Sites of Reaction of the Gastric H,K-ATPase with Extracytoplasmic Thiol Reagents*

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The vesicular gastric H,K-ATPase catalyzes an electroneutral H for K exchange allowing acidification of the intravesicular space. There is a total of 28 cysteines present in the α subunit of the gastric H,K-ATPase, of which 10 are found in the predicted transmembrane segments and their connecting loop, and 9 are present in which 10 are found in the predicted transmembrane present in the troncatalytic H for K exchange allowing acidification of the extracytoplasmic domain. Of the 10 cysteines present in the membrane and extracytoplasmic domain, only three are exposed sufficiently to allow reactivity with these cationic thiol reagents. The binding to cysteine 813 defines the location of the extracytoplasmic loop between TM5 and TM6 and places the carboxylic acids 820 and 824 conserved between the gastric H,K- and the Na,K-ATPases in TM6, consistent with their assumed role in cation binding.

The gastric H,K-ATPase is a member of the P type ATPase family. Transport of H$_3$O$^+$ outward in exchange for K$^+$ transport inward is coupled to a cycle of phosphorylation and dephosphorylation. In conjunction with parallel K$^+$ and Cl$^-$ conductances, this ATPase is responsible for the elaboration of HCl into the secretory canaliculus of the parietal cell or into isolated purified gastric vesicles, the enclosed space reaching a pH of about 1 (1).

A number of chemical reagents have been useful in analyzing several aspects of structure function in this P type ATPase and the Na,K-ATPase (1, 2). For example, DCCD$^+$ is a hydrophobic reagent that reacts with a carboxyl group in the membrane domain of either pump in a K protectable manner (3, 4) and thereby interfering not only with ATPase activity but also with Rb occlusion. Fluorescein isothiocyanate reacts with a cytoplasmic lysine in these pumps, providing a fluorescent marker (5, 6) for Na- and K-induced conformations. Thiol reagents have been used to advantage in the Na,K-ATPase to define reactive cysteines in the membrane or extracytoplasmic domain (7, 40). Sided reagents are relatively rare. Ouabain, partially K-competitive inhibitor of the Na,K-ATPase, binds to the extracytoplasmic surface of the Na,K-ATPase. Since this ligand is non-covalent, mutagenesis has been used to establish that it binds or interacts with the first, second, fifth, and sixth transmembrane domains (8, 9).

The essential contribution of the H,K-ATPase to acid secretion by the stomach has resulted in the synthesis of compounds that inhibit this enzyme selectively (10). One class consists of substituted 2-(2-pyridylmethylsulfanyl)-1H-benzimidazoles. These compounds are protonatable weak bases of pKa about 4.0

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§ The abbreviations used are: DCCD, dicyclohexylcarbodiimide; PVDF, polyvinylidene difluoride; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; NEM, N-ethylmaleimide; omeprazole, 5-methoxy-2-[(4-methoxy-3,5-dimethyl-2-pyridyl)methylsulfanyl]-1H-benzimidazole; lansoprazole, 2-[(4-trifluoromethoxy)-3-methyl-2-pyridyl]methylsulfanyl]-1H-benzimidazole; pantoprazole, 5-difluoromethoxy-2-[3,4-methoxy-2-pyridyl]methylsulfanyl]-1H-benzimidazole (rabeprazole), under acid transporting conditions. All of these compounds are weak bases that accumulate in the acidic space generated by the pump and undergo an acid catalyzed rearrangement to a cationic sulfenamide, which forms disulfides with accessible cysteines. The relative rates of acid activation of these compounds corresponded to the relative rates of inhibition of ATPase activity and acid transport. Fragmentation of the enzyme by trypsin followed by SDS-polyacrylamide gel electrophoresis showed that omeprazole bound covalently to one of the two cysteines in the domains containing the fifth and sixth transmembrane segments and their extracytoplasmic loop and to cysteine 892 in the loop between the seventh and eighth transmembrane segments, but inhibition correlated with the reaction with cysteines in the fifth and sixth domain. Lansoprazole bound to the cysteines in these two domains as well as to cysteine 321 toward the extracytoplasmic end of the third transmembrane segments. Pantoprazole bound only to either cysteine 813 or 822 in the fifth and sixth transmembrane region. The inhibition of Rabeprazole correlated also with its binding to this part of the protein, but this compound continued to bind after full inhibition, eventually binding also to cysteines 321 and 892. No binding was found to any of the cysteines in the seventh to tenth transmembrane segments. Thermolysin digestion of the isolated omeprazole-labeled fifth and sixth transmembrane pair showed that cysteine 813 was the site of labeling. It is concluded that binding of these sided reagents to cysteine 813 in the loop between transmembrane (TM5 and TM6 is sufficient for inhibition of ATPase activity and acid transport by the gastric acid pump. Of the 10 cysteines present in the membrane and extracytoplasmic domain, only three are exposed sufficiently to allow reactivity with these cationic thiol reagents. The binding to cysteine 813 defines the location of the extracytoplasmic loop between TM5 and TM6 and places the carboxylic acids 820 and 824 conserved between the gastric H,K- and the Na,K-ATPases in TM6, consistent with their assumed role in cation binding.
or 5.0. This pH and the membrane permeability of the unprotonated form result in their selective accumulation in the acidic space of the secretory canaliculus of the active parietal cell or in the acid transporting gastric-derived vesicles studied here. Following accumulation, the compounds undergo an acid-catalyzed rearrangement to a cationic sulfenamide in the extracytoplasmic space (11, 12). This cationic sulfenamide is relatively membrane impermeant and is able to react with thiol groups within the catalytic subunit of the H,K-ATPase to form relatively stable disulfides (13, 24, 30). Since the thiophilic cation is relatively stable in acidic solution, the compounds protonate and accumulate in an acid space and undergo an acid-catalyzed conversion to a tetracyclic sulfenamide, which then reacts with cysteines of the α subunit of the H,K-ATPase that are accessible from the extracytoplasmic surface.

The membrane domain of this ion pump, as for the sarco/ eradicate reticulum Ca$^{2+}$-ATPase, the Na$^{+}$,K-ATPase, and the Mg$^{2+}$-ATPase of *Salmonella typhimurium* as well as the H$^{+}$-ATPase of Neurospora or Saccharomyces, is thought to consist of ten transmembrane segments connected by five extracytoplasmic loops (15–19). Mutagenesis studies carried out largely on the sarco/ eradicate reticular Ca$^{2+}$-ATPase and the Na$^{+}$,K-ATPase place ion binding sites within this membrane domain, in particular in the fourth, fifth, sixth, and eighth transmembrane segments (9, 20, 21). The conserved carboxylic acids in the putative fifth and sixth transmembrane segments are placed within these segments because mutagenesis affects ion transport. Proof that they are in the membrane segments rather than in the extracytoplasmic loop has not been provided.

Four substituted 2-(2-pyridylmethylsulfinyl)-1H-benzimidazoles were used in this study, namely omeprazole, lansoprazole, pantoprazole, and rabeprazole, to identify the luminal accessible cysteines and those relevant to inhibition of the H,K-ATPase by these compounds. Their structures are shown in Fig. 1, as is their probable chemical mechanism of activation. They are shown as accumulating in acid due to protonation. This is followed by acid activation to the sulfenamide, which in turn results in binding to cysteines of the gastric H,K-ATPase. The sulfenamide is a permanent cation, restricting its membrane permeability and is, therefore, an extracytoplasmic thiol reagent.

The compounds differ in their acid stability. Pantoprazole is the most stable at neutral pH, omeprazole and lansoprazole have about equal stability, and rabeprazole is the least stable at neutral pH. All four compounds convert to the sulfenamide more rapidly at pH 3.0 and below but retain their relative rates of conversion. The $t_{1/2}$ of conversion of omeprazole or lansoprazole at pH 5.0 is about 0.3 h, whereas that of pantoprazole is about 1.2 h and rabeprazole is about 0.01 h (35). After acid activation, the compounds are unstable at neutral pH, converting to the inactive sulfide and other substances (22), but are relatively stable in acidic solution.

The effect of these four compounds was studied on vesicular preparations of the hog gastric H,K-ATPase under acid transporting conditions, in terms of the rate and level of inhibition of enzyme activity, acid transport, and the sites of labeling of the enzyme. The rate of inhibition of ATPase activity and of proton transport correlated with the degree of acid stability, rabeprazole being faster than omeprazole, which was equal to lansoprazole being slower. Labeling of different cysteines was found. In general, the first cysteines labeled were those contained within the fifth and sixth transmembrane domains and the connecting extracytoplasmic loop, and this labeling corresponded to the onset of inhibition. Pantoprazole labeled only these cysteines, omeprazole labeled mainly the fifth and sixth transmembrane segments, and lansoprazole labeled, in addition to the cysteines labeled by omeprazole, the cysteine present in the domain contained within the third and fourth transmembrane segments and the connecting loop. Rabeprazole labeled the fifth and sixth transmembrane segment, re-

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2 J. Senn-Billfinger, personal communication.
sulting in full inhibition of ATPase activity and acid transport but continued to label the enzyme after full inhibition with a pattern of labeling eventually similar to that of lansoprazole. Thermolysin digestion of the isolated omeprazole-labeled tryptic fragment followed by separation and sequencing of the peptide labeled with omeprazole showed that cysteine 813 was the cysteine reacting in the TM5/TM6 domain.

**EXPERIMENTAL PROCEDURES**

**ATPase Preparation**—Hog gastric vesicles were prepared as described previously (23). The ion tight fraction laying on top of a 7.5% Percoll solution in 250 mM sucrose on the density gradient was used in all the experiments.

**ATPase Activity**—The average basal activity in the experiments described here was 5 μmol of ATP hydrolyzed/mg of protein/h. 10 μmol of ATP/mg was hydrolyzed in the presence of 20 mM KCl and 120 μmol/mg/m in the presence of KCl and nigericin or in the presence of 100 mM NH4Cl. Accordingly, 90% or more of the vesicles were ion tight. K+-stimulated activity with 20 mM KCl in the absence of ionophores is due to leaky vesicles. The ATPase activity in the presence of K+ plus nigericin or of 100 mM NH4Cl was considered to reflect the total ATPase activity (24), whereas the difference between the latter measurements and the K+-stimulated ATPase in the absence of ionophore reflects the contribution of ion tight vesicles. NH4+ activates the ATPase by permeating as NH3 and then forming NH4+. This is then transported by the H,K-ATPase out of the vesicles as a K+ surroage resulting in cycling of the enzyme without the formation of a proton gradient (25). P, released was measured by the method of Yoda and Hokin (26) and protein by the Lowry method (27).

**Acid Transport**—Acidification of the gastric vesicles was measured by the quenching of acridine orange as described previously (28). Briefly, the vesicles at 10 μg/ml were suspended in a medium containing 250 mM sucrose, 150 mM KCl, 1 μM acridine orange, 5 mM Tris/HC1 buffer, pH 6.8, and 1 μg/ml valinomycin. The inhibitors, when present, were added at 5 μM final concentration. Transport was initiated by the addition of 2 mM MgATP, pH 6.8, and the fluorescence of acridine orange was measured as a function of time in a Spex fluorimeter with excitation at 480 nm and emission at 530 nm.

**Benzimidazole Inhibition and Labeling of the ATPase**—Inhibition of the ATPase by the compounds was evaluated during the labeling reaction and also in the absence of radioactive material but under otherwise identical conditions. In summary, the enzyme under acid transporting conditions was incubated with the drug, and samples were taken at time zero and various other time points for measurement of labeling and of enzyme activity. Inhibition of enzyme activity was obtained by comparing the phosphate present in the samples at any given time point with the phosphate released following the addition of ATP and NH4Cl to the samples as described previously (13, 24).

**The PVDF membrane was sprayed with ENHANCE (DuPont) and exposed to x-ray film in the cold for 8–14 days.** The film was developed, and the bands corresponding to the radioactive activity on the autoradiogram were cut out, a portion was taken for counting, and the rest were subjected to microsequencing in a microsequencing facility.

**Isolation of [3H]Omeprazole-labeled TM5/TM6 Segment and Thermolysin Digestion**—[3H]Omeprazole-labeled vesicles were extensively digested with 0.2 mg of trypsin in a buffer (1 ml) composed of 0.25 M sucrose, 50 mM Tris/HC1, pH 8.2, for 30 min. The reaction was stopped by adding 2 μg 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 dissolved in a buffer composed of 30 mM Tris/HC1, pH 8.0, 0.05% SDS at 120 K constant voltage for 3 h. It was necessary to reduce the concentration of SDS and salt of the eluate for further digestion. The electroelution buffer was carefully removed and replaced with a new buffer composed of 10 mM Tris/HC1, pH 8.0, 0.01% SDS. Electroelution was carried out in a buffer composed of 30 mM Tris/HC1, pH 8.0, 0.03% SDS at 120 K constant voltage for 3 h. After digestion, samples were concentrated up to 90 μl by Speed-Vac and combined with 10 μl of 2 M sucrose and 0.25% bromophenol blue. Samples were electrophoresed using a 16.5% (17:1 acrylamide/methylene bisacylamide) 1.5-mm gradient slab Tricine gel (29). Each lane had 50 μl of samples. The gel was run in the cold room (4 °C) for 18 h at 90 K constant voltage, along with a lane for prestained molecular mass (Bio-Rad, 106–18 kDa) standards and CNBr fragments of horse myoglobin (Sigma, 17–25 kDa). 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.

**SDS-Gel Separation**—The membrane digests dissolved in 25% volume of sample buffer were placed on top of a 10% (34:1 acrylamide/methylene bisacylamide) to 21% (17:1 acrylamide/methylene bisacylamide) 1.5-mm gradient slab gel using the Tricine buffer method (29). The gel was run in the cold room (4 °C) for 18 h at 90 K constant voltage along with a lane for prestained molecular mass (Bio-Rad, 106–18 kDa) standards and CNBr fragments of horse myoglobin (Sigma, 17–25 kDa). 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)) as a function of relative mobility were used to estimate the M, of the peptide products of digestion. The accuracy of the M, weight determination appeared to be within 10% based on predicted tryptic cleavage sites within the primary sequence of catalytic subunit of the enzyme. 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, 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. The PVDF membrane was sprayed with EN HANCE (DuPont) and exposed to x-ray film in the cold for 8–14 days. The film was developed, and the bands corresponding to the radioactive activity on the autoradiogram were cut out, a portion was taken for counting, and the rest were subjected to microsequencing in a microsequencing facility.

**Covalent Inhibition of the Gastric H,K-ATPase**
Covalent Inhibition of the Gastric H,K-ATPase

Fig. 2. The rate of inhibition of the gastric H,K-ATPase under acid-transporting conditions by rabeprazole, omeprazole, lansoprazole, and pantoprazole. The vesicles were incubated in 150 mM KCl, with 1 μg/ml valinomycin in buffer at pH 6.8 in the presence of 10 μM compound and the reaction started by the addition of MgATP as detailed in text. Aliquots were withdrawn at different times, and remaining ATPase activity was measured as above (see “Experimental Procedures”). Rabeprazole inhibited faster than omeprazole and lansoprazole, and pantoprazole, which in turn were faster than omeprazole.

Fig. 3. A, the rate of inhibition of acid transport by the H,K-ATPase by the four compounds as measured by the quenching of acridine orange fluorescence. These compounds (20 μM) were added at 0 time, and ATP was added 1 min later. Incubation conditions are detailed in the text. For omeprazole, lansoprazole, and pantoprazole, the initial rate of acid transport was the same as in the absence of compounds, whereas with rabeprazole, there was an immediate reduction in the rate of acidification. After a lag phase, all compounds were able to inhibit acidification of the vesicle interior. B, the 1/2 of inhibition of acid transport in three separate experiments carried out on different vesicle preparations by the four compounds as measured by the restoration of acridine orange fluorescence following the addition of ATP (n = 3 ± S.E.).

with a lane for CNBr fragments of horse myoglobin ( Sigma, 17–2.5 kDa). The peptides were transferred electrophoretically to PVDF membranes (Bio-Rad, 0.2 μm pore size) for 6 h in the cold room (4 °C) in a tank transfer apparatus (Idea Scientific Company) at 12 V constant voltage, in a transfer buffer of 15 mM CHAPS/NaOH, pH 11.0, and 10% ethanol. The PVDF membrane was rinsed with distilled water. One lane was sliced for radioactivity counting and the other lane was for microsequencing.

Stability of Labeling—With all the compounds tested, 10 mM dithiothreitol removed all counts found in the SDS-solubilized membranes showing that the stable labeling observed was due to the presence of a disulfide linkage between the compound and the enzyme.

Materials—Omeprazole and radioactive omeprazole were a gift of Astra Hassle, Sweden; lansoprazole and radioactive lansoprazole were a gift from Takeda, Japan; pantoprazole and labeled pantoprazole were a gift of Byk Gulden, Germany; and rabeprazole and labeled rabeprazole (E3810) were a gift of Eisai Ltd., Tokyo, Japan. All other chemicals were analytical grade or better.

RESULTS

Inhibition of Enzyme Activity

In the absence of ATP, there was some inhibition of enzyme activity as expected from the pH-dependent activation kinetics of the various compounds, in particular in the presence of rabeprazole (data not shown). Following ATP addition, the data on the time course of inhibition allowed a comparison of the rate of inhibition of the ATPase of the different compounds under acid transporting conditions. In the presence of nigericin, where no acid gradient is formed, the addition of MgATP did not produce additional inhibition, showing that acidification is necessary for ATP-induced inhibition of enzyme activity by these compounds (13, 24, 30).

Fig. 2 shows that the rate of inhibition of the ATPase by rabeprazole was faster than with the other drugs and that shown by pantoprazole was slower than that seen with the other compounds. In fact, rabeprazole inhibited the enzyme fully at 5 min. Pantoprazole showed only slight inhibition at 5 min, and inhibition had only reached 50% at the end of the experiment. Omeprazole and lansoprazole provided the same degree of inhibition as rabeprazole but only after 30 min of incubation, and these compounds showed essentially equal rates of inhibition. The experiments illustrated were carried out on the same population of vesicles.

Inhibition of Acid Transport

When ATP is added to gastric vesicles in the presence of KCl and valinomycin, there is rapid transport of acid into the vesicle interior, with consequent accumulation and quenching of the fluorescent weak base, acridine orange (28). Fig. 3A shows a set of typical results when ATP is added in the presence of the drugs. In the presence of omeprazole, lansoprazole, and pantoprazole, there is almost the same initial rate and amount of quenching of acridine orange as in the absence of the drugs, but then, in contrast to the control, there is restoration of fluorescence beginning at about 200 s for omeprazole and lansoprazole. This is due to the acid activation of the compounds triggering inhibition of ATPase activity with then elevation of the intravesicular pH due to leak of HCl from the vesicle interior. Inhibition by rabeprazole is faster than with the other compounds so that the maximal quench reached is less than the control. With pantoprazole, it takes about 1000 s for the reversal of the pH gradient to become evident. Fig. 3B shows the t1/2 for inhibition of 50% of the acid gradient obtained in three independent experiments for the four compounds. The rate of inhibition of acid transport is therefore fastest with rabeprazole, equal with omeprazole and lansoprazole, and slowest with
pantoprazole. This correlates with the relative rates of inhibition of enzyme activity by the four drugs.

**Analysis of Membrane Peptide Pairs**

In this and in previous work, sequencing from the PVDF membrane has shown that tryptic cleavage of the enzyme results in the production of detectable quantities of four transmembrane segment pairs. Numbering of the amino acids in the sequence of the α subunit omits the initial methionine since the natural sequence begins with glycine. The terminal amino acid is defined by the presence of a Lys or Arg residue in the cDNA-derived sequence calculated to give the appropriate molecular weight of the fragment.

Sequencing of the fragments detected in this work gave results similar to those reported previously (13, 31). Tryptic digestion in the presence of ATP and SCH 28080 provided an N-terminal fragment of 68 kDa and a C-terminal fragment of 33 kDa. Major labeling was found in the smaller C-terminal fragment for all the compounds, with lesser labeling in the N-terminal fragment in the case of lansoprazole and rabeprazole (data not shown), indicating that the reaction important for inhibition was likely to be with cysteines in the C-terminal one-third of the enzyme (13).

At about 11.2 kDa, the sequence detected corresponds to the seventh and eighth transmembrane segments with their connecting loop, beginning at Leu-Val-Asn at position 853 and continuing, based on relative molecular mass, to presumably Arg at position 946 or Lys at position 947. At about 7.5 kDa, the sequence begins Thr-Pro-Ile, at position 291, ending probably at Lys at position 358. This reflects the sequence of the third and fourth transmembrane segments and their connecting extracellular loop. At about 6.5 kDa, the peptide band contains two sequences corresponding to two pairs of transmembrane segments. The first sequence begins with Gln-Leu-Ala at position 104 and probably ends with Lys at position 162, and the second sequence begins with Leu-Ile-Phe at position 776 and probably ends at Lys in position 835, reflecting the sequence of the M3 and M4 pair and the M5 and M6 pair, respectively. The lowest relative molecular mass fragment, of about 5.9 kDa, begins with the sequence, Asn-Ile-Pro, that is at position 792–794 and also presumably ends at position 855, which is a shorter version of the M5 and M6 pair found at 6.5 kDa. In the region of the fifth and sixth transmembrane segments, sequencing yielded between 5 and 8 pmol of peptide, allowing an approximation of the stoichiometry of binding of the various compounds in this region.

**Labeling of the Enzyme**

The autoradiograms and count profiles shown are typical of at least three independent experiments. See “Experimental Procedures” for the specific activity of each of the compounds.

**Omeprazole**—Omeprazole labeling at 10 and 45 min is shown in Fig. 4A. The autoradiogram and counts are displayed as well as the percentage inhibition of ATPase activity found in the experiment illustrated. At 10 min, with 70% inhibition, the autoradiogram and counts showed an intense band corresponding to the fifth and sixth transmembrane segments. The band of radioactivity had a relative molecular mass of 6.2 kDa. As inhibition increased to 73%, the amount of label and intensity of the autoradiogram increased with about a 2-fold increase in cpm at the major peak. Sequencing of this peak showed that the N-terminal sequence contained Leu-Ile-Phe at a yield of about 8 pmol. An additional peak of counts was observed at 11.2 kDa, corresponding to the region contained within the seventh and eighth transmembrane segments and their connecting loop, as well as a small additional peak also found at about 7.5 kDa, corresponding to the sequence containing the third and fourth transmembrane segments and their connecting extracellular loop. Minor labeling of this region has been described before (13). Inhibition by omeprazole appears to coincide with the labeling of the region containing the fifth and sixth transmembrane domain and occurs prior to major labeling of the higher M₃ peak. There was some variation noted in the relative degree of labeling of the 11.2- and 6.5-kDa peptides using different batches of the [³H]omeprazole, perhaps due to radiation damage to the molecule. In every instance, however, the 6.5- and 11.2-kDa peptides were the major bands labeled. In the fifth and sixth transmembrane segments, as inhibition approached 100%, the stoichiometry of the reaction was about 1 mol compound bound/mol peptide.

**Lansoprazole**—The binding of lansoprazole is shown in Fig. 4B. At 10 min, radioactivity was seen in the region of the fifth and sixth transmembrane segments with 66% inhibition already being evident. At 45 min, this inhibition had increased to 100%, with the major band of labeling now being found in the region of the seventh and eighth transmembrane segments and additional minor labeling in the region of the third and fourth transmembrane segments, a labeling pattern generally similar to data observed earlier (32). The initial labeling in the fifth and sixth transmembrane region increased as inhibition progressed to 100%, the counts increasing about 2.5-fold. The kinetics of labeling of the domain containing the seventh and eighth transmembrane segments did not correspond to the inhibition kinetics since most of the inhibition was already evident prior to significant labeling of this region. The stoichiometry of labeling at the fifth and sixth transmembrane region was slightly greater than 1 mol of compound/mol of peptide.

**Pantoprazole**—The reaction of pantoprazole with the acid transporting vesicles is shown in Fig. 4C. At 10 min, with only 20% inhibition evident, labeling is seen essentially only in the region of the fifth and sixth transmembrane segments. After 45 min of incubation, where 49% inhibition was found, two regions of labeling were found, one corresponding also to the major peak seen with omeprazole, with the sequence Leu-Ile-Phe at 6.5 kDa. A second band is also seen, at a relative molecular mass of 5.9 kDa, beginning with the sequence Asn-Ile-Pro also contained within the fifth and sixth transmembrane segments. There was approximately a 5-fold increase in the measured counts. The relatively slow labeling of the enzyme is consistent with the slower inhibition found by this compound. Further, there is no labeling evident for any other region of the protein other than the domain involving the fifth and sixth transmembrane segments. Hence, inhibition by this particular substituted pyridyl methylsulfinyl benzimidazole is due to binding only in this region of the catalytic subunit of the H,K-ATPase. Similar conclusions have been published previously (33). The stoichiometry of labeling in the fifth and sixth transmembrane region, at 49% inhibition, was close to 0.5 mol compound/mol peptide.

**Rabeprazole**—Fig. 4D displays the results obtained with rabeprazole. This compound inhibited the enzyme fastest of the four compounds tested, as discussed above, with inhibition reaching close to 100% by 5 min. This also corresponded to inhibition of acid transport. As inhibition reached maximum at 5 min, mostly the domain containing the fifth and sixth transmembrane segments showed evidence of labeling, with only minor labeling of other membrane peptide domains. Sequencing of the radioactive peaks showed two sequences, starting at either Leu-Ile-Phe (6.5 kDa) or Asn-Ile-Phe (5.9 kDa). As incubation was continued, however, labeling increased 2-fold in these regions. The higher molecular mass peak was labeled more, but now significant labeling was also found in regions...
corresponding to the domains containing the third and fourth and the seventh and eighth transmembrane segments with an additional peak at about 8 kDa. For this compound, as for pantoprazole, there is a clear correlation of inhibition and labeling in the domain including the fifth and sixth transmembrane segments. The initial stoichiometry of labeling of these domains was about 1 mol of compound/mol of peptide, corresponding to 100% inhibition. With continuing incubation, the doubling of labeling in this region could be because of reaction with both of the cysteines (813 and 822) in the TM5/TM6 region.

**Effect of Omeprazole Sulfide—** The sulfide of omeprazole is unable to inhibit the enzyme in vitro and does not affect acidification of the vesicle interior at the concentrations used in these experiments since this compound cannot form the sulfonamide. However, the presence of 10 μM omeprazole sulfide significantly reduced the inhibition by omeprazole from 80 to 40% at 45 min. The autoradiogram, as shown in Fig. 5 shows that there was a large reduction in labeling of the transmembrane segments and their connecting loop, and this reduction in labeling reflected the loss of inhibition by omeprazole.

**Identification of the Cysteine Labeled by Omeprazole in the**

**FIG. 5.** The inhibition and labeling of the ATPase by omeprazole in the presence of omeprazole sulfide. The enzyme was incubated under acid transporting conditions with omeprazole alone or in the simultaneous presence of omeprazole and its sulfide, and then the procedure was the same as for the other labeling and inhibition studies.
TM5/TM6 Domain—[3H]Omeprazole-labeled vesicles were extensively digested for obtaining as short a TM5/TM6 segment as possible to avoid contamination from the TM1 and TM2 domain. From the extensive 30-min digestion at a 1:4 ratio of trypsin per protein, TM5/TM6 domain was split into two fragments at 6.5 and 5.9 kDa. As above, the 5.9-kDa band has only the TM5/TM6 segment with N-terminal sequence Asn-Ile-Pro, while the 6.5-kDa band has TM1/TM2 and TM5/TM6 segments as described above. Gel pieces in the molecular mass range from 5.5 to 6.2 kDa were electrophoresed. The sample was divided, and one-half digested with thermolysin and run side by side on SDS-PAGE as detailed under “Experimental Procedures.” The lanes containing the undigested and thermolysindigested material are shown in Fig. 6 along with distribution of [3H]omeprazole labeling. The undigested fragment lane (control lane) showed counts peaking at 5.9 kDa, and the digested fragment lane shows peaks at 2.5 kDa without any fluorescence. There are two cysteines in the TM5/TM6 domain, and the FMI labeling in the undigested fragment shows that there are residual –SH groups present after the reaction with omeprazole, presumably the cysteine that did not react with omeprazole. After thermolysin digestion, the total recovery of counts at 2.5 kDa and the absence of fluorescence shows that good separation was achieved between the cysteine that had reacted with omeprazole and the unreacted cysteine that had been subsequently labeled with FMI. The band at 2.5 kDa has the same N-terminal sequence, NIPETLTPY, . . . , as the sequence of the 5.9-kDa band. Based on this N-terminal sequence and the relative molecular mass, the 2.5-kDa peptide should extend to no further than Ile-814 or Ile-816. Therefore, Cys-813 is the covalent omeprazole binding site and Cys-822 does not react with omeprazole as a function of inhibition of either ATPase activity or proton transport. On Fig. 6, it can be seen that there is no fluorescence of the radioactive band after digestion with thermolysin. Hence, Cys-813 has fully reacted with omeprazole.

DISCUSSION

The compounds used in this study react with the gastric H,K-ATPase as extracytoplasmic thiol reagents. These reagents, therefore, give information on the cysteines accessible from the outside of the enzyme, provide data on membrane topology, and bind to regions of the extracytoplasmic domain involved in maintaining catalytic function.

The lag time for inhibition of ATPase activity and acid transport is probably due to the requirement for acid-catalyzed conversion of these compounds to the active sulfenamide. The initial rate of vesicle acidification is the same as the control in the presence of omeprazole, lansoprazole, and pantoprazole, and inhibition of transport requires about 60 s to become evident after acidification is initiated by the addition of ATP. When a non-acid-dependent inhibitor is present, such as the imidazo-pyridine, SCH 28080, no delay of inhibition of acidification is seen upon the addition of ATP (34).

The differences in the rate of inhibition of enzyme activity and of acidification by these compounds under acid transport conditions correlates with their relative acid stability (35, 36). Their pKₐ and hydrophobicity determine the level of accumulation in the acid space of the parietal cell. The rate of formation of the tetracyclic sulfenamide determines the rate of inactivation of the enzyme. Pantoprazole, by virtue of having a difluoromethoxy substituent on the benzimidazole, has a lower pKₐ on the benzimidazole N, which probably accounts for its greater acid stability, given equal nucleophilicity of the pyridine N. The activation at close to neutral pH that occurs more with rabeprazole than the other compounds may result from the higher pKₐ and nucleophilic reactivity of the pyridine N allowing reaction with the 2C of the benzimidazole at neutral pH. Hence the different rates of reaction with the enzyme, with pantoprazole the slowest and rabeprazole the fastest, can be explained by the rate of sulfenamide formation (12, 22, 35).

Although each compound forms a similar thiophilic sulfenamide, there are distinctive labeling patterns demonstrated in the experiments described here. The four compounds have similar hydrophobicities, and this property cannot account for the selectivity found here. Nor can the rate of acid activation explain the differences between the cysteines reacting with omeprazole or lansoprazole.

The majority of the gastric vesicles will achieve an internal pH much less than 3.0, based on the degree of acridine orange quenching (28). It can, therefore, be assumed that the majority of the compound accumulated in the acid space is converted to the sulfenamide. If the reaction with available cysteines depends only on the reactivity of the tetracyclic sulfenamide, which should not vary significantly between the drugs, then the compounds should react with similar cysteines. The reaction with free –SH groups is also diffusion-limited (22). The finding that omeprazole, lansoprazole, pantoprazole, and rabeprazole first label one of the cysteines in the TM5-loop-TM6 domain, shown to be Cys-813 for omeprazole, but then that omeprazole also labels Cys-892 and lansoprazole and rabeprazole label Cys-321 in addition, suggests that there may be structural specificity in interaction of the compounds with the enzyme surface prior to covalent disulfide formation. For example, there may be binding of the protonated species to the enzyme surface prior to conversion to the sulfenamide, allowing for selective reaction with the cysteine in the TM5-loop-TM6 region before the other cysteines. To determine whether prior binding of omeprazole or its sulfenamide to the enzyme was relevant to inhibition or the labeling pattern, inhibition and labeling were carried out in the simultaneous presence of omeprazole and its inactive sulfide. Since the reaction with the sulfenic acid generated from the sulfenamide is irreversible, only if there were competition for an important binding step would the presence of the unreactive sulfide affect the rate and extent of inhibition. The sulfide delayed the inhibition by omeprazole and significantly altered labeling of the peptide segments. Hence, it seems that there is binding of protonated omeprazole or of the sulfenamide to a region of the enzyme surface prior to the formation of the disulfides and this binding is reduced by the protonated omeprazole sulfide. This prior binding may account in part for the differing reaction pattern seen with the different benzimidazoles if the protonated species are able to bind at different regions of the enzyme.

In planar bilayer experiments it has been found that omeprazole inhibits the ATPase following photolysis of caged ATP.
in the absence of $K^+$, hence in the absence of net acid transport (37). The reaction pattern also allows for the possibility that protonation of the benzimidazole might occur on the enzyme surface itself, presumably in the region of proton transport such at the TM5-loop-TM6 domain, a concept consistent with the bilayer reconstitution results.

Since the reactive species is cationic, it seems likely that the more rapid binding correlating to inhibition is to a relatively exposed cysteine on the outside surface of the enzyme. Hence binding to a cysteine in the extracytoplasmic loop between TM5 and TM6 is responsible for the inhibition by pantoprazole and rabeprazole. Binding to this cysteine also appears to be important for inhibition by lansoprazole and omeprazole since inhibition corresponded to binding to the fifth and sixth transmembrane segments rather than to Cys-892 between the seventh and eighth or Cys-321 in the third transmembrane segment. Although Cys-892 is predicted to be in the large loop between TM7 and TM8, its rate of reaction is slower than that of Cys-813, the cysteine shown to react with omeprazole in the work described above. The short TM5-loop-TM6 domain would suggest lesser accessibility of Cys-813 to the cation than that of Cys-892. Either the long loop between TM7 and TM8 is relatively buried, or the structure of the TM5-loop-TM6 domain provides greater exposure than its linear sequence would suggest. In vitro translation of this region of the H,K-ATPase has not been successful in demonstrating membrane insertion of this region of the enzyme sequence (14), perhaps substantiating an aberrant structure of this part of the enzyme sequence.

The reaction of DCCD with the Na,K- and H,K-ATPases and inhibition of activity by DCCD demonstrate a role for carboxylic acids in the membrane domain in transport by these enzymes (3, 4, 38, 39). Site-directed mutations of the sarcoplasmic reticular Ca$^{2+}$-ATPase and the Na,K-ATPase that affect ion binding place the carboxylic acids of those pumps in this TM5/TM6 region in the membrane domain (40, 41). These mutations appear to affect cation interaction with the membrane domain, suggesting a role for these carboxylic acids in cation coordination placing the TM5-loop-TM6 domain within the ion transport pathway of these enzymes.

These pumps, although they have significant amino acid sequence differences, align well in terms of hydropathy with the gastric H,K-ATPase, and the three carboxylic acids in this region are conserved between the H,K- and Na,K-ATPases and two of the three between the H,K- and the sarcoplasmic reticular Ca$^{2+}$-ATPases, the other being replaced by asparagine. The alignment between the hog gastric H,K- and Na,K-ATPases is shown in Structure 1. This prediction and alignment would place cysteine 813 of the gastric H,K-ATPase in the extracytoplasmic loop between the fifth and sixth transmembrane segments and cysteine 822 within the membrane. The labeling with omeprazole followed by thermolysin digestion of the labeled TM5/TM6 peptide showed indeed that labeling was at cysteine 813. Given the cationic nature and bulk size of the transported sulfenamide (11, 12, 23, 24, 30), Cys-813 is probably more exposed at the extracytoplasmic surface. Fig. 7 illustrates a model for the binding of the various sulfenamides to cysteine 813, based on alignment with the current models for the Na,K- and sarcoplasmic reticular Ca$^{2+}$-ATPases and the labeling shown above.

It has been shown that extrusion of this region of the Na,K-ATPase from the membrane occurs after removal of K$^+$ from a tryptic digest carried out in the presence of K$^+$ (42), suggesting conformational mobility of this segment relative to the plane of the membrane. Binding of these sulfenamides to this cysteine results in inhibition of ATPase activity and acid transport by the gastric H,K-ATPase. The fifth and sixth transmembrane segments are relatively short, based on access of trypsin to Lys at position 791 and Lys at position 835, to give a relative molecular mass of 5.9 kDa. A fragment of this predicted relative molecular mass was often found in the different gels illustrated after tryptic digestion. Binding of a bulky cationic sulfenamide to this region would likely prevent conformational changes as well as blocking the ion transport pathway thought to include this region.

The conclusion that cysteines 321, 813, and 892 are accessible to these reagents after activation to a cationic sulfenamide on the luminal surface of the enzyme provides evidence for at least three pairs of transmembrane segments in this ATPase. Transmembrane segments 3 and 4 are defined by the binding to cysteine 321, transmembrane segments 5 and 6 are defined by the binding to cysteine 813, and transmembrane segments 7 and 8 are defined by the binding to cysteine 892. Tryptic digestion data (13), labeling with a K$^+$ competitive photoaffinity reagent (31), and in vitro translation of hydrophobic sequences in the presence of microsomes (14) combine to provide experimental evidence for a 10-transmembrane segment model for the membrane domain of this member of the alkali cation family of P-type ATPases.

Accessibility of cysteine residues at the extracellular surface of the Na,K-ATPase has also been studied in some detail with permeant and impermeant reagents (43). Cys-911, Cys-964, and Cys-981 were predicted to be at or near the extracellular surface of the membrane. Cys-981 at transmembrane segment 9 was modified with both the membrane-impermeable 4-acetamido-4'-maleimidylstilbene-2,3''-disulfonic acid and the membrane-permeable 7-diethylamino-3-(4'-maleimidyl)-4-methylcoumarin in the presence of Mg$^{2+}$ and P. Also, from the treatment of K$^+$-stabilized post-tryptic membrane digest of Na,K-ATPase, Cys-983 at M10 was labeled after removal of Rb$^+$ or K$^+$, with release of the transmembrane segments 5 and 6. These results showed that cysteines at the extracellular surface of the Na,K-ATPase are accessible in a certain conformation, and suggested that M5/M6 segment could be close to the M9/M10 segment. Simi-
larly, an association between the C-terminal 21-kDa fragment and M5/M6 segment was observed in the H,K-ATPase, suggesting that the cationic sulfenamide retains its sided reactivity and shows that only the catalytic subunit is labeled (47). It appears that the cationic sulfenamide retains its sided reactivity and membrane impermeability also in the intact cell or animal.

The cationic sulfenamide generated from acid transport activation of omeprazole (10) has a different labeling pattern of the enzyme when this is compared with labeling following acid activation of the omeprazole in the bulk solution. In the latter case, a large number of cysteines are derivatized (45). These in vitro data show that the cationic sulfenamide is relatively membrane impermeable. When radioactive omeprazole is added to rabbit gastric glands and the site of radioactivity is found only in the lumen of the active secretory canaliculi (46). Administration of radioactive omeprazole to animals and isolation of radioactive bands from the intact stomach shows that only the catalytic subunit is labeled (47). It appears that the cationic sulfenamide retains its sided reactivity and membrane impermeability also in the intact cell or animal.

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