Comparison of Covalent with Reversible Inhibitor Binding Sites of the Gastric H,K-ATPase by Site-directed Mutagenesis*

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The gastric H,K-ATPase is covalently inhibited by substituted pyridyl-methylsulfanyl-benzimidazoles, such as omeprazole, that convert to thiolphilic probes of luminally accessible cysteines in the acid space. The K⁺ competitive inhibitor, SCH28080, prevented inhibition of acid transport by omeprazole. In stably expressing HEK293 cells, the benzimidazole-reactive cysteines, Cys-321 (transmembrane helix (TM) 3), Cys-813 and Cys-822 (TM5/6), and Cys-892 (TM7/8) were mutated to the amino acids found in the SCH28080-resistant Na,K-ATPase and kinetic parameters of H,K-ATPase activity analyzed. Mutations of Cys-822 and Cys-892 had insignificant effects on the $K_{i}^{(app)}$ or $V_{max}$ but mutations of Cys-813 to threonine and Cys-321 to alanine decreased the affinity for SCH28080. Mutation of Cys-321 to alanine produced mixed kinetics of inhibition, still with higher affinity for the cation-free form of phosphoenzyme. Since the phenylmethoxy ring of the imidazo-pyridine inhibitors binds to TM1/2, as shown by earlier photoaffinity studies, and the mutations in TM6 (Cys-813 → Thr) as well as the end of TM3 (Cys-321 → Ala) decrease the affinity for SCH28080, the TM1/2, TM3, and TM6 helices lie within ~16 Å of each other based on the size of the active, extended conformation of SCH28080.

The H,K-ATPase, the enzyme responsible for generating gastric acid by pumping hydronium ions out of the parietal cell of the stomach in exchange for potassium, belongs to the family of P₂-type ATPases and has homology with other members of this family such as the Na,K-ATPases (65%) and Ca-ATPases (23%). By using several different techniques, such as trypsinization and peptide sequencing, biochemical labeling, and in vitro translation, the H,K-ATPase was shown to contain 10 transmembrane helices (TM) in the catalytic α subunit and a single TM in the accessory β subunit (1–6). Recent analysis of two-dimensional crystals of the SERCA-ATPase and the Neuros-

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The abbreviations used are: TM, transmembrane helix; G₁, hog gastric vesicles; $K_{i}^{(app)}$, apparent affinity for inhibitor; $K_{p}^{(app)}$, apparent affinity for cation (NH₄⁺); omeprazole, 5-methoxy-2-(4-methoxy-3,5-di-methyl-2-pyridyl) methylsulfinyl)-1H-benzimidazole; PIPES, pipera-

The gastric H,K-ATPase supports the presence of 10 transmembrane helices in these single subunit catalytic domains (7, 8).

Additional information about the structure of the membrane domain of the H,K-ATPase can be obtained from the investigation of the side chains of the protein that are involved in inhibitor binding, as originally exemplified by studies of residues important for high affinity ouabain binding to the Na,K-

The gastric H,K-ATPase is thus unique in the P₂ type ATPases in having quite small inhibitor molecules (molecular mass ~300 Da) specific for this enzyme.

The best studied class of proton pump inhibitors are substituted pyridyl methylsulfanyl benzimidazoles, characterized as thiol-reactive reagents, which covalently inhibit the H,K-

The gastric H,K-ATPase by forming a disulfide bond with luminally accessible cysteine side chains (10). Recent work in our laboratory showed that at least 4 cysteines, at positions 321, 813, 822, and 892, are labeled by one or more of these benzimidazoles (4, 10). Covalent binding to Cys-813 seemed to be responsible for inhibition by these drugs. A second class of inhibitors currently under develop-

ment for clinical use, the substituted imidazo-1,2a-pyridines, such as SCH28080 (11), and a photoaffinity derivative, Me-

DZIP⁻ (12), are K⁺-competitive, reversible inhibitors. The active form of both classes of inhibitors is either a permanent cation (sulfenamide in the case of benzimidazoles) (13) or the protonated or quaternary form in the case of the imidazo-

pyridines (14). Both classes bind to the membrane domain of the enzyme from the luminal surface. Their length is between 12 and 16 Å (Fig. 1).

SCH28080 prevented inhibition of acid transport by the benzimidazole, omeprazole (15), suggesting that SCH28080 and omeprazole binding sites overlap. Therefore, mutations of the benzimidazole-labeled cysteines were selected as targets for finding residues that are involved in the binding site of SCH28080. Since this is a reversible and not a covalent inhibitor, additional information about the orientation of the helices of the membrane domain can be obtained from knowledge of the side chains that interact with this compound.

Although the protonated form of SCH28080 and the counter-

transported ion (K⁺, Rb⁺, or NH₄⁺) are purely competitive in terms of ATPase kinetics, the fact that they are very different in size and shape suggests that luminal ion and inhibitor binding sites are not formed by the same set of amino acid side chains but that binding of one excludes binding of the other to give competitive kinetics. The SCH28080 binding pocket on the protein should be formed by a limited set of amino acid side chains from different membrane-spanning helices separated from each other by a distance corresponding to the size of SCH28080. Analysis of the SCH28080 binding pocket by site-

directed mutagenesis may be helpful in mapping the arrange-

ment of these helices. Thus, the presence of mutated variants affecting the apparent affinity of the inhibitor ($K_{p}^{(app)}$) but leaving the apparent affinity of the ion ($K_{i}^{(app)}$) unchanged

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by mutating single amino acids into conserved residues found in the Na,K-ATPase, which is insensitive to SCH28080. This approach should retain overall kinetic function of the enzyme with a concurrent increase of the apparent $K_i$ for SCH28080. As a first approach to side chain definition, the cysteines known to bind benzimidazoles were mutated and their effects on pump and inhibitor kinetics evaluated.

MATERIALS AND METHODS

Vectors Used for the Expression of the H,K-ATPase $\alpha$ and $\beta$ Subunits—The cDNA coding for the rabbit H,K-ATPase $\alpha$ subunit (16) (GenBank accession no. X64694) was inserted into the multiple cloning site of the mammalian expression vector pcDNA 3.1(Zeo) (Invitrogen, Carlsbad, CA) containing the eukaryotic selection marker Zeocin® (In-vitrogen). The vector was then called pcDNA3.1(Zeo)-H,K-$\alpha$. The cDNA coding for the rabbit H,K-ATPase $\beta$ subunit (17) (GenBank accession no. M55544) was inserted into the multiple cloning site of the mammalian expression vector pcDNA 3 (G418) (Invitrogen) containing the eukaryotic selection marker G418 for neomycin resistance (pcDNA3(G418)-H,K-$\beta$).

Site-directed Mutagenesis of Cys-321, Cys-813, Cys-822, and Cys-892—The mutation of Cys-813 to Thr, Cys-822 to Gly, and Cys-892 to Leu and the combination of these three residues were generated as reported previously (18). By using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA), we additionally generated the mutation Cys-321 → Thr and Cys-321 → Ala. For mutations Cys-321 → Thr/Ala, we used the sense primer GGCCATGTCAGCTTGTCACACCTCTCGT and the antisense primer TAGGCAATAGCATGTGGCCACTACAAAAAA.

The mutations were achieved by exchanging the underlined bases TG → AC/GC (sense) and CA → GT/GC (antisense) coding now for threonine or alanine respectively. All mutagenic cDNA sequences were excised using the EcoRI and Mrot restriction enzymes and ligated in the corresponding sites of the cDNA of the rabbit H,K-ATPase $\alpha$ subunit in the pcDNA3.1(Zeo)-H,K- $\alpha$ vector. Each final construct was sequenced before use in transfection.

Transfection of HEK293 Cells and Selection of Stable Cell Lines—Human embryonic kidney cells (HEK293-ATCC CRL 1573) were transfected with pcDNA3(G418)-H,K-$\beta$ as reported previously (18), and a stable cell line was selected by adding at 60 h after transfection the eukaryotic selection marker G418 at a concentration of 0.75 $\mu$g of G418/ml of medium. This concentration of G418 was maintained until single colonies appeared. A colony was isolated, expanded, and grown in the presence of 0.25 $\mu$g of G418/ml of medium. The H,K-ATPase $\beta$ subunit was stably expressed in this cell line, as confirmed by Western blot analysis using a monoclonal antibody against the H,K-ATPase $\beta$ subunit, and the antisense primer GATGCCAAATGCAATGGCCACTACAAAATA. For each mutation, several cell lines were selected and two clones with the best ratio of expressed H,K-ATPase to total protein were expanded for isolation of membranes. The maintenance concentration for Zeocin® was 0.1 mg/ml medium.

Preparation of Crude Membranes—Cell layers from stable cell lines, grown to confluence, were washed with phosphate-buffered saline. The cells were scraped and resuspended in buffer A (10 mM PIPES, 2 mM EGTA, 2 mM EDTA, pH 7.0). Buffer A was sodium-free. The cell suspension was homogenized with a tight Dounce homogenizer (Wheaton, Millville, NJ). The homogenate was centrifuged at 100,000 g for 5 min. The supernatant was collected, layered on 40% sucrose step gradient, and spun in a Beckman SW28 swinging bucket rotor at 25,000 rpm for 2.5 h at 4 °C. The fraction at the interface of sucrose/buffer was collected and diluted to a total volume of 15 ml in buffer A. The membrane fraction was collected by centrifugation in a Beckman 75Ti rotor (35,000 rpm, 4 °C, 30 min). The pellet was resuspended in buffer A and homogenized in a 2-ml Teflon homogenizer (Wheaton). The total protein concentration was determined by the method of Bradford (19) with immune globulin G as a standard according to the manufacturer (Bio-Rad). The total protein concentration was ~10 $\mu$g/ml. The membranes were aliquoted, flash-frozen, and stored in liquid nitrogen.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Anal-
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sis—Gel samples were prepared by mixing 1–5 μl of the above prepared membranes with 30 μl of gel sample buffer (4% SDS, 0.05% bromphenol blue (w/v), 20% glycerol, 1% β-mercaptoethanol (v/v) in 0.1 M Tris buffer pH 6.8). The samples were loaded on a 7% SDS-polyacrylamide gel. As a standard for native α and β subunits, 40–100 ng of purified gastric vesicles were loaded. Molecular weight standards (Bio-Rad) were loaded on each gel.

After SDS-polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose membranes (BioPlot-NC, Costar, Cambridge, MA). Membranes were washed twice with TBS (10 mM Tris, 150 mM NaCl, 0.05% Tween (v/v)) and incubated in TBS containing 1% bovine serum albumin (w/v). After 30 min, the membranes were washed with TBS to remove the primary antibody solution (monoclonal antibody 12.18 against amino acids 666–689 of the α subunit of the H,K-ATPase, diluted 1:10,000 in TBS; or monoclonal antibody 2B6 against amino acids 236–281 of the β subunit of the H,K-ATPase, diluted 1:1000 in TBS) (21, 22). After 1 h, membranes were washed twice with TBS and incubated with the secondary antibody solution (anti-mouse IgG conjugated to alkaline phosphatase (Promega, Madison, WI), diluted 1:4000 in TBS). Then, after 1 h, membranes were washed twice and incubated for 15 min in TBS. After a final wash, the membranes were incubated for 15 min in AP buffer (100 mM Tris, 100 mM NaCl, 5 mM MgCl₂, pH 9.5) containing 0.3% nitro blue tetrazolium solution (v/v) and 0.15% 5-bromo-4-chloro-3-indolyl-1-phosphate solution (v/v) according to the manufacturer’s instructions (Promega).

Quantification of Expressed H,K-ATPase (Fig. 3)—The content of expressed H,K-ATPase in the membrane fraction was quantified by using purified hog gastric vesicles containing ~85% H,K-ATPase (G1) (20). These vesicles were treated as described above, loaded, and run side by side on the same gel. Protein transfer on to nitrocellulose membranes and Western blot analysis was also similar. All blots were scanned using the AMBIS optical imaging system (AMBIS Inc., San Diego, CA), and the optical density of the bands was measured and a standard curve of nanograms of G1 versus integrated optical density was obtained, which was used for estimation of the content of G1 equivalent in the crude membrane protein. This then allowed an estimate of the specific activity of the expressed enzyme compared with hog gastric vesicles containing ~85% H,K-ATPase.

NH₂⁺-stimulated ATPase Activity—ATPase assays were performed by a modification of the method of Yoda and Hokin (23). 3 μl of membrane fraction were added to 122 μl of Na⁺-free reaction buffer (40 mM Tris-HCl (pH 7.4), 2 mM MgCl₂) containing 1 mM EGTA (Ca-ATPases), 500 μM ouabain (Na,K-ATPase), 1 μM oligomycin (mitochondrial ATPase), 10 mM bafilomycin (V-type ATPases) and 100 mM thapsigargin (SERCA-ATPases) to suppress the activities of known contaminating ATPases. ATPase activity was measured in triplicate for the unstimulated enzyme, stimulated enzyme with various NH₄Cl concentrations of total crude membrane protein was 3.5 ng/mg of G1/h and then converted to specific H,K-ATPase activity by subtracting the activity in the absence or presence of inhibitor. The content of total membrane protein (Fig. 3). All other crude membrane

The aKapp, 3 S.E. was determined graphically using the 1/Vmax versus inhibitor concentration replot. The intersection of the linear regression gives the negative aKapp, aKapp/Kapp gives the α-value, which reflects the relative affinity for the cation-free and cation-liganded form of the phosphoenzyme (29) (Figs. 4 and 5). To determine the significance of the determined aKapp/Kapp for the wild type H,K-ATPase versus the mutant enzymes, confidence intervals were calculated using the t value of Student’s t distribution.

ATP Affinity—ATPase activity was measured by the same method as described above using a fixed NH₄Cl concentration of about 4 times the Km(app) (9.5 mM for the wild type H,K-ATPase and the Cys-321 → Ala mutant (Thr)) and plotted versus the actual concentration of ATP (mean of initial and final ATP concentrations). The data were fitted by nonlinear regression to the Michaelis-Menten equation and the Km(app) ± S.E. for ATP obtained.

Immunofluorescence and Confocal Laser Scanning Microscopy—The cell lines were grown on coverslips in Falcon® six-well plates (Becton Dickinson) on Franklin Laidlaw 25-cm² flasks were fixed and printed out using Adobe Photoshop 2.01 (Adobe Systems Inc., San Jose, CA) imaging software and an Epson Inkjet printer to be visible in the plasma membrane fraction (Fig. 3). All other crude membrane

RESULTS

Confocal Microscopy and Content of Expressed H,K-ATPase in Membrane Fractions—With one exception, all cell lines expressed the normal and mutated H,K-ATPase α and β subunits as shown by Western blot (data not shown). Only the mutation Cys-321 → Thr did not yield a cell line expressing a detectable H,K-ATPase α subunit where Western blot screening of 24 cell lines was negative. The confocal microscopy images using the anti Na,K-ATPase α subunit antibody as a plasma membrane marker show clear plasma membrane localization of H,K-ATPase α subunits (Fig. 2). Therefore, the wild type H,K-ATPase and all expressed mutant H,K-ATPases are translated in the endoplasmic reticulum, processed, and exported to the plasma membrane as their major site of expression.

In order to estimate the amount of expressed H,K-ATPase α subunits, Western blots were quantitatively using mice 12.18, 40–120 ng of purified hog gastric vesicles (~85% H,K-ATPase, G1) yielding a linear calibration curve with values in the same range as compared with the densities obtained for the Expressed H,K-ATPase in crude membrane fractions. The average amount of expressed H,K-ATPase found in three different concentrations of total crude membrane protein was 3.5 ng/μg total membrane protein (Fig. 3). All other crude membrane
fractions were quantified using the same method and contained 2–8 ng of H,K-ATPase α subunits/μg of protein of the crude membrane fraction (Table I).

**NH_4^+**-stimulated ATPase Activity of Wild Type H,K-ATPase: Calculation of \( V_{\text{max}} \) for \( K_{\text{m(app)}}[\text{NH}_4^+] \) and \( K_{\text{i(app)}}[\text{SCH28080}] \) and comparison to hog gastric enzyme (G1) —3 μl of wild type H,K-ATPase containing crude membranes (30 μg of total protein) catalyzed a basal release of 11.7 ± 0.4 nmol of inorganic phosphate in 1 h. The addition of 37.3 mM NH_4^+ stimulated the enzyme activity to 17.9 ± 0.1 nmol of P_i/h (53% signal over background). The addition of 1 μM SCH28080 in the presence of 37.3 mM NH_4^+ inhibited the enzyme activity down to a release of 12.1 ± 0.3 nmol of P_i/h. The activities were converted to μmol of P_i/mg of G1/h using the conversion factor obtained from the quantitative Western blot analysis (Table I). By fitting the data points for various NH_4^+ concentrations (1–37.3 mM) in the absence or presence of various SCH28080 concentrations (125–250 nM) using the Michaelis-Menten non-linear fit, we found, for wild type enzyme, a \( K_{\text{m(app)}}[\text{NH}_4^+] \) of 2.6 ± 0.9 mM and a \( V_{\text{max}} \) of 68.9 ± 9 μmol/mg of G1/h. The \( K_{\text{m(app)}}[\text{NH}_4^+] \) and \( V_{\text{max}} \) for the assays in the presence of increasing SCH28080 concentrations (62.5–250 nM) were also determined, and the slopes of \( (K_{\text{m(app)}}[\text{SCH28080}] \) for each inhibitor concentration were plotted against the corresponding SCH28080 concentration (Fig. 4, upper inset). The inhibition kinetics of the expressed wild type H,K-ATPase was competitive, as characterized by a stepwise increase of the \( K_{\text{m(app)}}[\text{NH}_4^+] \) with an unchanged \( V_{\text{max}} \) as seen in the intercept of the lines in the \( 1/K_{\text{m(app)}} \) versus \( 1/V_{\text{max}} \) line plot (Fig. 4) on the y axis (\( V_{\text{max}} \)). The \( K_{\text{i(app)}}[\text{SCH28080}] \) for each data point was calculated using the Michaelis-Menten equation for competitive inhibition (25), and was 108 ± 12 nM. The expressed wild type H,K-ATPase displayed the same kinetic characteristics as previously reported (11, 26) for the hog gastric enzyme (G1 vesicles, namely \( K_{\text{m(app)}}[\text{NH}_4^+] 2.7 ± 0.06 \) mM, \( V_{\text{max}} 72.4 ± 0.5 \) μmol/mg/h, and \( K_{\text{i(app)}}[\text{SCH28080}] 107 ± 18 \) nM) (Table I).

\( V_{\text{max}} \), \( K_{\text{m(app)}}[\text{NH}_4^+] \), and \( K_{\text{i(app)}}[\text{SCH28080}] \) for the Expressed Mutant H,K-ATPases —Table I summarizes the results of the kinetic data for the selected mutants of the H,K-ATPase. All mutant enzymes were catalytically active. The \( V_{\text{max}} \) values for the mutant enzymes were either increased (Cys-892 → Ala, Cys-813 → Thr, Cys-822 → Gly) or significantly decreased (Cys-813 → Thr/Cys-822 → Gly/Cys-892 → Leu) as compared with the \( V_{\text{max}} \) of the wild type H,K-ATPase.

The \( K_{\text{m(app)}}[\text{NH}_4^+] \) was not significantly changed for the mutants Cys-321 → Ala, Cys-822 → Gly, and Cys-892 → Leu. In contrast, the \( K_{\text{m(app)}}[\text{NH}_4^+] \) was increased ∼3-fold for Cys-813 → Thr/Cys-822 → Gly, or significantly decreased (Cys-813 → Thr/Cys-822 → Gly/Cys-892 → Leu) as compared with the \( V_{\text{max}} \) of the wild type H,K-ATPase.

The effects on the \( K_{\text{i(app)}}[\text{SCH28080}] \) of the expressed wild type H,K-ATPase were studied with a basal release of 17.9 ± 0.1 nmol of P_i/h (53% signal over background) due to changes in the binding site for either inhibitor or translocation. The \( V_{\text{max}} \) values were either increased (Cys-321 → Ala, Cys-822 → Gly, and Cys-892 → Leu) or significantly decreased (Cys-813 → Thr/Cys-822 → Gly/Cys-892 → Leu) as compared with the \( V_{\text{max}} \) of the wild type H,K-ATPase.

**ATP Affinity for Wild Type H,K-ATPases, Cys-321 → Ala, and Cys-813 → Thr—**Alteration in inhibitory kinetics can be due to changes in the binding site for either inhibitor or translocation and is used in the ratio between \( E_0 \) and \( E_0 \)-P. As shown in Fig. 6, the dependence of activity on ATP concentration was similar for all three expressed enzymes. No significant change of the \( K_{\text{m(app)}} \) for ATP could be observed for the Cys-321 → Ala mutant (87 ± 16 μM) and the Cys-813 → Thr...
mutant (109 ± 16 μM) as compared with wild type H,K-ATPase (100 ± 6 μM), suggesting that there is no change in the E₁ to E₂ ratio in the mutants.

**DISCUSSION**

The successful expression of mammalian P₂-type ATPases adequate for kinetic analyses has proven to be challenging. Several strategies involving different expression systems have been devised (18, 27–31). Problems arising with some of the different expression systems are, for example, too little ATPase activity with too high a background or no correlation between the amount of expressed protein and the activity. In general, the activity data obtained from the different systems have not allowed kinetic analysis for the expressed wild type or mutant ATPase.

The present study utilizes HEK293 cells and successive
transfection of a cell line stably expressing the H,K-ATPase β subunit with wild type or mutant H,K-ATPase α subunits, to obtain stable cell lines with kinetically adequate expression levels of the ATPase. The expressed protein undergoes processing and transport to the plasma membrane, as shown by confocal microscopy (Fig. 2) and by Western blot analysis of the expressed β subunit (data not shown). If the amount of the expressed ATPase, as quantified by Western blot analysis using hog gastric vesicle enzyme as internal standard, is correlated to the measured ATPase activity of the total protein of the crude membranes, the specific H,K-ATPase activities are in the same range as those of hog gastric vesicles (G1). Thus, it appears that all antibody-labeled proteins in the Western blot analysis express the normal wild type ATPase activity.

In a previous study using a H,K-ATPase α-β fusion protein (18), we were only able to measure about 10–20% cation-stimulated release of Pi over background (Δ 10^5 cpm) allowed generation of reliable kinetic data for the wild type and the mutant enzymes. Various kinetic parameters can be obtained, such as the \( K_m \) and \( V_{max} \) of the enzyme.

Both the active form of SCH28080 and the sulfenamide of omeprazole are cations. While the SCH compound does not require acid activation, the sulfenamide of omeprazole only forms effectively in an acidic compartment. The only method to achieve specific inhibition of the enzyme with benzimidazoles in vitro is the addition of the uncharged parent compound (e.g. omeprazole) to acid-transporting cytoplasmic side out vesicles containing the gastric H,K-ATPase, which are stimulated by ATP and K^+ - valinomycin (13). The benzimidazoles accumulate inside the vesicles due to protonation, and an acid-catalyzed conversion to the highly reactive, cationic, and thiophilic sulfenamide occurs (either in bulk solution or in situ on the enzyme). The sulfenamide forms a covalent disulfide bond with luminal antibody labeled cysteine on the enzyme during inhibition.

FIG. 5. \( NH_4^+ \)-stimulated TPase activity of mutant H,K-ATPase Cys-321 → Ala. Lineweaver-Burk inverse plot of each assay in the absence or presence of various SCH28080 concentrations. Upper inset, \( K_m/V_{max} \) (the slopes of the inverse plot) replot versus SCH28080 concentration. The intersection with the x axis of the linear regression of these points shows the negative \( K_{i(app)} \). Lower inset, \( 1/V_{max} \) replot versus SCH28080 concentration. The intersection with the x axis of the linear regression of these points gives the \( K_{i(app)} \).

FIG. 6. Michaelis-Menten plot of ATPase activity versus ATP concentration of wild type and mutant ATPases. ■, wild type H,K-ATPase; ○, Cys-813 → Thr; ●, Cys-321 → Ala.
Cys-813 was found to be labeled by all benzimidazoles, and the degree of labeling of this cysteine correlates with the extent of inhibition of the enzyme, although other cysteines are labeled either as a function of time of incubation or as a function of the nature of the benzimidazole (10). Since the rate constants for accumulation and conversion of the inhibitor add to the inhibition mechanism and the species responsible for inhibition could be either the sulfenic acid or the sulfenamide bound to the enzyme prior to disulfide formation and since the inhibition is covalent, kinetic analysis of inhibition by these compounds does not yield information beyond identification of the cysteine residues derivatized.

Kinetic studies with SCH28080 have shown that this compound is a competitive inhibitor competing with $K^+$ for the binding to $E_2$ or $E_2P$ on the luminal side of the enzyme with a $K_{i(app)}$ of 56–80 nM (11, 32). The $K_i$ obtained for high affinity binding of SCH28080 to the phosphoenzyme formed in the presence of MgATP is similar to the $K_{i(app)}$ defined kinetically (ATPase turnover assay) (33). Only binding to $E_2P$ is a linear function of SCH28080 concentration; therefore, this is the preferred form of the enzyme for this imidazo-pyridine. The stoichiometry is one site per EP (32), and it is the protonated form of the compound that is active (11, 12, 32, 33). Experiments designed to define the structural site of inhibition of SCH28080 using a photoaffinity derivative of this inhibitor identified a region of the H,K-ATPase $\alpha$ subunit containing the TM1-loop-TM2 as being the binding site of the nitrile generated in the $para$ position of the phenyl methoxy group (12). This is the hydrophobic tail of the compound, whereas the charge resides in the imidazo-pyridine and is the region presumably responsible for the $K^+$ competitive inhibition. Thus, for the benzimidazoles, there is specific knowledge about the covalent binding sites, whereas, for the imidazo-pyridines, there is functional but little structural information about the binding site responsible for inhibition.

Evidence was presented that the inhibitor binding site for omeprazole and SCH28080 overlap, since the inhibition of omeprazole and SCH28080 is competitive at the concentrations of SCH28080 used here (15). There is also evidence that the inhibition of the H,K-ATPase by the benzimidazole, rabeprazole, has a $K^+$-sensitive component.2 Hence, residues involved in the inhibitory effect of benzimidazoles could contribute to the SCH28080 binding pocket. Since there are 4 cysteines found to be labeled by the different benzimidazoles (omeprazole, lansoprazole, pantoprazole, and rabeprazole) under acid transporting conditions, Cys-321 in TM3, Cys-813 and Cys-822 in TM6, and Cys-892 in the luminal loop between TM7 and TM8 (10), these residues were chosen as targets for mutational analysis. The large size and multiple binding possibilities for SCH28080 suggest that several residues will contribute to the binding site and that the overall affinity for the compound will change stepwise with each significant mutation.

Expression of mutant H,K-ATPase $\alpha$ subunits and measurement of the $K_{i(app)}$ (luminal ion) as well as the $K_{i(app)}$ [SCH28080] provide a means of finding the residues affecting inhibition of the H,K-ATPase by this inhibitor. We have interpreted the mutations that cause no or small changes in $K_{i(app)}$ but a significant change in $K_{i(app)}$ as selectively affecting the inhibitor binding region without inducing large conformational changes in the enzyme. In this work, we changed the cysteines of interest to the amino acids found in the Na,K-ATPase at that position, since this enzyme is both SCH28080 and omeprazole insensitive on its luminal surface. In the case of Cys-321, alanine had to be chosen as the substituting amino acid, since the Cys-321 → Thr mutation resulted in no detectable expression of the mutant enzyme in several cell clones.

The mutation of Cys-813 to threonine in TM6 of the H,K-ATPase $\alpha$ subunit decreased the inhibitor affinity for SCH28080 about 6-fold ($K_{i(app)} = 586$ nM). This is the same residue found to be responsible for benzimidazole binding and inhibition (10). Since the affinity for the luminal ion was also decreased ~ 3-fold ($K_{i(app)} = 6.6$) with no change in ATP affinity, there is probably an effect of this mutation on the cation binding region. Since cysteine is unlikely to be directly involved in ion binding (no oxygen-providing side chain) the effect is most likely conformational. If the second cysteine in TM6, Cys-822, predicted to be on the opposite side of TM6 to Cys-813 is mutated to glycine, no effect on the affinity for the luminal ion or for SCH28080 was found. Previous results using expression of a fusion protein had shown that mutation of Cys-822 to glycine abolished omeprazole inhibition of RB+ transport without affecting SCH28080 inhibition of cation flux (18), consistent with the lack of kinetic effects of the mutant described here. Although Cys-892 in the loop between TM7 and TM8 is labeled by the benzimidazoles omeprazole and lansoprazole, binding to this site did not correlate with the inhibitory effect of these compounds on the H,K-ATPase (10). This is in agreement with our observation that the mutation of Cys-892 to leucine had no effect on the affinity either for the luminal ion or for SCH28080, although there was a small increase in $V_{max}$.

If the above mutations were combined in a mutant containing Cys-813 → Thr/Cys-822 → Gly/Cys-892 → Leu, we obtained results similar to the single mutation, Cys-813 → Thr mutant alone ($K_{i(app)}$ [SCH28080] = 758, $K_{i(app)}$ = 5.1). In summary, while Cys-813 contributes to the inhibitor binding pocket on the H,K-ATPase for SCH28080, Cys-822 and Cys-892 apparently do not.

The benzimidazole, lansoprazole, labeled Cys-321 of TM3 (34) in addition to the above cysteines. The conserved mutation of Cys-321 to threonine did not produce any detectable ATPase signal on Western blot. This might be due to an effect of this mutation on protein folding and membrane insertion. Mutation of this residue to alanine allowed expression of a functional mutant, which had a moderate, 2.5-fold decrease of the affinity of SCH28080. Therefore, mutation of Cys-321 affects SCH28080 affinity but may not be directly in the binding pocket, given the small change in $K_{i(app)}$.

The mutation Cys-321 → Ala changed the kinetic nature of SCH28080 inhibition. As seen in the inverse plot for this mutation, the intersection of the lines occurs not at the y axis (1/$V_{max}$), but is shifted to the left (Fig. 5). The $V_{max}$ in the presence of increasing concentrations of SCH28080 decreased stepwise with increasing $K_{i(app)}$ values, giving mixed inhibition kinetics. At 500 nM SCH28080, the $V_{max}$ was decreased significantly, by 36%. Hence, SCH28080 and the luminal cation can bind to the mutant enzyme simultaneously (mixed-type inhibition), a critical kinetic change, since in the wild type H,K-ATPase, SCH28080 and cation binding are mutually exclusive at the concentrations of SCH28080 used here (competitive inhibition). Perhaps Cys-321 is involved in maintaining exclusive access of cation or imidazo-pyridine to their sites in the membrane domain. A small decrease in the $V_{max}$ of the fast $K^+$-dependent dephosphorylation reaction of the hog H,K-ATPase in the presence of a high concentration of SCH28080 (50 μM) has been described. This effect can be accounted for by a mechanism where SCH28080 binds to E$_2$P.K to form E$_2$P.K.SCH. The affinity for this form appeared to be very small ($aK_{i(app)} = 30$ μM) for hog enzyme and within the scatter of the reported data and would not be observed in the H,K-ATPase overall turnover assay (32). Our data show that the apparent

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2 W. A. Simon, unpublished observations.
affinity of SCH28080 for $E_2\cdot P\cdot K$ was increased in the mutation of Cys-321 → Ala ($K_{m(app)} = 938\ \text{nM}$) as compared with the wild type enzyme.

We investigated the possibility of a change of $E_2$ toward $E_1$ of the ATPase caused by a conformational change due to the mutations thus affecting the $K_{m(app)}$ for SCH28080 (Cys-321 → Ala and Cys-813 → Thr) since the inhibitor binds to the $E_2$ form of the enzyme. This change could decrease the affinity for the inhibitor if the enzyme is stabilized in $E_1$. Since no significant change in the $K_{m(app)}$ for ATP for the Cys-321 → Ala and Cys-813 → Thr mutants compared with the wild type ATPase was observed (Fig. 6), a change in the $E_2/E_1$ equilibrium as an explanation for the $K_{m(app)}$ effects of these mutant enzymes is unlikely.

In summary, the present data provide an explanation for the overlap between covalent inhibition by the benzimidazoles and competitive inhibition by SCH28080 since the mutation of Cys-813 has an effect on the SCH28080 affinity. Since the phenyl ring of the imidazo-pyridine inhibitors binds to TM1/2 and the mutation in TM5/6 (Cys-813) decreases the affinity for SCH28080, these helix pairs may be within ~16 Å of each other. Further, the end of TM3 may also be close to these transmembrane helices since the mutation of Cys-321 also affects the affinity for SCH28080. Cys-822 and Cys-892 lie outside the inhibitor binding region. This implies that TM1/2, TM3, and TM5/6 lie within ~16 Å of each other.

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