Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 68 (2004) 2117–2127
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Differences in binding properties of two proton pump inhibitors on the gastric H^+,K^+-ATPase in vivo

Jai Moo Shin[a,b], George Sachs[a,b,*]

[a]Department of Physiology and Medicine, University of California, Los Angeles, CA 90073, USA
[b]VA Greater Los Angeles Healthcare System, 11301 Whlshire Blvd., Building 113, Los Angeles, CA 90073, USA

Received 10 June 2004; accepted 27 July 2004

Abstract

Restoration of acid secretion after treatment with covalently-bound proton pump inhibitors may depend on protein turnover and on reversal of inhibition by reducing agents such as glutathione. Glutathione incubation of the H^+,K^+-ATPase isolated from omeprazole or pantoprazole-treated rats reversed 88% of the omeprazole inhibition but none of the pantoprazole inhibition. The present study was designed to measure binding properties of omeprazole or pantoprazole in vivo. Rats were injected with ^{14}C-omeprazole or ^{14}C-pantoprazole after acid stimulation. The specific binding to the gastric H^+,K^+-ATPase was measured at timed intervals as well as reversal of binding by glutathione reduction. The stoichiometry of omeprazole and pantoprazole binding to the catalytic subunit of the H^+,K^+-ATPase was 2 moles of inhibitor per mole of the H^+,K^+-ATPase phosphoenzyme. Omeprazole bound to one cysteine between transmembrane segments 5/6 and one between 7/8, pantoprazole only to the two cysteines in the TM5/6 domain. Loss of drug from the pump was biphasic, the fast component accounted for 84% of omeprazole binding and 51% of pantoprazole binding. Similarly, only 16% of omeprazole binding but 40% of pantoprazole binding was not reversed by glutathione. The residence time of omeprazole and pantoprazole on the ATPase in vivo depends on the reversibility of binding. Binding of pantoprazole at cysteine 822 is irreversible whereas that of omeprazole at cysteine 813 and 892 is reversible both in vivo and in vitro. This is consistent with the luminal exposure of cysteine 813 and 892 and the intra-membranal location of cysteine 822 in the 3D structure of the H^+,K^+-ATPase.

Keywords: The proton pump inhibitor; The residence time; The gastric H^+,K^+-ATPase; Binding sites; Glutathione; Stoichiometry

1. Introduction

Proton pump inhibitors (PPIs) are now widely used for treatment of both erosive and non-erosive gastro-esophageal reflux disease (GERD and NERD) [1]. These current PPIs are substituted pyridyl methyl sulfinyl benzimidazole prodrugs that accumulate in the acidic secretory canaliculus of the parietal cell (luminal surface of the gastric ATPase) due to protonation of the pyridine [2,3]. In this compartment they then undergo an acid-catalyzed chemical rearrangement that is necessary for their activity that follows a second protonation on the benzimidazole at a much lower pK_a [3]. This result in rearrangement to the active form of these compounds which is a thiophilic cation, which then reacts with one or more cysteines accessible from the exoplasmic surface of the pump to form a covalently inhibited enzyme. The reactive species may be either a sulfenic acid or the sulfenamide that is formed by dehydration of the sulfenic acid [2,3]. The sulfenamide is the form that can be isolated in acidic solutions but the sulfenic acid is a necessary intermediate in the formation of the sulfenamide [3,4]. Recent chemical evidence suggests that the protonated PPI binds to the pump and then undergoes activation due to the second protonation to the sulfenic acid [3]. Since the rate of this activation differs between different PPIs, different sites of reaction with cysteines available on the luminal surface of the H^+,K^+-ATPase are possible.

Identification of the cysteines in the catalytic α-subunit of the pump protein that react with the various proton pump inhibitors produced the rather surprising result that different proton pump inhibitors react with different cysteines

Abbreviations: PPI, proton pump inhibitor; GSH, glutathione; NEM, *N*-ethyl maleimide; F-MI, fluorescein-5-maleimide; DTT, 1,4-dithio-DL-threitol; TM, transmembrane segment; H2RA, H2-receptor antagonist; AUC, area under the curve; TCEP, tri(carboxyethyl)phosphine

* Corresponding author. Tel.: +1 310 268 4672; fax: +1 310 312 9478.
E-mail address: gsachs@ucla.edu (G. Sachs).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.07.035

[5–7]. The catalytic subunit of the pump has ten transmembrane segments and several cysteines are possibly accessible from the exoplasmic surface, the site of activation of the PPIs. While all PPIs reacted with cysteine 813 at the exoplasmic entry into 6th transmembrane segment (TM6) of the alpha subunit of the pump, omeprazole was also able to bind to cysteine 892 in the loop between TM7 and TM8, lansoprazole with cysteine 321 at the end of TM3 and pantoprazole with cysteine 822 now known to be deeper within the membrane domain of TM6, by about 2.5 turns of the alpha helix [5–10]. Labeling of cysteine 813 correlated with inhibition of the enzyme by omeprazole whereas that of cysteine 892 did not [11].

Two factors are considered relevant in terms of their efficacy and their biological target, the gastric acid pump that is the final step in acid secretion and their covalent binding to cysteines of the acid pump that provides duration of action much longer than that predicted from their plasma half-life. This covalent binding was thought to irreversibly inhibit the ATPase and recovery of acid secretion then to depend entirely on de novo pump biosynthesis [12]. However, acid secretion and H^+,K^+-ATPase activity after omeprazole treatment returned with a half-life of only 15–20 h in rat [13–15] and ~17 h in dog [16], about three times faster than the half-life of the α-subunit of the pump which is 54 h [13]. Acid secretion in man also returns faster than expected from protein turnover for omeprazole. However, in people, pantoprazole has duration of action compatible with recovery due only to pump biosynthesis [17,18].

It is therefore possible that the more rapid restoration of acid secretion in the case of omeprazole depends on the tertiary structure of the inhibitory complex and that the rate of reversal of the inhibition by glutathione or other reducing agents in the parietal cell could be different for omeprazole and pantoprazole due to their different cysteine binding sites. The concentration of glutathione in the rat stomach is about 3.3 μmole per gram of wet tissue [19]. Access of these agents to the sites of inhibition on the pump could occur during membrane recycling or glutathione could be secreted into the canaliculus of the parietal cell. In studies in isolated rabbit gastric glands various disulfide reducing agents, such as cysteine and glutathione were able to reverse omeprazole inhibition [15]. Moreover, reduction of cellular glutathione levels by cylohexene-1-one enhanced omeprazole inhibition [20].

Previously, we had investigated the reversal of in vivo inhibited gastric H^+,K^+-ATPase by treating the enzyme isolated from PPI-treated rats with disulfide reducing agents and measuring recovery of ATPase activity as a function of time of incubation in vitro. It was observed that incubation with 10 mM GSH at 37 °C reversed the inhibition due to prior treatment with omeprazole, *R*-omeprazole, *S*-omeprazole, lansoprazole, and rabeprazole. 88% of omeprazole inhibition was reversed by glutathione. In contrast, neither DTT nor glutathione were able to reverse pantoprazole inhibition in either resting or stimulated membranes [21].

There have been several pharmacokinetic and pharmacodynamic analyses of proton pump inhibition [17,22,23]. The essential parameters for covalent inhibition are not as well defined as those for the more usual class of reversible inhibitor, where the plasma level is the most important determinant of activity. The most important parameters for PPI inhibition were thought to be either the area under the curve or more recently plasma residence time above threshold since the covalent nature of the inhibition results in a duration of action longer than the plasma residence time [24–26]. A property that has not been considered is the stability of covalent binding of the PPI on the pump. Thus, we have now investigated the kinetics of omeprazole or pantoprazole binding to the gastric H^+,K^+-ATPase at timed intervals after intravenous administration of labeled compounds to fasted rats.

2. Materials and methods

2.1. Materials

^{14}C-omeprazole and ^{14}C-pantoprazole sodium salt were gifts of Wyeth Pharmaceutical Company. All reagents were analytical grade or higher.

2.2. Animals

The animal study was approved by the Animal Care and Use Committee of VA Greater Los Angeles Healthcare System and fulfilled National Institutes of Health guidelines for use of animal subjects. Male rats (Sprague–Dawley, 280–310 g) were used.

2.3. Preparation of crude gastric membrane containing rat gastric H^+,K^+-ATPase labeled by ^{14}C-omeprazole and ^{14}C-pantoprazole

Rats were fasted for 24 h with free access to water. Rats were maximally stimulated by subcutaneous histamine (40 mg/kg) and carbachol (20 μg/kg) injection and radioactive ^{14}C-omeprazole or ^{14}C-pantoprazole was administered by IV injection at 1 mCi/kg of animal with a dosage of 20 μmole/kg (N = 4–6 per each group) through the tail vein. The rats were sacrificed at 0.5, 1, 2, 6, 12, and 24 h. At each time point the stomachs were opened and washed with a phosphate buffered saline (pH 7.4) buffer, and kept in a 15 ml borosilicate bottle on ice, and then used for scraping the gastric mucosa. From each rat, the corpus mucosa was scraped and the tissue was then resuspended in 3.5 mL of a homogenization buffer composed of 0.25 M sucrose, 2 mM *N*-ethyl maleimide (NEM, to prevent secondary labeling of unreacted cysteines), 2 mM EDTA, 2 mM EGTA, and 10 mM Pipes/Tris, pH 7.4. The mucosal

suspension was homogenized with 15 strokes of Teflon pestle at 2000 rpm in a Potter–Elvehjem homogenizer. All operations were done at 2–4 °C. The homogenate was centrifuged at 1600 × *g* for 30 min at 4 °C, and the pellet was discarded. The supernatant was centrifuged at 100,000 × *g* at 4 °C for 60 min to give the crude membrane pellet. This membrane pellet was resuspended in 2 ml of a buffer composed of 0.25 M sucrose, 10 mM Pipes/Tris, pH 7.4.

2.4. Quantification of the H^+,K^+-ATPase in the crude gastric membranes

The amount of gastric H^+,K^+-ATPase in each crude membrane fraction was quantified by the method described previously [21]. Briefly, an aliquot of the membrane fraction (1 μg) was run on mini slab SDS-gel (8%) together with given amounts of hog gastric H^+,K^+-ATPase membranes (0.05–0.3 μg) which are about 85% pure. The quantity of H^+,K^+-ATPase was determined by Western-blotting of the membranes with the monoclonal antibody Ab12.18 and by comparing the staining intensity in the membrane fraction with the known amounts of hog gastric H^+,K^+-ATPase. Staining intensity was analyzed by a software, Image J (version 1.29x, Wayne Rasband, National Institutes of Health, USA).

2.5. Omeprazole and pantoprazole binding to the H^+,K^+-ATPase

The specificity of protein binding was determined by SDS-gel electrophoresis. An aliquot of crude membrane fraction was run on a tricine gel (10–16.5% gradient). One lane of the gel was sliced at 2 mm width and each slice was dissolved in a tissue dissolving solution (DuPont Solvable™, NEN research products) and the counts, measured. In another method, the same amount of rat membranes was precipitated as described below and the precipitated protein was counted. From the gel, all counts were recovered on the H^+,K^+-ATPase band, which showed the same counts as obtained from the precipitated protein.

The crude membrane fraction (200 μg) from every stomach were isolated and counted to give the total amount of compound accumulated in the membranes at given time. The amount of omeprazole or pantoprazole covalently bound to the enzyme was determined by either SDS-gel analysis as described above or by protein precipitation. Here, the same amount of crude membranes (200 μg) from each stomach was precipitated by a nine-fold excess of ice-cold methanol on ice for 20 min, and then centrifuged at maximum speed of an Eppendorf centrifuge for 3 min. The supernatant was removed and the pellet was again resuspended in 0.8 ml of ice-cold methanol, and centrifuged to remove trace amounts of non-bound PPI. Precipitated materials were dissolved in 0.3 ml of 50 mM Tris–HCl, 0.2% SDS, pH 7.0, and 1 mM NEM, and counted for ^{14}C isotope content, which reflects the PPI level that is covalently protein-bound. The absolute amount of drug bound per mg of gastric H^+,K^+-ATPase was calculated by dividing the pmol compound bound to the 97 kDa protein on the gel by the ng of H^+,K^+-ATPase present on the gel at that molecular weight as defined by Western analysis.

2.5.1. Effect of reducing agents on binding

An aliquot of enzyme was resuspended in a buffer composed of 50 mM Tris–HCl, pH 7.4, in the presence or absence of 10 mM glutathione (GSH) or dithiothreitol (DTT), at a H^+,K^+-ATPase protein concentration of 0.1 mg/ml. These enzyme aliquots were incubated at 37 °C. At timed intervals, an aliquot containing 15 μg of the H^+,K^+-ATPase was taken out and precipitated by adding nine-fold excess of ice-cold methanol and kept on ice for 20 min, then, centrifuged at maximum speed of Eppendorf centrifuge for 2 min. The precipitated pellet was resuspended in 0.3 ml of ice-cold methanol, and centrifuged again to remove trace of non-bound PPI. Precipitated materials were dissolved in 0.3 ml of 50 mM Tris–HCl, 0.2% SDS, pH 7.0, and 1 mM NEM, and counted ^{14}C isotope content.

The binding refractory to reducing agents was determined by treatment of with the strong reducing agent, tri-(carboxyethyl)phosphine (TCEP) as follows. An aliquot was incubated at 37 °C for 1 h in the presence or absence of 10 mM TCEP. An aliquot of ∼15 μg of the H^+,K^+-ATPase was taken out and precipitated by adding nine-fold excess of ice-cold methanol and counted. About 6% binding was not reversed by this very strong reducing reagent for both omeprazole and pantoprazole showing that a small fraction was bound by non-disulfide bonds.

2.5.2. Identification of regions of binding by trypsin cleavage

Crude membranes containing 100 μg of the H^+,K^+-ATPase were diluted up to total 0.5 mL by adding 0.25 M sucrose, 0.2 M Tris–HCl, pH 8.2. Trypsin 80 μg in dissolved in 80 μl of 0.1 M Tris–HCl, pH 8.2, was added and the mixture was incubated for 30 min at 37 °C. The samples were kept on ice and soybean trypsin inhibitor 800 μg dissolved in 100 μl of 50 mM Tris–HCl (pH 8.2) was added. The membranes were centrifuged at 100,000 × *g* for 1 h (at 4 °C). The pellet was briefly washed with 1 ml of 0.25 M sucrose, 0.1 M Tris–HCl, pH 7.4, then, dissolved in 400 μl of 0.01 mM NEM, 0.1 M Tris–HCl, pH 7.4 with 0.2% SDS, To this solution, 100 μl of sample buffer composed of 40% sucrose, 0.01% phenol blue, and 0.1 M Tris–HCl, pH 7.4 was added. 100 μl of sample having 25 μg of the H^+,K^+-ATPase was applied on a 10–20% tricine big slab gel and run at 50 mA constant. G1 vesicles were treated by the same method as described above and run on one lane next crude membrane digest for comparing transmembrane segments. This method has been previously described in detail and has allowed identification of the different sites of labeling by proton pump

inhibitors [5,7]. The gel was transblotted on a PVDF membrane as previously described [27]. A film was developed to obtain the autoradiogram from the PVDF blot.

2.5.3. Identification of pantoprazole-labeled TM5/segment by thermolysin digestion

To confirm the dual site of labeling by pantoprazole as compared to omeprazole in the TM5/6 region, further digestion experiments are necessary. Here, [^{14}C] pantoprazole-labeled gastric vesicles were prepared as previously described [5,8] and used for identification of the pantoprazole-binding sites. [^{14}C] Pantoprazole-labeled gastric vesicles (0.8 mg) were extensively digested with trypsin (0.2 mg) in a buffer (1 ml) composed of 0.25 M sucrose, 50 mM Tris–HCl, pH 8.2, for 60 min. The reaction was stopped by adding 2 mg of soybean trypsin inhibitor. The membrane digest was spun in a Beckman L5 centrifuge using a Ti-65 rotor at 100,000 × *g* for 60 min. The pellet was labeled with fluorescein-5-maleimide (F-MI) by dissolving in a buffer (0.8 ml) composed of 20 mM Tris–HCl, pH 6.7, 0.2 mM fluorescein-5-maleimide, and 0.5% SDS. The F-MI is used here to identify any unreacted cysteines. The membrane digest sample was combined with an electrophoresis sample buffer and applied on a 10–20% tricine big slab gel and run at 50 mA constant current as previously described [5,8]. The lowest region at 4.5–6.2 kDa molecular weight range contained a fluorescent band representing the TM1/2 segment and the [^{14}C] pantoprazole-labeled TM5/6 segment. This band was sliced from the gel. The labeled peptide fragments were electroeluted from the gel slices using Bio–Rad Electroeluter Model 422 equipped with a Membrane Caps (molecular cut-off 3.5 kDa). Electroelution was carried out in a buffer composed of 30 mM Tris–HCl, pH 8.0, 0.03% SDS at 120 V constant voltage for 6 h. The electroelution buffer was carefully removed and replaced with a new buffer composed of 10 mM Tris–HCl, pH 8.0, 0.01% SDS. Electrodialysis was carried out at 100 V constant voltage for 1 h. The eluate containing the TM1/2 and TM5/6 segments, which was above the membrane caps, was separated and diluted by adding three-fold excess volume of a buffer composed of 10 mM Tris–HCl, 3 mM $CaCl_2$, pH 8.0. The eluate was concentrated by Amicon Ultra Centrifugal Filter Device-4500 MWCO (Millipore) down to 0.2 mL. The eluate was divided into two portions. One portion served as a control, and the other portion was digested with 30 μg of thermolysin (Sigma, Protease Type X) at 37 °C for 24 h to further cleave TM5/6. After digestion, the samples were combined with 10 μl of 2 M sucrose and 0.25% bromphenol. The samples were placed on top of a 1.5 mm gradient slab gel using the Tricine buffer method [8]. The upper half of the gel consisted of 10% (34:1 acrylamide/methylene bisacrylamide) to 21% (17:1 acrylamide/methylene bisacrylamide) gradient gel and the lower half of the gel consisted of 21% (17:1 acrylamide/methylene bisacrylamide). The gel was run in the cold room (4 °C) for 18 h at 45 mA constant current along with a lane for CNBr-cleaved fragments of horse myoglobin (Sigma, 17–2.5 kDa) as molecular weight standards.

The peptides were transferred electrophoretically to PVDF membranes (Millipore) for 18–24 h in the cold room (4 °C) in a tank transfer apparatus at 120 mA constant current, in a transfer buffer of 150 mM glycine, 20 mM Tris–HCl, and 20% methanol. A sandwich of three sheets of Whatman 3 mm filter paper was placed on either side of the gel, which had a prewetted PVDF membrane on the anode side. After transfer, the blots were rinsed twice in distilled water and stained with 0.1% Coomassie Blue in 10% glacial acetic acid and 45% methanol.

In every case, a duplicate lane was run to provide material for sequencing as well as for either counting or autoradiography. Reducing agents were absent in all experiments since these remove the bound benzimidazole. Standard curves of ln (M_r) as a function of relative mobility were used to estimate the M_r of the peptide products of digestion. The accuracy of the M_r determination appeared to be within 10% based on predicted tryptic cleavage sites within the primary sequence of catalytic subunit of the enzyme.

3. Results

We administered 20 μmole/kg of labeled omeprazole or pantoprazole intravenously after histamine and carbachol stimulation to induce full stimulation of acid secretion thereby maximizing binding of the labeled inhibitors to the H^+,K^+-ATPase [15,23,28,29].

3.1. Labeling of intact stomach

After intravenous injection, both proton pump inhibitors were rapidly accumulated in total gastric tissue and then the counts decreased. After 2 h, elimination of the PPIs from the stomach slowed markedly. The loss of counts over the first two hours probably represents elimination of a large fraction of drug that was not covalently bound to the pump, whereas the slow phase represents loss of covalently bound counts. There was no significant difference of total distribution to the stomach between omeprazole and pantoprazole.

3.2. Quantification of the H^+,K^+-ATPase in gastric membranes

Coomassie staining and Western blot analysis identified the position of the catalytic subunit of the H^+,K^+-ATPase at 97 kDa. The intensity of Coomassie staining at 97 kDa was a little higher than that of the Western blot as previously reported [30]. Since Western blot analysis with antibody12.18 is specific for the gastric H^+,K^+-ATPase [31],

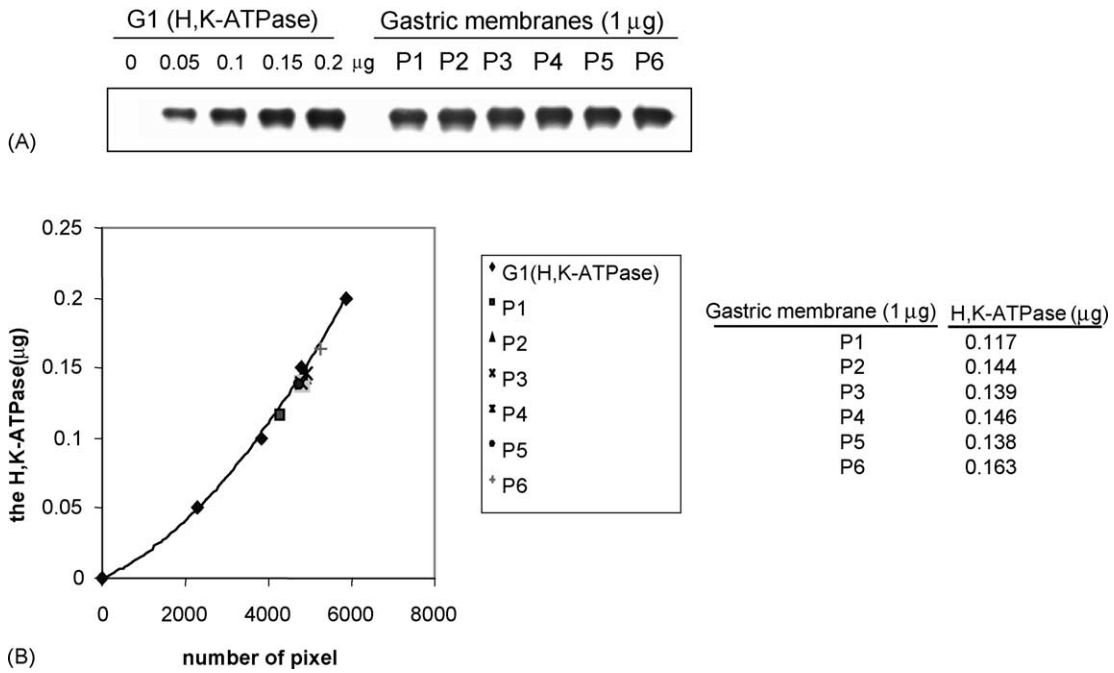

Fig. 1. Quantitative analysis of the gastric H^+,K^+-ATPase in the gastric membranes. The quantity of H^+,K^+-ATPase was determined by Western blot analysis with Ab12.18 and comparing the intensity with the given amounts of hog gastric H^+,K^+-ATPase. The typical example in this figure shows that six different pantoprazole labeled gastric membranes (P1–P6) prepared from six different rats have 11.7–16.3% of H^+,K^+-ATPase in the membrane fraction. Similar data were obtained with omeprazole.

the amount of the H^+,K^+-ATPase in the gastric membrane was determined by immunoblot quantification [21].

Fig. 1 shows a typical quantitative analysis for the gastric H^+,K^+-ATPase for a preparation of pantoprazole-treated membranes prepared at a 24 h time point. The relationship between the staining for H^+,K^+-ATPase and pixel numbers generated from Western blot of hog gastric H^+,K^+-ATPase fitted a binomial equation,

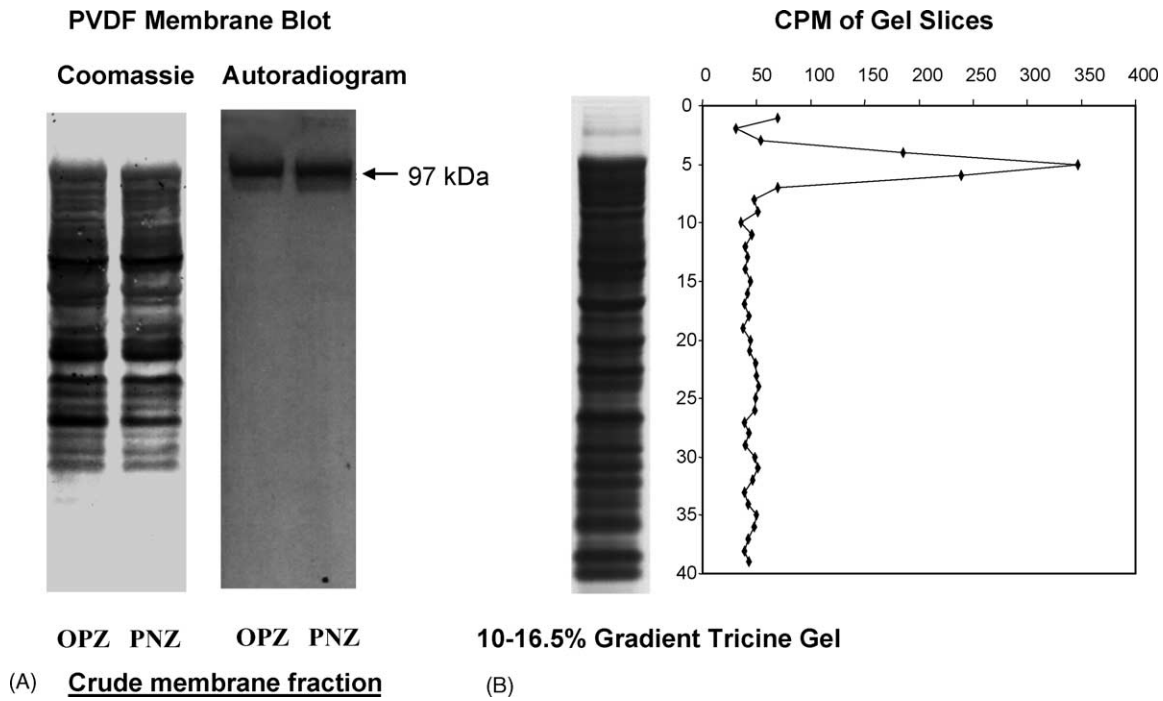

Fig. 2. Site of covalent binding. (A) An aliquot of crude gastric membrane fraction from omeprazole or pantoprazole treated rat was run on a tricine gel (10–16.5% gradient) and immunoblotted. Omeprazole (OPZ) and pantoprazole (PNZ) bind at a protein band of 97 kDa, which is the gastric H^+,K^+-ATPase α-subunit. (B) An aliquot of crude membrane from omeprazole-treated rat was run in 10–16.5% gradient gel. One lane of the gel was sliced at 2 mm width and each slice was dissolved in a tissue dissolving solution (DuPont Solvable™, NEN research products), and radioactivity determined. Similar data were obtained with pantoprazole.

$y = 4 \times 10^{-09} \times x^2 + 10^{-05} \times x - 6 \times 10^{-05}$ where x is the number of pixel and y is the amount of the H^+,K^+-ATPase.

Each group (N = 4–6) of crude membranes prepared at given time was analyzed by this method using hog gastric H^+,K^+-ATPase standards (50–300 ng) in the same gel and Western blot. One set of typical data is shown in Fig. 1.

3.3. Compound binding to the H^+,K^+-ATPase

The proteins in the crude membranes were separated by SDS–PAGE to identify the binding site of the two PPIs. Fig. 2A and B shows that the proton pump inhibitors bind only to the protein band at 97 kDa confirming previous data [5,7,8].

From the gel, all counts were present at the position of the H^+,K^+-ATPase α-subunit, and the radioactivity found here was the same as that obtained from the precipitated protein (deviation was <1.5%). For example, in Fig. 3B, the sum of the counts at the H^+,K^+-ATPase was 772 cpm. When the same amount of crude membrane was methanol precipitated as described in experimental procedure, the precipitated protein provided 783 cpm.

3.4. The residence time of omeprazole and pantoprazole on the gastric H^+,K^+-ATPase in fasting rats

Previous in vitro data have shown that omeprazole binds two omeprazole molecules per phosphoenzyme [32]. About 2.6 nmole of omeprazole was bound to 1 mg of isolated hog gastric H^+,K^+-ATPase-containing vesicles under acid transporting conditions. One milligram of ATPase contains about 1.3 nmole of phosphoenzyme

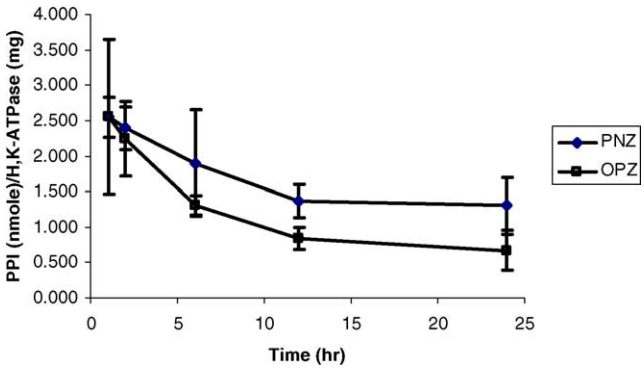

Fig. 3. Time course of loss of omeprazole or pantoprazole binding to the H^+,K^+-ATPase. At timed intervals, crude gastric membranes were collected and ^{14}C-labeling of the H^+,K^+-ATPase measured as described in experimental procedure using fasted rats. Radioactive ^{14}C-omeprazole or ^{14}C-pantoprazole was administered by IV injection at 1 mCi/kg of animal with a dosage of 20 μmole/kg (N = 4–6 per each group) through the tail vein. The rats were sacrificed at 1, 2, 6, 12, and 24 h. At each time point the stomachs were opened and the binding amounts of proton pump inhibitor were measured per mg of the H^+,K^+-ATPase. Diamonds (◆) represent average amounts of pantoprazole (PNZ) binding per mg of the H^+,K^+-ATPase at each given time point and open squares (□) represent average amounts of omeprazole (OPZ) binding per mg of the H^+,K^+-ATPase. Error bars represent the standard deviation of amounts of PPI binding (n = 4–6 for each time point).

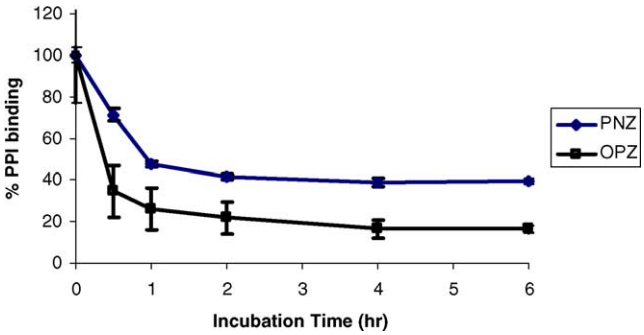

Fig. 4. Removal of omeprazole or pantoprazole binding by GSH. Crude gastric membranes, prepared at 2 h after IV injection of ^{14}C-labeled drug, were incubated with 10 mM GSH and the amount of labeled H^+,K^+-ATPase was determined as described in the experimental procedure. The % labeling at the different times of GSH incubation is shown. The points are average of % PPI binding from five experiments at each given time point and the error bars at each time point represent the standard deviation.

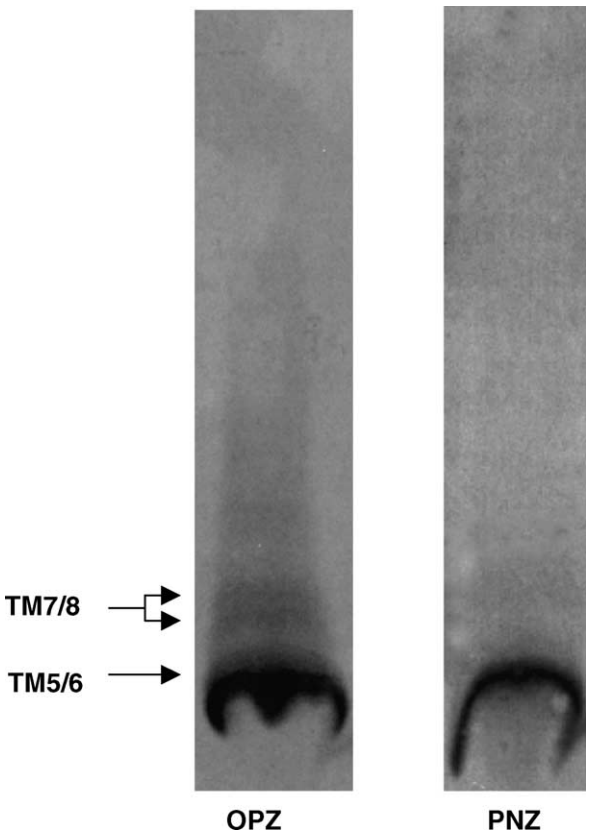

Fig. 5. Autoradiogram of trypsin digest of the gastric membrane prepared at 1 h after PPI treatment in vivo. After isolation of the labeled membranes, they were digested by trypsin as described in Section 2 and the digest separated by SDS–PAGE to identify the labeled region of the ATPase. OPZ and PNZ represent trypsin digest of omeprazole and pantoprazole-treated gastric membranes, respectively. TM7/8 represents a peptide fragment containing 7th and 8th transmembrane segments containing Cys892 and TM5/6 represents a peptide fragment containing the 5th and 6th transmembrane segments, which contain Cys813 and 822. Only the OPZ lane shows labeling of TM7/8 and an additional minor band below TM7/8, which is a TM7 fragment due to further trypsin cleavage.

[2,33]. Here also, based on the immunoblot analysis, a maximum binding of 2.7 nmole of omeprazole or pantoprazole per mg of the H^+,K^+-ATPase was obtained at 1 h as shown in Fig. 3 Thus, in vivo, omeprazole and pantoprazole seem to have same stoichiometry, namely, 2 moles of PPI per mole phosphoenzyme.

Full inhibition was reported to be obtained at ~1 h after intravenous administration [22,34]. Maximum binding of PPI at 1 h in this experiment fits well with the known full inhibition obtained at 1 h. The rate of loss of both omeprazole and pantoprazole from the H^+,K^+-ATPase was biphasic with a distinct first fast phase and a distinct subsequent slow phase, each showing first-order kinetics.

3.5. Removal of omeprazole or pantoprazole binding by glutathione and other reducing agents

It has been shown that glutathione (GSH) can reverse the inhibition by omeprazole in gastric glands and of the gastric H^+,K^+-ATPase [20,21,32,35]. Similarly, GSH can reverse the inhibition of rabeprazole [36] and lansoprazole [37]. Fig. 4 shows that GSH can remove either omeprazole or pantoprazole from the H^+,K^+-ATPase following in vivo labeling.

However, reversal of covalent binding had different characteristics for omeprazole or pantoprazole as shown in Fig. 5 for GSH treatment of enzyme isolated after 2 h labeling in vivo. Only 16% of omeprazole counts were not removed by GSH whereas 40% of pantoprazole counts remained stable under identical conditions. These data are similar to the spontaneous loss of counts determined above.

However, about 6% of omeprazole and pantoprazole binding was not removed by tri(carboxyethyl)phosphine (TCEP), a strong reducing agent, which suggests that about 6% of in vivo PPI binding is not bound via a disulfide bond. Probably this refractory, non-reducible binding must be a carbon–sulfur or nitrogen–carbon bond on the enzyme after the reactive pyridinium sulfenic acid undergoes a substitution of pyridinium group with enzyme thiol as a minor reaction pathway. It is known that there is a minor reaction pathway of activation where the pyridinium sulfenic acid or sulfenamide forms a thioamide, which would not be affected by reducing agents [38,39], presumably accounting for these data.

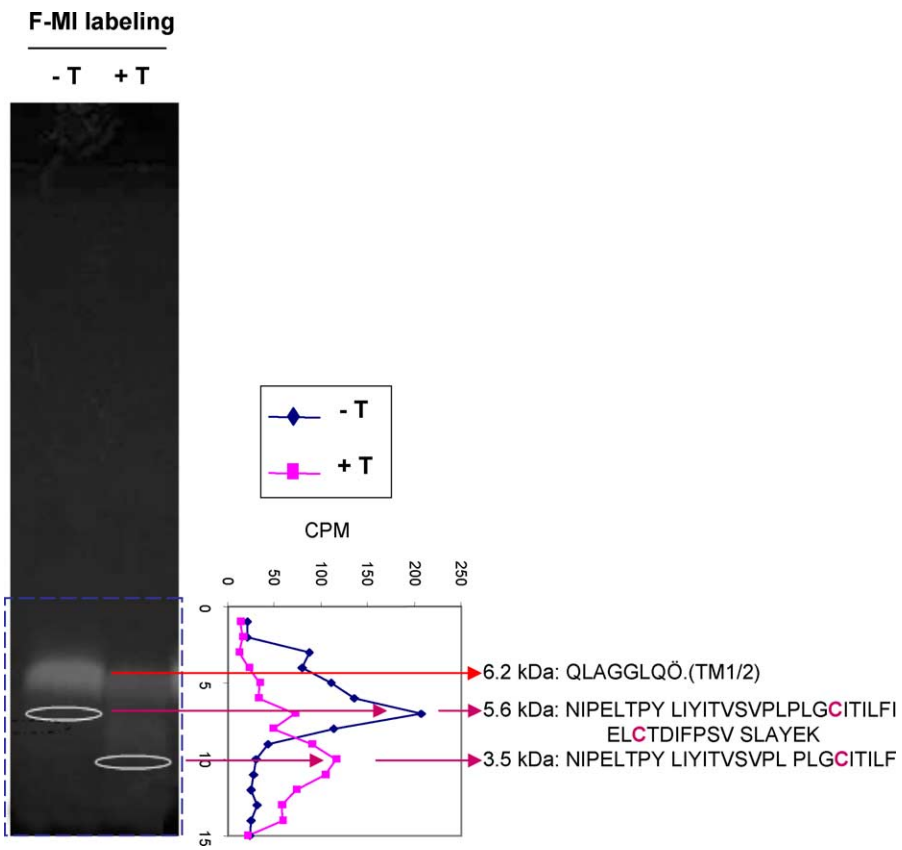

Fig. 6. Thermolysin digestion of fifth and sixth transmembrane segment. The left panel represents fluorescein-5-maleimide (F-MI) fluorescence image of the gel. (−T) represents undigested TM1/2 and TM5/6 segments and (+T) represents the thermolysin digest. The rectangle highlights the regions of interest. The band of F-MI fluorescence contained the TM1/2 segment given the N-terminal sequence, QLA (first arrow). The rectangular insert to the right shows the radioactivity of the corresponding regions of the left panel. The band labeled by pantoprazole at 5.6 kDa is the TM5/6 segment which starts with a N-terminal sequence, NIPE shown by the second arrow. The band at 3.5 kDa shown by the third arrow has also as its N-terminal sequence, NIPE, showing that loss of counts is due to cleavage prior to cysteine 822 with retention of the counts bound to cysteine 813 as shown by the sequences defined with cysteine highlighted in red. The radioactivity at each cysteine appears to be about equal.

3.6. The binding sites of omeprazole or pantoprazole

As found from studies of labeling of the isolated hog gastric ATPase [7] omeprazole binds to both the TM5/6 and the TM7/8 membrane domains as shown in the tryptic digest pattern of Fig. 5. Pantoprazole does not bind to the TM7/8 domain but only to TM5/6 as also shown in Fig. 5 [5].

Omeprazole binding at Cys813 has been identified by thermolysin cleavage of omeprazole-labeled TM5/6 [8]. Here, pantoprazole binding to both Cys813 and Cys822 was also confirmed by thermolysin cleavage. After trypsin digestion followed fluorescein-5-maleimide (F-MI) labeling in the presence of SDS, all transmembrane segments were labeled with either [^{14}C]pantoprazole or F-MI. On first gradient gel, the pantoprazole-labeled TM5/6 segment overlapped with the TM1/2 segment labeled with F-MI. These fragments were not detected by Coomassie staining, but only by [^{14}C]pantoprazole or F-MI fluorescence. These fragments were separated on a second gel after a second thermolysin digestion. [^{14}C] pantoprazole-labeled TM5/6 was then separated from F-MI labeled TM1/2 as shown in Fig. 6. The [^{14}C]pantoprazole-labeled TM5/6 segment did not show any F-MI labeling, which shows that both cysteines (cysteine 813 and 822) in the TM5/6 had been labeled by [^{14}C]pantoprazole [5]. Further thermolysin digestion of these fragments resulted in approximately half of the label being lost. The N-terminal sequence of the labeled peptides at both 5.6 and 3.5 kDa was NIPE [8]. The tryptic fragment of 5.6 kDa (TM5/6) contains two cysteines, Cys813 and Cys822, and the thermolysin-digested 3.5 kDa fragment contains only one cysteine, Cys813, and has lost the sequence, L^{821}CTDIFPSV-SLAYEK. Hence, the loss of counts following thermolysin digestions is due to cleavage before the Cys 822 as shown in Fig. 6 The radioactivity of this C terminal fragment is lost due to loss of this small peptide since it cannot be retained on the PVDF. These results strongly suggest that both Cys813 and Cys822 were labeled by pantoprazole. These data are consistent with the prolonged residence time of pantoprazole on the ATPase in vivo, where Cys 822 is not accessible to glutathione in contrast to Cys 813.

4. Discussion

The binding of omeprazole and pantoprazole to the ATPase depends on accumulation in the acidic space (secretory canaliculus) of the active parietal cell due to protonation of the pyridine (p$K_a \sim 4.0$), which is followed by protonation of the benzimidazole nitrogen at the very low pH of the secretory canaliculus of the stimulated parietal cell, which results in activation of the C-2 position of the benzimidazole which then reacts with the fraction of the pyridine which is unprotonated [3].

It seems likely that the monoprotonated drug binds to the enzyme. Then both drugs then convert to a cationic sulfenic acid, which reacts with cysteines in the pump. In solution the sulfenic acid dehydrates to form the cyclic sulfenamide [4,40]. The rate of activation at acidic pH is significantly slower for pantoprazole than omeprazole, which probably accounts for their binding to different cysteines on the pump. Omeprazole binds to cysteine 813 in the loop between TM5/6 and to cysteine 892 in the loop between TM7/8 [7]. Pantoprazole binds also to cysteine 813 but also to cysteine 822 in TM6 [5,8]. With the availability of 3D crystals of the homologous SERCA Ca-ATPase [41,42] it can be inferred that cysteine 813 is located within a luminal vestibule of the pump between TM5 and TM6 and that cysteine 822 on TM6, is located 2.5 turns of the alpha helix into the membrane domain. Hence, the slower activation of pantoprazole allows access of the protonated drug to both cysteine 813 and 822 followed by sulfenic acid induced disulfide bond formation. Both of these residues are in the transport domain of the enzyme. Cysteine 813 is also in the binding region of K^+-competitive inhibitors of the H^+,K^+-ATPase such as the imidazopyridine, SCH 28080, that are more effective also in their protonated form [43,44]. Cysteine 892 is on the luminal surface of loop between TM7 and TM8 outside the transport domain. This is consistent with the demonstration that binding to cysteine 892 does not affect pump activity [11].

From the predicted 3D structure, cysteines 813 and 892 should be accessible to reducing agents whereas cysteine 822 should not be. This concept was verified by showing that H^+,K^+-ATPase activity following in vivo inhibition was restored after DTT or GSH treatment of the inhibited enzyme in the case of omeprazole but not after pantoprazole treatment [21].

The inhibition of acid secretion by omeprazole has a longer duration of action than that expected from its plasma half-life due to the covalent inhibition of the pump than that expected based on its plasma half-life [45,46]. However, the recovery of acid secretion is much faster than that expected from the half-life of the pump protein alone which is about 54 h. [13,16-18]. The duration of action of pantoprazole appears to be prolonged as compared to omeprazole [17,18,22,34]. In rats, after full inhibition by intravenous administration of PPI, ~15% of acid secretion activity was restored from pantoprazole-treated rats at 3.5 h [22,34], while ~40% of activity was restored from omeprazole-treated rats at 3.5 h [23,34]. The hypothesis investigated here was that this longer duration of action could be correlated with the differences in binding sites on the pump of the two inhibitors. Thus far, no detailed investigation has been carried out on the labeling of the H^+,K^+-ATPase in vivo.

The binding stoichiometry assay of in vivo labeled enzyme showed that about 2 moles of both omeprazole and pantoprazole were bound per mole phosphoenzyme of the H^+,K^+-ATPase, as has been found for isolated enzyme

under acid transporting conditions [2]. This finding would be consistent with binding to two cysteines per mol active enzyme, cysteine 813 and 892 for omeprazole and cysteine 813 and 822 for pantoprazole on each molecule of inhibited pump.

Analysis of the residence time of omeprazole and pantoprazole on the ATPase catalytic subunit over 24 h showed that there was a fast phase with a steep decline in the first 12 h and then a prolonged slow phase from 12–24 h. The loss of counts was therefore biphasic, with both components showing first order kinetics.

The biphasic mode of pantoprazole decay can be approximately fitted to the equation

$$C_{(\text{panto})} = 1.39e^{-0.1193t} + 1.35e^{-0.0139t}$$

and that of omeprazole fitted to

$$C_{(\text{omep})} = 1.39e^{-0.1193t} + 1.35e^{-0.0139t}$$

Pantoprazole labeling at 3.5 h reported here is 85% of maximal binding, which is well fit with the finding that ~15% of acid secretion activity was restored in pantoprazole-treated rats at 3.5 h [22,34]. Pantoprazole binding at TM5/6 as shown in Fig. 5 is known to inhibit the acid secretion [5,8]. In the case of omeprazole, 78% of maximal labeling was found at 3.5 h while ~40% of activity was reportedly recovered from omeprazole-treated rats at 3.5 h [23,34], which suggests that a significant fraction of omeprazole labeling is not responsible for inhibition. It is likely, based on earlier results [8,11], that this is due to labeling at Cys892 in TM7/8, which is not related to inhibition of acid secretion.

From the analysis of the biphasic mode of PPI decay, the fast component of omeprazole accounts for 84% of the total omeprazole bound and the slow component for only 16%. In contrast, the fast and slow components of pantoprazole are approximately equal accounting for 51 and 49% of binding, respectively.

A similar difference in stability of binding of omeprazole or pantoprazole to the pump in vivo was also found when labeled pump was treated with GSH in vitro. Only 16% of the omeprazole counts were resistant to GSH treatment corresponding to the slow phase of dissociation in vivo whereas 40% of pantoprazole labeling resisted GSH treatment. This also approximates the amount of slow dissociating inhibitor found in vivo with pantoprazole labeling.

The simplest explanation for the biphasic kinetics and the difference between omeprazole and pantoprazole is that omeprazole labeling at cysteine 813 and cysteine 892 is reversed in vivo by access of reducing agents such as GSH to the luminal surface of the pump whereas pantoprazole binding at cysteine 813 is also reversible. Loss of drug from these sites would account for the fast phase of loss of either of the bound drug. The rapid phase of in vivo reversal of binding could be explained by the secretion of GSH by the stomach [19,36] or may occur as the pumps cycle between the resting and stimulated state from canaliculus to vesicular tubules and vice versa.

The slow phase of pantoprazole dissociation is due to the irreversible binding at cysteine 822, approximately equal to its binding at cysteine 813. The binding to either cysteine 813 or 822 is sufficient for inhibition of acid secretion since these cysteines are located in the ion transport pathway enclosed by TM4, TM5 and TM6. In the case of omeprazole, removal of the drug from cysteine 813 allows acid secretion to be restored. However, since cysteine 822 is in the transport domain of the pump close to the ion binding sites at 820 and 824 [47], failure to remove pantoprazole from this site by reduction in vivo will result in a longer dwell time and more stable inhibition of acid secretion after a single dose of this drug as compared to omeprazole.

There is a distinction in the rate of activation of omeprazole or pantoprazole in acidic solutions, where the activation of omeprazole is faster than that of pantoprazole [3]. Based on this kinetics of acid activation of two drugs, it seems that the initial step is binding of the mono-protonated drug close to cysteine 813 in the luminal vestibule of the pump. Incubation with omeprazole sulfide, which cannot be activated but is structurally similar to omeprazole competes with omeprazole, resulting in reducing omeprazole-labeling at cysteine 813 [7]. This strongly suggests that indeed mono-protonated omeprazole binds to the pump probably close to cysteine 813. If there is rapid activation to the sulfenic acid at this site, then there will be rapid covalent labeling at cysteine 813, preventing further reaction at cysteine 822. When activation to the sulfenic acid is slower, the protonated drug can reach cysteine 822 before reaction has occurred at cysteine 813 [3]. Pantoprazole thus can react with both cysteines in this region of the enzyme.

Under in vitro acidifying conditions where there is a less acidic pH than in vivo, there does not appear to be reaction with omeprazole at cysteine 822 [7]. However, the data obtained in vivo in terms of labeling and reversal in terms of dissociation of counts or restoration of ATPase activity suggest, that at the higher acidity obtained in vivo, that there is indeed some relatively minor access of the slowly reacting fully di-protonated omeprazole to cysteine 822 and activation at this site, accounting for the 12% irreversibility of ATPase activity ex vivo and the 16% of counts bound to the enzyme that are not removed by GSH. This additional binding would be accounted for by the presence of the more stable di-protonated form of omeprazole [3] at the pH of 0.8 of the secretory canaliculus which is not found at the ~pH 3.0 of acidified gastric vesicles in vitro.

The clinical implication of these data suggest that the inhibition by pantoprazole may be longer lasting than that of omeprazole, which might imply better night-time control of acidity by the former. However, this has not been evaluated in any clinical trials to date.

Acknowledgements

This work was supported by USVA and NIH grant numbers DK46917, 53462 and 41301.

References

[1] Colin-Jones DG. The role and limitations of H2-receptor antagonists in the treatment of gastro-oesophageal reflux disease. Aliment Pharmacol Ther 1995;9(Suppl 1):9–14.
[2] Lorentzon P, Jackson R, Wallmark B, Sachs G. Inhibition of ($H^+ + K^+$)-ATPase by omeprazole in isolated gastric vesicles requires proton transport. Biochim Biophys Acta 1987;897(1):41–51.
[3] Shin JM, Cho YM, Sachs G. Chemistry of Covalent Inhibition of the Gastric H,K-ATPase by Proton Pump Inhibitors. J Am Chem Soc 2004;126(25):7800–11.
[4] Lindberg P, Nordberg P, Alminger T, Brandstrom A, Wallmark B. The mechanism of action of the gastric acid secretion inhibitor omeprazole. J Med Chem 1986;29(8):1327–9.
[5] Shin JM, Besancon M, Simon A, Sachs G. The site of action of pantoprazole in the gastric H^+/K^+-ATPase. Biochim Biophys Acta 1993;1148(2):223–33.
[6] Sachs G, Shin JM, Besancon M, Prinz C. The continuing development of gastric acid pump inhibitors. Aliment Pharmacol Ther 1993; 7(Suppl 1):4–12. discussion 29–31.
[7] Besancon M, Shin JM, Mercier F, Munson K, Miller M, Hersey S, et al. Membrane topology and omeprazole labeling of the gastric H^+,K^+-adenosinetriphosphatase. Biochemistry 1993;32(9):2345–55.
[8] Besancon M, Simon A, Sachs G, Shin JM. Sites of reaction of the gastric H,K-ATPase with extracytoplasmic thiol reagents. J Biol Chem 1997;272(36):22438–46.
[9] Toyoshima C, Nomura H, Sugita Y. Crystal structures of Ca^{2+}-ATPase in various physiological states. Ann NY Acad Sci 2003;986:1–8.
[10] Munson K, Vagin O, Sachs G, Karlish S. Molecular modeling of SCH28080 binding to the gastric H,K-ATPase and Mg-ATP interactions with SERCA-and Na,K-ATPases. Ann NY Acad Sci 2003; 986:106–10.
[11] Lambrecht N, Munson K, Vagin O, Sachs G. Comparison of covalent with reversible inhibitor binding sites of the gastric H,K-ATPase by site-directed mutagenesis. J Biol Chem 2000;275(6):4041–8.
[12] Sachs G, Shin JM, Briving C, Wallmark B, Hersey S. The pharmacology of the gastric acid pump: the H^+,K^+-ATPase. Annu Rev Pharmacol Toxicol 1995;35:277–305.
[13] Gedda K, Scott D, Besancon M, Lorentzon P, Sachs G. Turnover of the gastric H^+,K^+-adenosine triphosphatase alpha subunit and its effect on inhibition of rat gastric acid secretion. Gastroenterology 1995;109(4): 1134–41.
[14] Wallmark B, Larsson H, Humble L. The relationship between gastric acid secretion and gastric H^+,K^+-ATPase activity. J Biol Chem 1985;260(25):13681–4.
[15] Im WB, Blakeman DP, Davis JP. Irreversible inactivation of rat gastric (H^+-K^+)-ATPase in vivo by omeprazole. Biochem Biophys Res Commun 1985;126(1):78–82.
[16] Abelo A, Holstein B, Eriksson UG, Gabrielsson J, Karlsson MO. Gastric acid secretion in the dog: a mechanism-based pharmacodynamic model for histamine stimulation and irreversible inhibition by omeprazole. J Pharmacokinet Pharmacodyn 2002;29(4):365–82.
[17] Katashima M, Yamamoto K, Tokuma Y, Hata T, Sawada Y, Iga T. Comparative pharmacokinetic/pharmacodynamic analysis of proton pump inhibitors omeprazole, lansoprazole and pantoprazole, in humans. Eur J Drug Metab Pharmacokinet 1998;23(1):19–26.
[18] Dammann HG, Burkhardt F, Wolf N. The effects of oral rabeprazole on endocrine and gastric secretory function in healthy volunteers. Aliment Pharmacol Ther 1999;13(9):1195–203.
[19] Hung CR. Importance of histamine, glutathione and oxyradicals in modulating gastric haemorrhagic ulcer in septic rats. Clin Exp Pharmacol Physiol 2000;27(4):306–12.
[20] Im WB, Blakeman DP, Sachs G. Reversal of antisecretory activity of omeprazole by sulfhydryl compounds in isolated rabbit gastric glands. Biochim Biophys Acta 1985;845(1):54–9.
[21] Shin JM, Sachs G. Restoration of acid secretion following treatment with proton pump inhibitors. Gastroenterology 2002;123(5):1588–97.
[22] Ferron GM, McKeand W, Mayer PR. Pharmacodynamic modeling of pantoprazole's irreversible effect on gastric acid secretion in humans and rats. J Clin Pharmacol 2001;41(2):149–56.
[23] Katashima M, Yamamoto K, Sugiura M, Sawada Y, Iga T. Comparative pharmacokinetic/pharmacodynamic study of proton pump inhibitors, omeprazole and lansoprazole in rats. Drug Metab Dispos 1995; 23(7):718–23.
[24] Lindberg P, Keeling D, Fryklund J, Andersson T, Lundborg P, Carlsson E. Esomeprazole-enhanced bio-availability, specificity for the proton pump and inhibition of acid secretion. Aliment Pharmacol Ther 2003;17(4):481–8.
[25] Kromer W, Horbach S, Luhmann R. Relative efficacies of gastric proton pump inhibitors: their clinical and pharmacological basis. Pharmacology 1999;59(2):57–77.
[26] Andersson T, Rohss K, Bredberg E, Hassan-Alin M. Pharmacokinetics and pharmacodynamics of esomeprazole, the S-isomer of omeprazole. Aliment Pharmacol Ther 2001;15(10):1563–9.
[27] Hall K, Perez G, Anderson D, Gutierrez C, Munson K, Hersey SJ, et al. Location of the carbohydrates present in the H,K-ATPase vesicles isolated from hog gastric mucosa. Biochemistry 1990;29(3):701–6.
[28] Larsson H, Carlsson E, Junggren U, Olbe L, Sjostrand SE, Skanberg I, et al. Inhibition of gastric acid secretion by omeprazole in the dog and rat. Gastroenterology 1983;85(4):900–7.
[29] Wallmark B, Sachs G, Mardh S, Fellenius E. Inhibition of gastric ($H^+ + K^+$)-ATPase by the substituted benzimidazole, picoprazole. Biochim Biophys Acta 1983;728(1):31–8.
[30] Scott DR, Besancon M, Sachs G, Helander H. Effects of antisecretory agents on parietal cell structure and H/K- ATPase levels in rabbit gastric mucosa in vivo. Dig Dis Sci 1994;39(10):2118–26.
[31] Smolka A, Alverson L, Fritz R, Swiger K, Swiger R. Gastric H,K-ATPase topography: amino acids 888–907 are cytoplasmic. Biochem Biophys Res Commun 1991;180(3):1356–64.
[32] Fryklund J, Gedda K, Wallmark B. Specific labelling of gastric H^+, K^+-ATPase by omeprazole. Biochem Pharmacol 1988;37(13):2543–9.
[33] Keeling DJ, Fallowfield C, Underwood AH. The specificity of omeprazole as an ($H^+ + K^+$)-ATPase inhibitor depends upon the means of its activation. Biochem Pharmacol 1987;36(3):339–44.
[34] Kromer W, Postius S, Riedel R, Simon WA, Hanauer G, Brand U, et al. BY 1023/SK&F 96022 INN pantoprazole, a novel gastric proton pump inhibitor, potently inhibits acid secretion but lacks relevant cytochrome P450 interactions. J Pharmacol Exp Ther 1990;254(1): 129–35.
[35] Im WB, Sih JC, Blakeman DP, McGrath JP. Omeprazole, a specific inhibitor of gastric (H^+-K^+)-ATPase, is a H^+-activated oxidizing agent of sulfhydryl groups. J Biol Chem 1985;260(8):4591–7.
[36] Fujisaki H, Shibata H, Oketani K, Murakami M, Fujimoto M, Wakabayashi T, et al. Inhibitions of acid secretion by E3810 and omeprazole, and their reversal by glutathione. Biochem Pharmacol 1991; 42(2):321–8.
[37] Nagaya H, Satoh H, Maki Y. Possible mechanism for the inhibition of acid formation by the proton pump inhibitor AG-1749 in isolated canine parietal cells. J Pharmacol Exp Ther 1990;252(3): 1289–95.
[38] Sturm E, Kruger U, Senn-Bilfinger J, Figala V, Klemm K, Kohl B, et al. (H^+-K^+)-ATPase Inhibiting 2-[(2-pyridylmethyl)sulfinyl]benzimidazoles, 1. Their reaction with thols under acidic conditions. Disulfide containing 2-pyridiniobenzimidazolides as mimics for the inhibited enzyme. J Organic Chem 1987;52:4573–81.

[39] Brandstorm A, Lindberg P, Bergman N-A, Tekenbergs-Hjelte L, Ohlson K. Chemical Reactions of omeprazole and omeprazole analogues IV: reaction of compounds of the omeprazole system with 2-mercaptoethanol. Acta Chemica Scandinavica 1989;43:577–86.

[40] Brandstrom A, Lindberg P, Bergman N-A, Alminger T, Ankner K, Junggren U, et al. Chemical reactions of omeprazole and omeprazole analogues I: a survey of the chemical transformations of omeprazole and its analogues. Acta Chemica Scandinavica 1989;43:536–48.

[41] Toyoshima C, Nakasako M, Nomura H, Ogawa H. Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution. Nature 2000;405(6787):647–55.

[42] Toyoshima C, Nomura H. Structural changes in the calcium pump accompanying the dissociation of calcium. Nature 2002;418(6898): 605–11.

[43] Vagin O, Denevich S, Munson K, Sachs G. SCH28080, a K^+-competitive inhibitor of the gastric H,K-ATPase, binds near the M5-6 luminal loop, preventing K^+ access to the ion binding domain. Biochemistry 2002;41(42):12755–62.

[44] Wallmark B, Briving C, Fryklund J, Munson K, Jackson R, Mendlein J, et al. Inhibition of gastric H^+,K^+-ATPase and acid secretion by SCH 28080, a substituted pyridyl(1,2a)imidazole. J Biol Chem 1987; 262(5):2077–84.

[45] Larsson H, Mattson H, Sundell G, Carlsson E. Animal pharmacodynamics of omeprazole. A survey of its pharmacological properties in vivo. Scand J Gastroenterol Suppl 1985;108:23–35.

[46] Wallmark B. Omeprazole: mode of action and effect on acid secretion in animals. Methods Find Exp Clin Pharmacol 1989;11(Suppl 1): 101–6.

[47] Vagin O, Munson K, Denevich S, Sachs G. Inhibition kinetics of the gastric H,K-ATPase by K-competitive inhibitor SCH28080 as a tool for investigating the luminal ion pathway. Ann NY Acad Sci 2003; 986:111–5.