted in

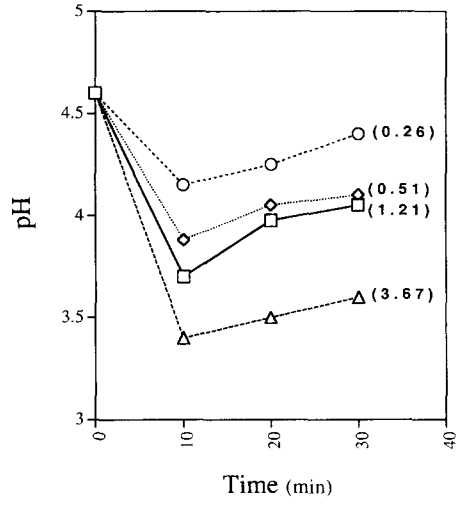

Fig. 5. In vivo inhibition by omeprazole of glucose-dependent medium acidification and correlative effects on H^+-ATPase activity. Glucose-induced medium acidification was measured in 100 ml suspensions of SN236 cells (A_{595nm} ~ 7.5) treated with the indicated amounts of acid-activated omeprazole for 10 min. Plasma membranes were isolated from the treated cells after a 30 min incubation with drug and the specific activity (μmol P_i /min per mg) of the associated H^+-ATPase is indicated in parentheses. All activity measurements were performed on duplicate samples with less than 10% difference between like samples. The cell treatments were 0 omeprazole (△), 0.36 mM omeprazole (□), 0.72 mM omeprazole (◇), 1.1 mM omeprazole (○).

a mutant enzyme that was significantly less sensitive to omeprazole than either the wild type or the C148S mutant. The parent G158D mutant is pH sensitive and cannot be assayed under these conditions. However, it shows identical behavior to the double mutant with other types of sulfhydryl-modifying reagents (not shown). These results confirm previous assertions that this region of the H^+-ATPase is conformationally sensitive [19,20] and further suggest that the region around C148 is important for inhibition of the enzyme and hence, yeast cell growth.

The differential effects of omeprazole on the growth of the cysteine-deficient *pma1* mutants were seen in both whole cell proton pumping assays and in determinations of plasma membrane-bound H^+-ATPase activity in vitro. Analysis of glucose-dependent medium acidification by wild type and *pma1* mutant strains showed that strain C148S, but not strains C312S and C867A, was substantially resistant to omeprazole-induced inhibition (Fig. 6B). Although proton pumping was partially inhibited in the C148S mutant by 0.36 mM omeprazole, growth was barely affected at this concentration (Fig. 6A). This observation probably reflects the more extensive degradation of activated omeprazole during growth assays, which were carried out over a much longer time-course than the proton pumping assays (24 h versus 40 min). When purified plasma membranes from the wild type and the C148S mutant were recovered after in vivo inhibition of proton pumping by omeprazole, a comparison of residual H^+-ATPase activities showed that the mutant enzyme was only modestly inhibited. This result correlated with the limited omeprazole inhibition of proton pumping in the mutant cells (Fig. 7). In contrast, treatment of detergent-extracted enzymes with omeprazole in vitro, at concentra-

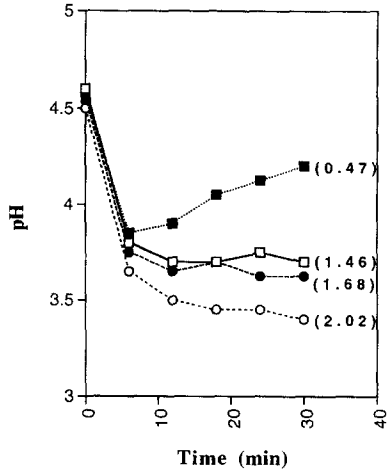

Fig. 7. Comparative inhibition by omeprazole of medium acidification and H^+-ATPase activity in C148S. (A) In vivo inhibition by acid-activated omeprazole of glucose-dependent medium acidification and plasma membrane H^+-ATPase activity of SN236 and C148S. Medium acidification was measured by point determinations of 100 ml cell suspensions of SN236 (□, ■) or C148S (○, ●) that were either untreated (open symbols) or treated with 0.36 mM acid-activated omeprazole (closed symbols) for 10 min. Plasma membranes were isolated from treated cells after 30 min and the residual plasma membrane H^+-ATPase activity (μmol P_i/min per mg) is indicated in the parentheses. Each activity represents the average obtained from duplicate experiments, with replicates differing by less than 10%.

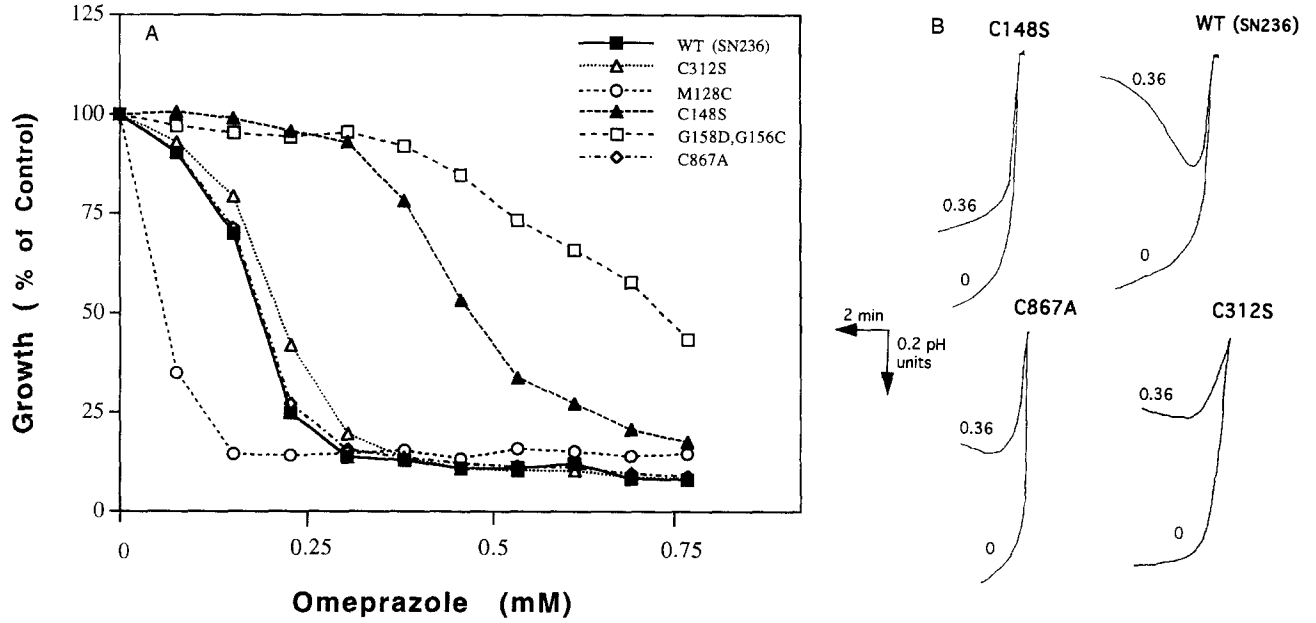

Fig. 6. Effects of omeprazole on growth and glucose-dependent proton pumping in wild type and *pma1* mutant strains of *S. cerevisiae*. (A) Effect of omeprazole on the growth of the wild type strain SN236 and *pma1* mutants altered in membrane sector amino acids. The *pma1* mutant strains examined contained the following mutations C148S, C312S, C867A, M128C and G158D/G156C, as indicated. All strains were measured at pH 3.5 in the standard drug challenge growth assay described in Section 2. (B) Effect of acid-activated omeprazole on glucose-dependent medium acidification by SN236 and *pma1* mutants with altered membrane sector cysteine residues. Glucose-dependent proton pumping was measured in the presence and absence of 0.36 mM acid-activated omeprazole as described in Section 2.

tions several-fold below those required to inhibit the enzyme in vivo, extensively inhibited both the wild type and *pma1* mutant H^+-ATPases (not shown). In fact, the ATPase from the C148S mutant was slightly more sensitive than the ATPase from the wild type strain. The in vitro sensitivity of the H^+-ATPase indicates that the enzyme contains additional omeprazole sensitive sites that are not accessible in vivo. Such sites are not without precedent and include three cytoplasmically located sulfhydryl groups, including an NEM-reactive site [37] within the ATP binding site. Consistent with a catalytic site origin for omeprazole inhibition in vitro, Mg-ATP was found to strongly protect the enzyme from inhibition (data not shown). This high affinity cytoplasmic inhibitory site is unlikely to be related to the site affected by omeprazole inhibition in vivo.

4. Discussion

An ideal antifungal target should be both essential to the cell and accessible to selective inhibitors that interact with the extracellular surface of the cell under acidic growth conditions. The plasma membrane H^+-ATPase of fungi has these attributes. The reagent omeprazole was chosen to probe the H^+-ATPase as an antifungal target because conserved cysteine residues are located near the cell surface [2] and because the drug is activated under acidic conditions, forming a positively charged highly specific cysteine-reactive species that does not readily penetrate the lipid bilayer. Omeprazole interacts with cell surface residues of the H^+,K^+-ATPase in the lumen of the parietal cell, irreversibly blocking gastric acidification and thereby aiding in the treatment of gastric ulcers [4,28]. We have shown that omeprazole inhibits the growth of the fungi *S. cerevisiae* and *C. albicans* in a pH-dependent manner between pH 2.5 and 4.5 and that there is stoichiometric protection by β-mercaptoethanol of growth inhibition by acid-activated omeprazole at pH 3.5 (Figs. 1 and 2). These data are consistent with the expected formation of a sulfenamide derivative as the active growth-inhibitory species. The inability of acid-activated omeprazole to inhibit growth at pH 5.0 is consistent with the known instability and rapid deactivation of the sulfenamide at pH > 4.0 [29]. The primary growth-inhibiting target for omeprazole in *S. cerevisiae* is the plasma membrane H^+-ATPase, and the conserved residue C148 of transmembrane segment 2 may be an important site of interaction. Several complementary lines of evidence support these conclusions.

First, omeprazole blocks both growth and glucose-dependent proton pumping, and the inhibitory effect on proton pumping correlates with the relative activity of plasma membrane ATPase recovered from such cells (Fig. 5). The residual glucose-activated H^+-ATPase activity recovered from cells treated with omeprazole at a suboptimal level shows the expected decrease in V_{max} but no change in the $K_{0.5}$ for ATP or sensitivity to the inhibitor sodium vanadate was observed (Fig. 5). These data strongly suggest that the H^+-ATPase is the principal target for omeprazole-induced inhibition of cell growth while the systems responsible for either glucose uptake or the glucose-dependent regulation of the H^+-ATPase are unaffected.

Second, omeprazole inhibits yeast growth via cell surface sites, since the levels of omeprazole required to inhibit growth were several times greater than those needed to inhibit the plasma membrane ATPase in vitro. The active form of omeprazole is a positively charged sulfenamide and is therefore unlikely to cross the plasma membrane. Any sulfenamide that crosses the membrane should be rapidly inactivated by the neutral cytoplasmic pH (pH ~ 7.1) and by the presence of a significant concentration of intracellular glutathione. In addition, Mg-ATP strongly protects against the action of omeprazole in vitro, implying that the high affinity inhibitory site(s) observed under these conditions is cytoplasmically located. The well characterized NEM binding site in the ATP-binding region of the cytoplasmic kinase domain [37] is a likely site of omeprazole action on the in vitro treated enzyme.

Third, omeprazole inhibition of in vivo proton pumping appears to require the enzyme to proceed through its reaction cycle, with omeprazole binding site becoming accessible during the reaction cycle (Fig. 4). This is consistent with the known modes of action of SCH28080 and ouabain on the gastric H^+,K^+-ATPase and the Na^+,K^+-ATPase, respectively. These drugs both favor the E_2 enzyme conformation of the ATPase for binding [3,5].

Fourth, the C148S mutation in the *PMA1* gene makes *S. cerevisiae* less sensitive to omeprazole inhibition of growth and glucose-dependent proton pumping, while the plasma membrane ATPase from cells treated with omeprazole in vivo has a residual activity commensurate with the level of resistance shown by proton pumping (Fig. 7). The C148S mutant H^+-ATPase is known to be present at a wild type level in the plasma membrane and displays a normal sensitivity to vanadate [18]. The differential sensitivities of the C148S mutant and wild type to omeprazole in growth assays occurred over a relatively narrow concentration range (Fig. 6A). This effect was expected since the reactive sulfenamide species should scavenge free -SH groups including exposed cysteine residues on the H^+-ATPase, on other membrane proteins and in the cell wall. These data suggest that in this concentration range, the H^+-ATPase is the principal inhibitory target and that the C148 region may be an important initial site of interaction with omeprazole. Direct labeling studies and more detailed mutagenesis experiments are in progress to better assess this point.

Inhibition of the H^+-ATPase during glucose-dependent proton pumping appears to require catalytic turnover (Fig. 4) which is consistent with a major conformational change occurring during the reaction cycle. While C148 of the

yeast plasma membrane ATPase is distant in primary sequence from the membrane sector residues (in the C-terminal portion) of gastric H^+,K^+-ATPase which covalently bind omeprazole and pantoprazole [4,5], omeprazole seems to bind to the same transmembrane loop as SCH28080 does in the gastric enzyme [15]. The binding of SCH28080 requires an E_2 conformation and is competitively affected by binding of K^+. SCH28080 protects the gastric H^+,K^+-ATPase against omeprazole binding, suggesting that the SCH28080 and omeprazole binding sites may partially overlap [5]. The differences in the amino acid targets between the yeast ATPase and in the gastric H^+,K^+-ATPase does not exclude the possibility that comparable loci are important for initial sulfenamide binding, with the ultimate effectiveness of the drug depending on covalent attachment to accessible reactive cysteine residues.

The present study adds to the growing body of evidence implicating transmembrane segments 1 and 2 of the fungal ATPase as conformationally important elements of the catalytic reaction cycle. Over 30 mutations mapping to this region have now been analyzed and most modify the function of the ATPase [2,19,20]. The importance of C148 to ATPase structure/function in the region of transmembrane segment 2 is consistent with the conservation of C148 [38]. Other mutations, M128C and G156C, G158D which are expected to perturb the structure near C148, also substantially modify the sensitivity of *S. cerevisiae* to omeprazole (Fig. 6A,B). The G158D, G156C mutations probably alter the conformation or environment of transmembrane segment 2 in *S. cerevisiae*, restricting the accessibility of C148 to attack by the sulfenamide of omeprazole. M128 and C148 are conserved residues predicted to lie on the same face of transmembrane segments 1 and 2 [19]. An M128C mutant enzyme is more sensitive to omeprazole than the wild-type enzyme (Fig. 6A). The M128C mutation could either provide a more accessible target or an enhanced function-altering target for the drug. Whether the introduced cysteine at M128 or the residue C148 is the principal target for drug action in this mutant has yet to be determined.

The growth inhibitory and fungicidal effects of omeprazole on *S. cerevisiae* suggests that the plasma membrane H^+-ATPase could to be developed as a cell surface antifungal target. The conformationally active transmembrane segments 1 and 2 and the short intervening turn contain a number of important residues which are conserved among the fungi and which either deleteriously effect yeast growth or are lethal when genetically modified [19]. Such structural constraints offer potential recognition and targeting features. Drugs targeted to this region do not have to cross the plasma membrane and therefore will not be susceptible to the action of multidrug resistance pumps [39]. An understanding of fungal plasma membrane proton pump structure and how the enzyme modulates cell growth will expedite the development of directed antifungal screening procedures and expand the possibilities for the rational design of agents directed at this novel cell surface target. This approach and the power of yeast molecular genetics have the potential to contribute to our understanding of the mode of action of existing therapeutics and simplify experimental approaches to the more general problem of P-type ATPase structure/function. It should also facilitate the development of new reagents that are targeted at other P-type ATPases, including those of other parasites and pathogens, and those differentially expressed in animal tissues.

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