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Exploring an antifungal target in the plasma membrane H^+-ATPase of fungi

Donna Seto-Young [a], Brian Monk [b], A. Brett Mason [b], David S. Perlin [a,*]

[a] *Public Health Research Institute, 455 First Avenue, New York, NY 10016, USA*
[b] *Department of Oral Biology and Oral Pathology, University of Otago, Dunedin, New Zealand*

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Abstract

The plasma membrane H^+-ATPase is a promising new antifungal target that is readily probed with the sulfhydryl-reactive reagent omeprazole. Inhibition of the H^+-ATPase by omeprazole is closely linked to cell killing, and it has been suggested that enzyme inhibition may result from a covalent interaction within the first two transmembrane segments (M1 and M2) (Monk et al. (1995) Biochim. Biophys. Acta 1239, 81–90). In this study, the molecular nature of this interaction was examined by screening a series of 26 well-characterized *pma1* mutations residing in the first two transmembrane segments of the H^+-ATPase from *Saccharomyces cerevisiae*. Only two *pma1* mutants, A135G and G158D,G156C, were found to significantly decrease the sensitivity of cells for omeprazole. In contrast, enhanced sensitivity was observed at a number of positions, with D140C(A) and M128C producing the most significant increases in sensitivity. The introduction of cysteine at various locations within this region only marginally affected omeprazole sensitivity, suggesting that this region was not a direct site of covalent modification. Rather, its conformation influences omeprazole binding at some other locus. In order to determine the sidedness of the omeprazole interaction, a novel in vitro assay system was exploited that utilized liposomes co-reconstituted with the H^+-ATPase and the light-driven proton pump bacteriorhodopsin. Omeprazole was found to completely inhibit proton transport by the H^+-ATPase at 50 μM in this system. An asymmetrically-distributed chemical trap system involving glutathione was used to demonstrate that this inhibition appears localized to the extracellular portion of the enzyme. This work indicates that omeprazole can inhibit the H^+-ATPase from its extracellular face, and this inhibition is influenced by changes in the M1, M2 region of the protein.

Keywords: ATPase, H^+-; Antifungal target; Omeprazole

1. Introduction

Opportunistic fungal infections are widespread in HIV and other immunosuppressed individuals, and are a growing problem for the management of such patients. *Candida albicans*, which causes both mucosal and invasive disease, is the predominant organism associated with fungal disease, although other fungal pathogens such as *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Pneumocystis carinii*, *Histoplasma capsulatum*, and other *Candida* species are increasingly important [1,2]. Mucosal fungal infections can be treated with existing azole-based antifungals, although fewer treatment options are available for invasive disease, which usually requires ther-

* Corresponding author. Fax: +1 (212) 5780804.
E-mail: perlin@phri.nyu.edu

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apy with the highly nephrotoxic polyene antibiotic amphotericin B [3]. In addition to the problem of invasive disease, a major concern is the increasing number of azole-related treatment failures due to both induction of resistance in *Candida albicans* and to colonization by less susceptible *Candida* species [4–6]. There exists a critical need to identify novel antifungal targets that can be used to develop new mechanistic classes of therapeutics, which can effectively circumvent such problems.

The plasma membrane proton pumping H^+-ATPase is an important new target for therapeutic intervention [7]. The H^+-ATPase is a predominant membrane protein that belongs to the P-type ATPase family of ion translocating ATPases. This essential enzyme plays a critical role in fungal cell physiology by maintaining the large transmembrane electrochemical proton gradient necessary for nutrient uptake and by regulating intracellular pH [8]. It is one of the few antifungal targets which have been demonstrated to be essential by gene disruption [9]. In addition to its role in cell growth, the H^+-ATPase has been implicated in fungal pathogenicity through its effects on dimorphism [10,11], nutrient uptake, and medium acidification [12].

The family of P-type ion pumps includes the Na^+,K^+-ATPase and H^+,K^+-ATPase, which are molecular targets for several clinically-important therapeutics [13,14]. These drugs are enzyme-specific antagonists, which inhibit via an interaction with the extracellular surface of their respective target enzyme. These precedents suggest that it should be possible to develop similar types of antagonists specific for the fungal H^+-ATPase. Highly specific H^+-ATPase antagonists capable of acting from outside the cell should be extremely valuable as antifungals. This is especially pertinent given the increasing clinical problem of drug resistance due to multi-drug resistance pumps, such as CDR1 and BEN^r [4]. Extracellularly-directed reagents do not cross the cellular membrane, and therefore are not substrates for these high capacity drug pumps.

Recently, we demonstrated the importance of the H^+-ATPase as an antifungal target by showing that inhibition of the enzyme by the sulphydryl-reactive reagent omeprazole was closely correlated with inhibition of cell growth by *Candida albicans* and *Saccharomyces cerevisiae*, and that omeprazole-induced inhibition of the proton pump was fungicidal [15]. Although not suitable as a therapeutic antifungal agent, omeprazole showed well defined and controlled properties that made it a valuable probe to examine the role of the H^+-ATPase as a molecular target. Consistent with studies of the action of omeprazole on the gastric H^+,K^+-ATPase, omeprazole-induced inhibition of the fungal H^+-ATPase was suggested to result from a drug interaction with the extracellular surface of the enzyme. Mutant analysis of the *Saccharomyces cerevisiae* enzyme indicated that amino acid residues in M1 and M2 may be important for omeprazole-induced inhibition [15,16]. In this work, we explore in more detail the molecular interaction of omeprazole with the H^+-ATPase.

2. Materials and methods

2.1. Cell strains and cell growth

Halobacterium halobium strain S9 [17] was grown at 37°C for one week in 175 ml of growth medium consisting of 1% bacto-yeast extract, 2.5% bacto-tryptone, 4.4 M NaCl, 40 mM $MgSO_4$, 67 mM KCl, and 1.3 mM $CaCl_2$. A 10-ml volume of this culture was used to inoculate 2 liters of growth medium in a 2.8-l Fernbach flask. The cultures were grown 2–3 weeks at 37°C on a gyrator shaker at very slow speed (25 rpm) to limit aeration. All *Saccharomyces cerevisiae* strains utilized in this study were isogenic derivatives of *Y55 (HO gal3 MAL1 SUC1)* [18] and most have been described [19–21]. *pma1* strains containing cysteine mutations were prepared as previously described [19]. The wild-type strain GW201 *(HO ade6-1 trp5-1 leu2-1 lys1-1 ura3-1PMA1::URA3)* was used as a control strain. All yeast cell cultures were grown at 22°C in YPD medium containing 1% yeast extract, 2% peptone and 2% dextrose (pH 5.7). In vivo drug sensitivities were determined as described by Monk et al. [15].

2.2. Preparation of bacteriorhodopsin membranes

Halobacterium cells were collected by centrifugation at $10\,000 \times g$ for 10 min. The cells were washed by resuspension with 40 ml *wash buffer* containing 10 mM Tris-HCl (pH 7.0) and 4.0 M NaCl and then

centrifugation, as above. The cells were osmotically lysed in 120 ml *lysis buffer* containing 10 mM Tris-HCl (pH 7.0), 100 mM NaCl and 1 mM NaN_3 and 0.13 μg/ml DNAse I. The suspension was centrifuged at $5000 \times g$ for 10 min, and the supernatant was removed and centrifuged at $40\,000 \times g$ for 15 min. The pellets were washed by resuspension and centrifugation, as above. Purple membranes were resuspended with *storage buffer* consisting of 10 mM Tris-HCl (pH 7.0), 100 mM NaCl, 1 mM NaN_3 and 20% glycerol, and stored at -80°C.

2.3. Isolation and reconstitution of yeast H^+-ATPase

Microsomal membranes, prepared by the method of Perlin and Brown [22], were extracted with 0.48% (w/v) deoxycholate in *solubilization buffer* containing 10 mM Hepes-KOH (pH 7.0), 0.1 M KCl, 45% (v/v) glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mg/ml asolectin and 1 mg/ml brain extract (Folch Fraction III containing 80–85% phosphatidyl-serine; Sigma Co.). The extracted membranes were resuspended with 25 ml of *solubilization buffer* supplemented with 0.5 M KCl, and centrifuged at $250\,000 \times g$ for 1 h. The KCl-washed membranes were resuspended at 0.5 mg/ml in 1 ml *solubilization buffer* containing 20 mg/ml asolectin. Deoxycholate was added with gentle stirring to a final concentration of 0.48% (w/v) by dropwise addition from a 10% (w/v) stock solution. The mixture was placed on ice for 5 min and then diluted into 50 ml of ice-cold *dilution buffer* containing 5 mM Hepes-KOH (pH 7.0) and 100 mM KCl. The reconstituted vesicles were recovered by centrifugation at $250\,000 \times g$ for 1 h. The reconstituted liposomes were resuspended in 25 ml *dilution buffer* and then pelleted by centrifugation, as above. The final pellets were resuspended in 0.2 ml of *dilution buffer*.

2.4. Co-reconstitution of bacteriorhodopsin and H^+-ATPase

A 50 μl volume of reconstituted H^+-ATPase-containing vesicles (100–200 μg) were mixed with 10 μl of bacteriorhodopsin membranes (20 μg) and 60 μl of 2 M NaCl. The mixture was frozen on dry ice for 10 min and then thawed at 22°C for 10 min. This procedure was repeated twice. The vesicles were briefly sonicated for 10 s in a sonic bath (Model G112PIT, Laboratory Supplies Co., Inc., at 80 kHz and 80 W).

2.5. Proton transport measurements

Proton transport in reconstituted vesicles was followed using the acridine orange fluorescence quenching assay [23]. The assay medium consisted of 2 ml of 5 mM Hepes-KOH (pH 7.0), 1 M NaCl, 4 μM acridine orange and 100–200 μg of the reconstituted vesicles. Light driven proton transport was initiated by applying high-intensity actinic light (Fiber-Lite, Dolan-Jenner Industries, Inc) to activate the bacteriorhodopsin pump, as described by Oesterhelt and Stoeckenius [17]. ATP driven proton pumping was initiated by the sequential addition of 5 mM ATP and 1 mM $MgCl_2$. Relative fluorescence was monitored with a Perkin-Elmer LS-5B Luminescence Spectrophotometer using excitation and emission wavelengths of 419 and 520 nm, respectively. An interference filter-transmitting light of λ less than 555 nm was placed in front of the detector and a filter blocking actinic light above 597 nm was placed in front of the sample. The magnitude of the pH gradient formed in light by bacteriorhodopsin was determined with the indicator dye dicarboxy-dichlorofluorescein (CDCF). Co-reconstituted bacteriorhodopsin and H^+-ATPase liposomes were resuspended in 2 ml of buffer containing 5 mM Hepes-KOH (pH 7.0), 1 M NaCl, and 0.05 μM CDCF. Bacteriorhodopsin-induced proton pumping was initiated by the addition of light (see above) for 10 min. Calibration of pH was accomplished by evaluating the shift in the fluorescence spectrum for CDCF as a function of pH [24]. Vesicles were pre-equilibrated at varying pH values (pH 3.0–7.0) in the presence of 10 mM NH_4Cl to collapse any residual pH gradients. A series of spectra reflecting the different pH values was obtained by varying the excitation wavelength from 400 to 500 nm at a fixed emission wavelength of 510 nm [24].

2.6. Other procedures

ATP hydrolysis measurements were performed in a 96-well plate assay in a 100 μl volume containing 10 mM Mes-Tris (pH 6.4), 25 mM NH_4Cl, 5 mM

ATP, 5 mM $MgCl_2$, 0.5 mM NaN_3, and 1 μg yeast plasma membranes, as described by Seto-Young et al. [19]. Protein concentration was determined by a modified Lowry method [25].

3. Results

3.1. Amino acid modulation in (M1) and (M2) alters cell growth inhibition by omeprazole

It was previously demonstrated that omeprazole-induced antagonism of the plasma membrane H^+-ATPase was influenced by structural changes in M1 and M2 [15]. To further investigate the role of this region in omeprazole-induced antagonism, a series of *Saccharomyces cerevisiae pma1* mutants with well characterized mutations in M1, M2 [19,20] were screened for drug-induced changes in growth inhibition profiles. Cells were grown in YPD at pH 3.5 with omeprazole from 0 to 250 μM, which was previously shown to impart selectivity for the H^+-ATPase [15]. Fig. 1A summarizes growth inhibition data for the various mutants. It can be seen that mutations at several positions in M1, M2 increased the sensitivity of cells to omeprazole. In contrast, only two mutations, A135G and a double mutant, G158D,G156C, were found to significantly decrease the sensitivity of cells for omeprazole, as previously reported [15]. Many mutations were without effect. The enhanced sensitivities observed were not due to deficiencies in growth, since all the mutants had growth rates comparable to wild-type (not shown). These results suggest that modification of amino acid side groups in M1, M2 result in a modulation of omeprazole-induced inhibition of the H^+-ATPase.

Omeprazole is highly reactive with the -SH side group moiety of cysteine, although it does not appear to interact with C148 in M2 [16]. To examine whether cysteine at other positions in this region could interact with omeprazole, a series of *pma1* mutants was constructed that included I124C, M128C, A132C, A136C, G137C, S139C, D140C and W141C. Fig. 1B shows that introduction cysteine at various positions enhanced the sensitivity of cells to omeprazole. The most significant enhancement was observed with D140C, which lies on the external loop region linking M1 and M2. This suggested that mutant enzyme containing C140 interacts more strongly with omeprazole than wild-type. However, it is not likely

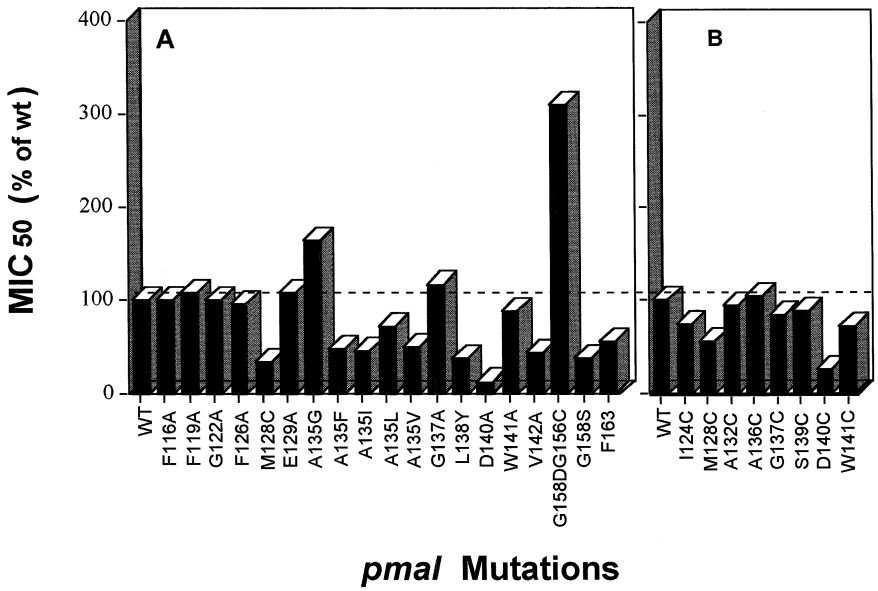

Fig. 1. Growth effect of omeprazole on *pmal* mutants. A: A series of well-characterized mutations in the M1 and M2 [19–21] were screened for changes in omeprazole-dependent growth inhibition by inoculating log-phase cells in YPD medium at pH 3.5 containing increasing amounts of omeprazole (0–250 μM). B: A set of cysteine mutants were constructed, as described in Section 2, and were screened as described above. The inhibition profile for wild-type was taken as the control.

to occur directly at this position because the introduction of alanine at D140 produced a similar effect (Fig. 1A). Three cysteines, C148, C312 and C867, are predicted to lie either within the membrane sector or near it [26]. Yet, none of these residues appear to be responsible for omeprazole interactions because each can be converted to either alanine or serine without altering omeprazole sensitivity [15]. This conclusion was further supported by showing that simultaneous conversion of two cysteines (C148S,C312S; C148S,C867A; C312S,C867A) or all three cysteines (C148S,C312S,C867A) had no effect on omeprazole sensitivity (data not shown). Thus, other cysteine residues may be involved in omeprazole-induced enzyme antagonism.

3.2. Assessing sidedness of omeprazole action

An important aspect of developing the H^+-ATPase as an antifungal target is that inhibition of the enzyme should be possible from the extracellular surface of the enzyme. Omeprazole is a good reagent to explore this possibility because it has properties which allow for interactions in selective environments. In the presence of acid, omeprazole forms a short-lived and highly reactive sulfenamide moiety that reacts in its local environment. This property has been important for selective inhibition of the gastric H^+,K^+-ATPase [14]. A novel in vitro assay adapted from Wach et al. [27] was used to investigate whether omeprazole-induced antagonism of the H^+-ATPase occurs from the extracellular portion of the enzyme. In this assay, which is illustrated in Fig. 2A, the light-driven proton pump bacteriorhodopsin (BR) is used to acidify the intravesicular space of liposomes co-reconstituted with BR and the H^+-ATPase, thereby activating omeprazole partitioned in this compartment. At neutral pH, omeprazole is unreactive and freely permeant across the bilayer. However, in the presence of acid, a charged sulfenamide species is formed that is essentially impermeant across the bilayer. Following a period of acid-activation, Mg-ATP is added to the reconstituted vesicles to assess the efficiency of ATP-driven pH gradient formation by the H^+-ATPase, as illustrated by the quenching of acridine orange fluorescence (Fig. 2B). Even though the H^+-ATPase reconstitutes in an equal orientation, only those enzymes with the active site (cytoplasmic portion of the enzyme) on the extracellular surface of the vesicles are active in this assay. Thus, it is possible to observe sidedness properties of the activated compound. The relative level of intravesicular acidification was determined by using the pH-sensitive probe dicarboxy-dichlorofluorescein (CDCF). CDCF is sensitive to pH at wavelengths above 450 nm with optimal differential sensitivity at 480–490 nm [24]. The probe was calibrated by observing the fluorescence emission spectrum in vesicle preparations equilibrated in the pH range 3.5 to 7.0. In the presence of actinic light, the intravesicular space of the co-reconstituted BR-H^+ATPa vesicles were acidified down to approx. pH 3.9–4.3.

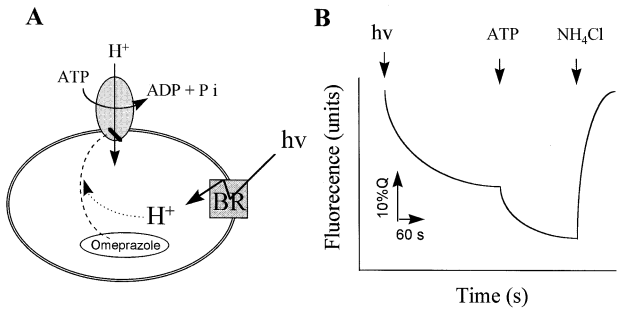

Fig. 2. Schematic diagram of in vitro assay system. A: Diagram illustrates a liposome co-reconstituted with bacteriorhodopsin (BR) and the H^+-ATPase. The introduction of an actinic light pulse initiates proton pumping by BR that results in an interior acid pH gradient. The acidic interior pH activates omeprazole which then interacts with the H^+-ATPase. B: In a typical experiment, co-reconstituted vesicles are added to 2-ml buffer containing 5 mM Hepes (pH 7.0), 1 M NaCl, 5 mM ATP, and 4 μM acridine orange. In the presence of an actinic light, interior acid pH gradients are formed as shown by the quenching of acridine orange fluorescence. Proton transport by the H^+-ATPase is assessed by the addition of 5 mM $MgCl_2$. The pH gradients formed can be dissipated by the addition of 10 mM NH_4Cl, as indicated.

Fig. 3 shows the dose-response for omeprazole-induced inhibition of ATP-mediated proton transport by the H^+-ATPase following a 10-min actinic light pretreatment. The observed inhibition was fully dependent on acid conversion of omeprazole catalyzed by bacteriorhodposin, since dark pretreatment did not lead to specific inhibition of proton transport. Omeprazole was found to completely inhibit transport by the H^+-ATPase at 50 μM. There were only minor effects on light-induced proton pumping by bacteriorhodopsin under these conditions (not shown).

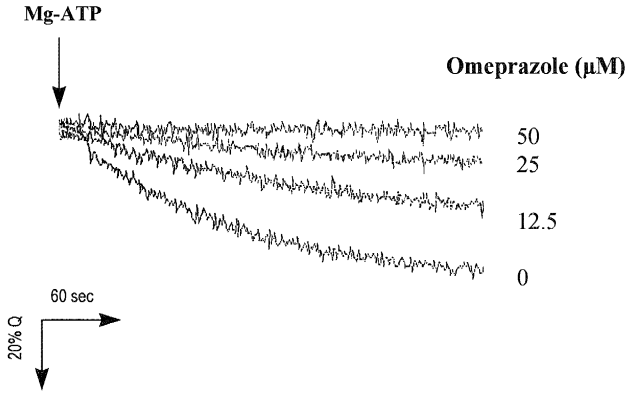

Fig. 3. Dose-dependent inhibition of proton transport of H^+-ATPase by omeprazole. Vesicles co-reconstituted with H^+-ATPase and bacteriorhodopsin were incubated with omeprazole at 0–50 μM, as indicated, in a buffer containing 5 mM Hepes (pH 7.0), 1 NaCl and 4 μM acridine orange. The vesicles were pretreated with actinic light for 10 min, left in the dark for 5 min, and then 5 mM Mg-ATP was used to initiated proton transport by H^+-ATPase.

The reactive sulfenamide produced as a result of acid activation of omeprazole should be localized to the intravesicular surface of the enzyme. To rule out the possibility that a small fraction of the reactive species crossed the bilayer and reacted at the catalytic site of the enzyme, a large molar excess (25-fold) of agarose-conjugated glutathione relative to activated omeprazole was added to the assay mixture. (It was assumed that less than 5% of the omeprazole present would be converted to the sulfenamide form at $\sim$ pH 4.0.) Glutathione effectively protects against the action of omeprazole, as previously demonstrated [15]. When coupled to agarose, it cannot cross the bilayer and is essentially localized to the extravesicular compartment of the reconstituted vesicles. If any active sulfenamide molecules crossed the bilayer, they would rapidly react with the large excess of glutathione, which served as a chemical trap. Fig. 4 shows that light-induced inhibition of the H^+-ATPase was completely unaffected by the presence of glutathione-agarose, even at a high level of omeprazole (100 μM), which caused a slight non-specific diminution of the pH gradient. In contrast, permeant sulphydryl reagents were completely effective in protecting the enzyme against omeprazole-induced inactivation (not shown). Typically, omeprazole-inhibited enzyme was

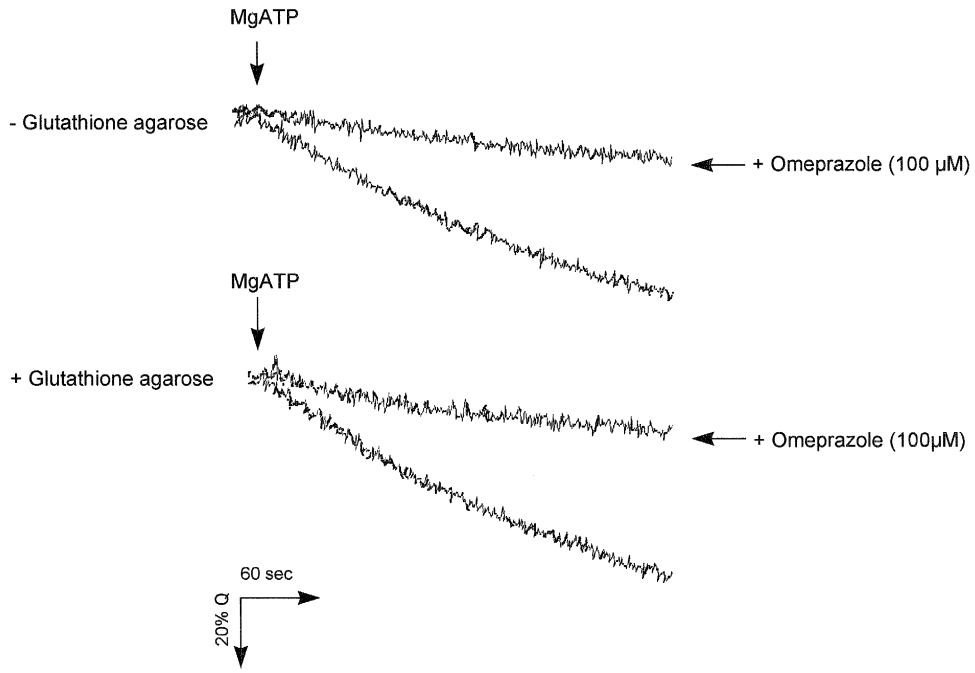

Fig. 4. Effect of Glutathione-agarose on omeprazole-induction inhibition of H^+-ATPase Co-reconstituted vesicles were assayed for ATP-mediated proton transport, as described in Fig. 3. The vesicles were incubated in the presence or absence of 100 μM omeprazole and 250 μM glutathione-agarose, prior to 10 min actinic light treatment, as shown.

only partially reactivated (< 40%) following treatment with 2-mercaptoethanol. Partial reactivation may have resulted from inhibited enzyme forming a highly perturbed conformation or from the modification of multiple sites with differential chemical accessibility once modified. The covalent nature of the inhibition was further demonstrated by showing that the enzyme remained fully inhibited following centrifugation and/or dilution with reaction buffer. These results suggest that inhibition of the H^+-ATPase most likely occurs from the intravesicular face of the enzyme, and it is mediated by covalent linkage to one or more cysteine residues.

4. Discussion

4.1. Enzyme inhibition is mediated by the extracellular portion of the H^+-ATPase

In this report, we provide evidence from a novel in vitro assay system that H^+-ATPase function can be blocked when a positively charged sulfenamide derived from omeprazole interacts with the extracellular portion of the H^+-ATPase. This result is consistent with the sided interaction of omeprazole and SCH28080 on the H^+,K^+-ATPase of gastric parietal cells [14,28] and of cardiac glycosides with the Na^+,K^+-ATPase of red blood cells [29]. The P-type ATPases in the plasma membrane expose only a small portion (< 10%) of their total mass at the extracellular surface [30]. Nonetheless, therapeutics which target these enzymes are highly selective. Omeprazole-induced inhibition of the fungal H^+-ATPase does not involve the same local interaction site as the gastric H^+,K^+-ATPase, since the cysteine residues involved in inhibition of that enzyme are not conserved in the fungal enzyme [15]. Our experiments raise the distinct possibility that water-soluble antagonists targeting the fungal enzyme can be identified. Such reagents would be desirable because inhibition of the enzyme from the extracellular surface of the membrane would effectively eliminate drug resistance problems due to high capacity drug efflux pumps, which are associated with azole resistance of clinical isolates of *Candida albicans* [4]. A clearer understanding of the mechanism of sulfenamide-dependent inhibition of the H^+-ATPase could help identify a cell surface target region suitable for drug discovery purposes.

4.2. M1 and M2 can modulate omeprazole-dependent inhibition of the H^+-ATPase

It is well established that modification of amino acid side groups in M1, M2 can alter the local structure of this region [19,20]. The role of local perturbations in M1, M2 on omeprazole-induced inhibition was examined by surveying a series of well characterized mutations in this region. Many of the mutations enhanced the sensitivity of cells to omeprazole (Fig. 1A), although the most prominent effects were observed for M128C in M1 and D140A or D140C in the extracellular loop region joining M1 and M2 (Fig. 1A,B). In contrast, only one mutant, G158D,G156C, near the cytoplasmic end of M2 was shown to confer significant insensitivity to omeprazole. It was not possible to examine this mutant enzyme in the in vitro assay system because it did not show reconstituted proton transport. These results suggest that perturbations in the hairpin region alter the omeprazole interaction with the H^+-ATPase. While it is not yet possible to determine whether these effects are direct or indirect, M1 and M2 are believed to be important regions of therapeutic interaction for cardiac glycosides in the Na^+,K^+-ATPase [31,32] and reversible inhibitors like SCH28080 in the gastric H^+,K^+-ATPase [28]. This region of the enzyme is highly conformationally active and appears to be coupled to the catalytic active site domain [33,34]. Thus, the hairpin region formed by M1 and M2 could represent an important interaction region on the fungal H^+-ATPase for therapeutic intervention, as has been observed for therapeutics to higher enzymes. The hairpin structure appears to be a conserved structural motif for all P-type ATPases. However, the specificity of the various therapeutics for individual enzymes, which may vary by 3–4 orders of magnitude [32], appears to lie in the turn region; this region varies between all the different eukaryotic enzymes [35]. While M1, M2 may affect an interaction domain for the omeprazole-generated sulfenamide, it does not appear to be the site of covalent modification. Conserved residue C148 in M2 had earlier been suggested to be a possible primary site of interaction for omeprazole [15]. It was recently shown

in a different strain background that C148S, as well as two other putative membrane sector sulfhydryl groups, C312 and C867, are not involved in omeprazole inhibition of cell growth [16]. In this study we showed that the incorporation of cysteine at various positions within this region had little impact, except at positions D140 and M128. However, there is no evidence for a direct interaction of omeprazole at these sites. Thus, it is appears more likely that the site of covalent interaction by omeprazole lies in a different portion of the external domain of the molecule. Identifying the region containing that residue(s) will be an important goal toward defining a useful drug target site on the H^+-ATPase.

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