The Cavity Structure for Docking the K\(^{+}\)-competitive Inhibitors in the Gastric Proton Pump

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2-Methyl-8-(phenylmethoxy)imidazo[1,2-\(\alpha\)]pyridine-3-acetonitrile (SCH 28080) is a reversible inhibitor specific for the gastric proton pump. The inhibition pattern is competitive with K\(^{+}\). Here we studied the binding sites of this inhibitor on the putative three-dimensional structure of the gastric proton pump \(\alpha\)-subunit that was constructed by homology modeling based on the structure of sarcoplasmic reticulum Ca\(^{2+}\) pump. Alanine and serine mutants of Tyr\(^{801}\) located in the fifth transmembrane segment of the gastric proton pump \(\alpha\)-subunit retained the \(^{86}\)Rb transport and K\(^{-}\)-dependent ATPase (K\(^{-}\)-ATPase) activities. These mutants showed 60–80-times lower sensitivity to SCH 28080 than the wild type in the \(^{86}\)Rb transport activity. The K\(^{-}\)-ATPase activities of these mutants were not completely inhibited by SCH 28080. The sensitivity to SCH 28080 was dependent on the bulkiness of the side chain at this position. Therefore, the side chain of Tyr\(^{801}\) is important for the interaction with this inhibitor. In the three-dimensional structure of the E\(_{2}\) form (conformation with high affinity for K\(^{+}\)) of the gastric proton pump, Tyr\(^{801}\) faces a cavity surrounded by the first, fourth, fifth, sixth, and eighth transmembrane segments and fifth/sixth, seventh/eighth, and ninth/tenth loops. SCH 28080 can dock in this cavity. However, SCH 28080 cannot dock in the same location in the E\(_{1}\) form (conformation with high affinity for proton) of the gastric proton pump due to the drastic rearrangement of the transmembrane helices between the E\(_{1}\) and E\(_{2}\) forms. These results support the idea that this cavity is the binding pocket of SCH 28080.

The gastric H\(^{+}\),K\(^{+}\)-ATPase is a proton pump that is responsible for gastric acid secretion (1). This pump consists of two kinds of subunits, the \(\alpha\)- and \(\beta\)-subunits. The \(\alpha\)-subunit is the catalytic subunit containing 10 transmembrane segments. The ATP binding site (2, 3) and the cation binding sites are located on this subunit (4–10). The \(\beta\)-subunit is the non-catalytic glycoprotein containing a single transmembrane segment and is involved in stabilization as well as targeting of the \(\alpha\)-subunit to the plasma membrane (11). This pump belongs to the family of type II P-type ATPases, which include sarcoplasmic reticulum (SR)\(^{1}\) Ca\(^{2+}\)-ATPase (Ca\(^{2+}\) pump) and Na\(^{+}\).K\(^{+}\)-ATPase (Na\(^{+}\) pump) (12).

At present, inhibitors specific for the gastric proton pump are classified into two groups. Irreversible inhibitors such as omeprazole, rabeprazole, and lansoprazole, termed the proton pump inhibitors, are the first group. They are activated in acidic lumens, modify the cysteine residues located on the luminal side of the proton pump, and inhibit its pump activity. They are clinically used for controlling hyperacidity. The second classified group of specific proton pump inhibitors are the reversible inhibitors such as SCH 28080 (13) and 3-amino-5-methyl-2-(2-methyl-3-thienyl)imidazo[1,2-\(\alpha\)]thieno[3,2-\(\alpha\)]pyridine (SPI-447) (Fig. 1) (14), which are described as acid pump antagonists (APAs). They bind to the E\(_{2}\) or E\(_{2}\)P forms (conformations showing a high affinity for K\(^{+}\)) and inhibit the gastric proton pump in a K\(^{-}\)-competitive manner (15, 16). Elucidation of the precise structure of the binding sites of the proton pump inhibitors and APAs is very important for generation of future drugs for acid-related diseases.

Cysteine residues covalently attached by the active molecules (sulfenamides) of the proton pump inhibitors were identified as Cys\(^{E15}\) and Cys\(^{793}\) (either in or close to the M6 segment) as well as Cys\(^{323}\) (in the M3/M4 loop) and Cys\(^{894}\) (in the M7/M8 loop) (the amino acid positions are given based upon the rabbit enzyme) (17). These cysteine residues are themselves not essential for the function of the proton pump, but the attachment of a bulky drug to the cysteine residues seems to disrupt the conformational change in the proton pump during the catalytic reaction cycles (18).

The binding site of SCH 28080 was first studied by photoaffinity labeling followed by site-directed mutagenesis. Although the first photoaffinity labeling study indicated that the first extracellular loop (the M1/M2 loop) is the direct binding site of SCH 28080 (19), our mutational and chimeric studies showed that the M1/M2 loop is not the direct binding site (6, 20). Several mutants showed lower sensitivity to SCH 28080: the M336I and V339I mutants (mutants in Met336 and Val339 in the M3/M4 loop), the M793S and E797D mutants (mutants in Lys\(^{793}\) and Glu\(^{797}\) in the M5 segment), and the C815T mutant (mutant in Cys\(^{815}\) either in or close to the M6 segment) (21–23). However, these mutants showed a less than 10-fold lower af-

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¶ The abbreviations used are: SR, sarcoplasmic reticulum; SCH 28080, 2-methyl-8-(phenylmethoxy)imidazo[1,2-\(\alpha\)]pyridine-3-acetonitrile; SPI-447, 3-amino-5-methyl-2-(2-methyl-3-thienyl)imidazo[1,2-\(\alpha\)]thieno[3,2-\(\alpha\)]pyridine; APA, acid pump antagonist; HEK, human embryonic kidney.
Cavity Structure of the Docking Site in Gastric Proton Pump

Fig. 1. Chemical structures of SCH 28080 and SPI-447.

finity for SCH 28080, and until recently there had been no reports on the amino acid residues that are critical for the interaction with this inhibitor. Very recently Vagin et al. (24) reported that the L811F mutant (mutant in Leu811 at the edge of the M5/M6 loop) showed a 90-fold decrease in the sensitivity to SCH 28080. Especially the Y801A and Y801F mutants showed a 1000- and 6000-fold decrease in their sensitivity to SCH 28080 and SPI-447, respectively. Very recently Vagin et al. (24) reported that the L811F mutant (mutant in Leu811 at the edge of the M5/M6 loop) showed a 90-fold decrease in the sensitivity to SCH 28080. Especially the Y801A and Y801F mutants showed a 1000- and 6000-fold decrease in their sensitivity to SCH 28080 and SPI-447, respectively.

We constructed the three-dimensional models of the gastric proton pump α-subunit by homology modeling based on atomic structures of SR Ca\(^{2+}\) pump as template molecules, and we propose a cavity structure found in the luminal side of the transmembrane domain to be the putative binding pocket of these drugs.

EXPERIMENTAL PROCEDURES

Materials—HEK-293 cells were a kind gift from Prof. Jonathan Lytton (University of Calgary, Calgary, Canada). The pcDNA3.1(+)(ZEO) and pcDNA3 vectors were obtained from Invitrogen. Effectene transfection reagent and EndoFree Plasmid Maxi and Mega kits were obtained from Qiagen (Tokyo, Japan). Pfu DNA polymerase was obtained from Stratagene (La Jolla, CA). Restriction enzymes and other DNA-modifying enzymes were from Toyobo (Osaka, Japan), New England Biolabs (Beverly, MA), or Amersham Biosciences. Collagen type I-coated tissue culture wares were obtained from Asahi Technoglass Co. SCH 28080 was a kind gift from Dr. Peter Chiu (Schering-Plough Co., Kenilworth, NJ). SPI-447 was a kind gift from Dr. Hironori Tanaka (Shionordon Pharmaceutical Co., Osaka, Japan). All other reagents were of molecular biology grade or the highest grade of purity available.

cDNAs of the α and β-subunits of the Gastric Proton Pump—cDNAs of the α and β-subunits were prepared from rabbit gastric mucosae as described elsewhere (4). The α- and β-subunit cDNAs were digested with EcoRI and XhoI. The fragments obtained were each ligated into the pcDNA3 vector for the β-subunit cDNA or pcDNA3.1(+)(ZEO) for the α-subunit cDNA treated with EcoRI and XhoI.

Site-directed Mutagenesis—A cDNA encoding the rabbit gastric proton pump α-subunit, which contains two SphI sites (nucleotide positions 2953 and 2954) was subjected to site-directed mutagenesis to remove the restriction site of SphI at nucleotide position 2954 (corresponding to Gly985 in the resulting cDNA (HK-αΔSphI)). The 5′-flanking nonamer “GCCATGC” was mutated to “GGATGC” without amino acid replacement. The subsequent introduction of site-directed mutations in the M5 segment of the gastric proton pump α-subunit was carried out by sequential PCR steps (6) in which appropriately mutated α-subunit cDNA sequences were inserted between the SphI sites of SCH 28080 (nucleotide 2833) and the AvrII site (nucleotide 2496) were prepared. Two kinds of flanking sequence primers were prepared: one is the 5′-flanking sense primer 5′-GAGAGCGGTGAGGAGGATGTCCTGCG-3′ (nucleotides 1948–1971), and the other is the 3′-flanking antisense primer 5′-CAAGGACACGGAGGGAAAAATGC-3′ (nucleotides 2476–2499). In addition, sense and antisense synthetic oligonucleotides, each 21 bases long containing one or two mutated bases near the center were designated as the sense mutating primer and the antisense mutating primer. In the first PCR amplification step, the HK-αΔSphI cDNA cloned in pBluescriptII SK(–) was used as a cDNA template. Two fragments were prepared in this step: one between the 5′-flanking sense primer and the antisense mutating primer and the other between the sense mutating primer and the 3′-flanking antisense primer. Each amplified fragment was purified by gel electrophoresis, combined, and incubated with the 5′-flanking sense primer and the 3′-flanking antisense primer in the second PCR amplification. The amplified fragment was purified by gel electrophoresis, cloned in pCR-Script Amp SK(–) (Stratagene), and sequenced. PCR was routinely carried out in the presence of a 300 μM concentration of each dNTP, 6 μM primers, 10 mM Tris-HCl (pH 8.8), 10 mM (NH\(_4\))\(_2\)SO\(_4\), 2 mM MgSO\(_4\), 0.1% Triton X-100, 100 μg/ml bovine serum albumin, and 2 units of Pfu DNA polymerase for 25 cycles. After sequencing, the amplified fragment in the second PCR was digested with SphI and AvrII and ligated back into the relevant position of the wild-type construct of the α-subunit. DNA sequencing was done by dideoxy chain termination method using a BigDye Terminator DNA sequencing kit and ABI Prism 3100 DNA sequencer (Applied Biosystems Inc.).

Cell Culture and Transient Expression—Cell culture of the HEK-293 cell line was carried out as described previously (4). For transient expression, co-transfection of the α- and β-subunit cDNAs was performed by the calcium phosphate method with 10 μg of purified DNA/10-cm dish. Cells were harvested 2 days after the DNA transfection.

Establishment of Stable Cell Lines—Stable cell lines co-expressing the α- and β-subunits were constructed as described previously (26). HEK-293 cells were first transfected with the β-subunit cDNA that was cloned in pcDNA3 (G418) by lipofection using Effectene transfection reagent, and stable cell lines were selected in the presence of 1 mg/ml Genetin (G418 sulfate) followed by screening for protein expression of the β-subunit by Western blot and immunofluorescence. Cell lines expressing the β-subunit were then subjected to the second transfection with the gastric proton pump α-subunit (either wild type or mutant) cDNA that was cloned in pcDNA3.1(+)(ZEO), selected, and cloned in a modified medium containing 0.5 mg/ml Geneticin and 0.2 mg/ml Zeocin. Mock cells were also constructed by successive transfection with vacant pcDNA3 and pcDNA3.1(+)(ZEO) vectors following the same method as for the construction of the cell lines expressing the α- and β-subunits.

Preparation of Membrane Fractions, SDS-Polyacrylamide Gel Electrophoresis, and Western Blot—Membrane fractions of HEK cells were prepared as postnuclear pellets as described previously (4). SDS-polyacrylamide gel electrophoresis was carried out as described elsewhere (27). Membrane preparations (30 μg) were incubated in a sample buffer containing 2% SDS, 2% β-mercaptoethanol, 10% glycerol, and 10 mM Tris-HCl, pH 6.8, at room temperature for 10 min and applied to the SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane. The membranes were blocked with a 4% BSA solution (Dainippon Pharmaceutical Co.). The proteins were detected with an anti-gastric proton pump α-subunit antibody, Ab1024, which recognizes the carboxyl terminal peptide (residues 1024–1034) (28). After incubation with a horseradish peroxidase-conjugated anti-rabbit...
and antibody, the bands were visualized with the ECLplus kit (Amersham Biosciences) and scanned using luminescent image analyzer LAS-1000plus (Fuji Film, Tokyo, Japan).

**Quantification of Expressed α-Subunit in the Membrane Fraction**

The content of α-subunit expressed in the membrane fraction was quantified by comparing with the α-subunit content in a pig gastric vesicle preparation as a standard. The α-subunit comprises about 60% of the protein content of the gastric vesicle preparation judged by the densitomeric analysis of SDS-polyacrylamide gel using ATTO densitographic software (ATTO, Tokyo, Japan). The membrane fractions of HEK cells as well as a series of diluted gastric vesicle preparations were run on the same SDS-polyacrylamide gel and subjected to Western blotting. The blots were scanned using optical scanning system Multi-Analyst (Bio-Rad). The signal intensity of the α-subunit bands of a series of diluted gastric vesicle preparations was plotted against the content of the α-subunit as a standard curve. The content of α-subunit in membrane fractions was estimated from the linear range of the standard curve of the gastric vesicle preparation as described previously (26).

**Assay of K⁺-ATPase Activity—K⁺-stimulated ATPase (K⁺-ATPase) activity** was measured in 1 ml of a solution containing 50 μg of membrane protein, 3 mM MgCl₂, 1 mM ATP, 5 mM NaF, 2 mM ouabain, and 40 mM Tris-HCl, pH 7.4, in the presence and absence of 15 mM KCl. After incubation at 37°C for 30 min, the inorganic phosphate released was measured as described elsewhere (29). The K⁺-ATPase activity was calculated as the difference between the activities in the presence and absence of KCl. Specific K⁺-ATPase activities observed in the membrane fractions of the stable cell lines were calculated by subtracting the K⁺-ATPase activity observed in mock cells and expressed in micromoles of Pi/milligrams of the α-subunit/hour. Protein was measured using the BCA protein assay kit from Pierce with bovine serum albumin as a standard.

**Rubidium Transport Assay**—The ⁸⁶Rb transport assay was performed as described previously (26). One day before the transport assay, cells were seeded in two sets of 6-well collagen type I-coated plates (Asahi Technoglass), one set for the ⁸⁶Rb transport assay and the other set for counting of cell number, and they were grown until confluence. For the ⁸⁶Rb transport assay, the cells were washed with 2 ml of ice-cold wash solution containing 144 mM NaCl and 5 mM HEPES-NaOH, pH 7.4. Cells in each well were incubated in 1 ml of a solution containing 144 mM NaCl, 5 mM HEPES, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 1 mM RbCl (3 x 10⁻³⁵ cm³⁻¹⁰⁴ Rb), 500 μM ouabain, and 10 μM furosemide at 37°C for 10 min. Rubidium transport was assayed in the presence and absence of various concentrations of SCH 28080 or SPI-447. After 10 min of incubation, the supernatant was removed, and each well was washed with 2 ml of ice-cold wash solution. Cells were solubilized with 2 ml of lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4. One milliliter of solution was mixed with 5 ml of an aqueous counting scintillant, ACS-II (Amersham Biosciences), and the radioactivity was counted. The ⁸⁶Rb transport activity was normalized by the number of the cells expressed in nanomoles of Rb₁₀⁶ cells/milliliter. In the case of omeprazole treatment, the cells were incubated with different concentrations of omeprazole in 144 mM NaCl, 0.5 mM MgCl₂, 5 mM glucose, and 2 mM HEPES, pH 5.8, at 37°C before the transport assay.

**Construction of M5 Mutants of the Gastric Proton Pump**

Amino acid Asp₅₉, with the predicted flexible region of M1 in the gastric proton pump, was mutated to Asp₅₉ (data not shown). The Tyr801 mutant was inhibited by only 20%. This Tyr801 residue is located near the luminal end of the M₅ transmembrane segment. In the immunofluorescence study, a major tyrosine was visualized with the ECLplus kit (Amersham Biosciences). The resulting alignments of M1 are identical to those previously reported by Sweadner and Donnet (32) and Zhang et al. (33). The three-dimensional models (E₅ form and E₄ form) of gastric proton pump α-subunit were constructed using the MODELLER9V2 program (34) based on the atomic model of the Ca²⁺-bound form (E₅ form, Protein Data Bank code 1EU1) and the thapsigargin-bound form (E₄(TG) form, Protein Data Bank code 1WIO) of rabbit SR Ca²⁺ pump, respectively, with restriction of partial structures. Structures of long insertion (Gly₂-Met₆ and Arg₁₀-Glu₅₁) were restricted to the predicted structures obtained by PredictProtein (35). Energy minimization was then carried out using the AMBER7 program (36). Identification and visualization of cavities within an obtained model were calculated by the PASS program (37). These models were visualized by the Turbo-Frodo program (38). All calculations were done using a Silicon Graphics OCTANE graphic work station.

**Docking of Inhibitors to the Gastric Proton Pump α-Subunit**—The docking of inhibitors to the α-subunit was performed by the AutoDock3.0 and AutoDockTools programs (39, 40). The three-dimensional coordinates of the inhibitor that has the least docked energy was chosen as the docked structure.

**RESULTS**

**Construction of M5 Mutants of the Gastric Proton Pump α-Subunit**—Table I shows the expression level of the mutant α-subunit and the K⁺-ATPase activity found in the membrane preparations from cells transiently co-expressing the mutant α-subunit and the wild-type β-subunit. The K793A mutant showed very low or no K⁺-ATPase activity as reported previously (22, 41). The Y798A mutant also lost the K⁺-ATPase activity. Alanine mutants of Asn⁷⁹⁴ and Glu⁷⁹⁷, which likely coordinate the transporting cation, showed low K⁺-ATPase activity as reported previously (9, 22). Among them, it is very interesting that the Y801A mutant showed low sensitivity to SCH 28080, although this mutant retained K⁺-ATPase activity almost comparable to that found in the wild type. Even in the presence of 50 μM SCH 28080, the K⁺-ATPase activity of this mutant was inhibited by only 20%. This Tyr⁸⁰¹ residue is located near the luminal end of the M₅ transmembrane segment (Fig. 2).

**Tyrosine as the Binding Site of SCH 28080**—To precisely study the role of Tyr⁸⁰¹ in the interaction with SCH 28080, stable cell lines expressing the Y801A, Y801S, Y801L, and Y801F mutants were constructed, and the ⁸⁶Rb transport activity of these mutants was measured. In the immunofluorescence study, a major portion of these mutant α-subunits was expressed at the cell surface, and there was no apparent difference in the intracellular distribution between the wild-type α-subunit and the Tyr⁸⁰¹ mutant (data not shown).

The cell lines expressing the Y801A mutant retained the

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**Table I**

| Mutant | Expression of α-subunit | K⁺-ATPase activity | SCH 28080-sensitive K⁺-ATPase activity |
|--------|-------------------------|--------------------|----------------------------------------|
| Wild type | 17.1 ± 1.9 | 91.1 ± 10.0 | 87.8 ± 8.0 |
| Y798A | 12.0 ± 0.5 | 1.5 ± 2.4 | 0.7 ± 2.2 |
| T790A | 10.3 ± 1.5 | 71.4 ± 9.9 | 69.8 ± 11.0 |
| T791A | 10.8 ± 2.3 | 19.4 ± 2.2 | 19.6 ± 7.1 |
| L792A | 9.9 ± 2.7 | 56.9 ± 1.1 | 55.0 ± 5.0 |
| K793A | 14.4 ± 2.3 | 2.8 ± 1.2 | 3.5 ± 1.4 |
| N794A | 14.8 ± 3.9 | 6.3 ± 2.1 | 6.6 ± 2.6 |
| I795A | 9.9 ± 2.9 | 53.8 ± 6.0 | 45.9 ± 7.9 |
| T796A | 17.1 ± 2.9 | 40.4 ± 6.8 | 39.0 ± 6.1 |
| E797A | 12.0 ± 11 | 11.7 ± 4.0 | 1.3 ± 4.2 |
| L798A | 8.1 ± 1.7 | 65.3 ± 17.7 | 61.8 ± 12.4 |
| T799A | 20.2 ± 4.6 | 72.0 ± 16.7 | 70.8 ± 15.8 |
| P800A | 17.0 ± 1.4 | 50.3 ± 6.3 | 47.8 ± 6.6 |
| Y801A | 11.8 ± 1.3 | 69.0 ± 12.4 | 13.8 ± 3.3 |
| L802A | 11.1 ± 1.3 | 42.1 ± 7.3 | 37.3 ± 6.7 |
| I803A | 11.8 ± 1.6 | 57.9 ± 11.7 | 54.3 ± 14.0 |
| Y804A | 7.6 ± 1.6 | 29.6 ± 9.5 | 27.0 ± 8.2 |
| I805A | 10.3 ± 1.5 | 48.3 ± 9.3 | 42.3 ± 11.5 |
| T806A | 11.8 ± 7.7 | 68.5 ± 17.0 | 69.5 ± 15.7 |
| V807A | 18.7 ± 6.0 | 63.6 ± 22.3 | 69.3 ± 26.1 |
| S808A | 11.0 ± 3.6 | 83.3 ± 18.7 | 83.5 ± 17.6 |
| V809A | 7.3 ± 0.9 | 32.3 ± 7.4 | 35.0 ± 9.0 |

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*H. Ogawa and C. Toyoshima, unpublished data.*
$^{86}$Rb transport activity (0.88 nmol/10^6 cells/min), which was almost identical to that found in the cells expressing the wild-type gastric proton pump (0.88 nmol/10^6 cells/min). The cell line expressing the Y801S mutant showed a lower $^{86}$Rb transport activity (0.45 nmol/10^6 cells/min), whereas the cell lines expressing the Y801L and Y801F mutants showed higher $^{86}$Rb transport activities (1.04 and 1.72 nmol/10^6 cells/min, respectively) than the cells expressing the wild type.

Mutants Y801A and Y801S showed much lower sensitivity to SCH 28080 than the wild type as shown in Fig. 3. The IC_{50} values of the wild type and mutants Y801A and Y801S were 1.2, 69, and 83 μM, respectively. The $^{86}$Rb transport activities found in these mutants were not completely inhibited even in the presence of 100 μM SCH 28080. Mutants Y801F and Y801L showed slightly lower sensitivity to SCH 28080 than the wild type. The IC_{50} values of mutants Y801F and Y801L were 3.1 and 5.5 μM, respectively. The finding that the Y801F mutant showed slightly lower sensitivity to SCH 28080 than the wild type is in good agreement with that reported by Vagin et al. (24). They reported that this mutant showed 1.4-fold lower sensitivity to SCH 28080 in the NH$_4$-stimulated ATPase activity. The behavior of the Tyr801 mutants to SCH 28080 in the inhibition of K$^+$-ATPase activity was comparable to that in the inhibition of $^{86}$Rb transport.

Non-gastric isoforms of H$^+$/K$^+$-ATPase are expressed in distal colon and kidney and are involved in K$^+$ reabsorption as well as acid secretion. They show lower or no sensitivity to SCH 28080. A human non-gastric H$^+$/K$^+$-ATPase, the ATP1AL1 gene product, showed lower sensitivity to SCH 28080 compared with the gastric proton pump, H$^+$/H$^+$/K$^+$-ATPase. The $^{86}$Rb transport activity of the ATP1AL1 gene product expressed in HEK-293 cells was inhibited by a high concentration of SCH 28080.
containing 144 mM NaCl, 5 mM HEPES-NaOH (pH 7.4), 0.5 mM MgCl2, collagen type I-coated plates were incubated in 1 ml of a solution.

Specific K<sup>i</sup>/H<sub>9251</sub> were 188, 163, 134, 58, and 72 for the Y801A (<i>E</i>c141) mutants. Y801L (<i>E</i>c141) and the lines expressing the wild-type gastric proton pump (<i>E</i>c141) and Y801F (<i>E</i>c141) and the Y801A (<i>E</i>c141) mutants.

The cells lines grown to confluence in 6-well collagen type I-coated plates were incubated in 1 ml of a solution containing 144 mM NaCl, 5 mM HEPES-NaOH (pH 7.4), 0.5 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, 1 mM RubCl (3 × 10<sup>5</sup> cpn 8<sup>6</sup>Rb), 500 µM ouabain, and 10 µM furosemide in the presence and absence of various concentrations of SCH 28080 at 37 °C for 10 min. The radioactivity of the cell lysate was counted, and the <sup>86</sup>Rb transport activities were expressed as nanomoles of <sup>86</sup>Rb transported/10<sup>6</sup> cells in 1 min. Specific <sup>86</sup>Rb transport activity was calculated by subtracting the <sup>86</sup>Rb transport activity observed in mock cells and expressed as the percentage of the control values measured in the absence of SCH 28080. The values are mean ± S.E. for four experiments.

![Effects of SCH 28080 concentrations on the <sup>86</sup>Rb transport activities of the wild-type gastric proton pump (●) and the Y801A (●), Y801F (●), Y801L (●), and Y801S (●) mutants. The K<sup>+</sup>-ATPase activities were measured as a function of SCH 28080 concentrations. Specific K<sup>+</sup>-ATPase activities were calculated by subtracting the K<sup>+</sup>-ATPase activity observed in mock cells and expressed as the percentage of the control values measured in the absence of SCH 28080. Control values of K<sup>+</sup>-ATPase activities in the absence of SCH 28080 were 188, 163, 134, 58, and 72 umol/mg of ω-subunit/h for the wild type and mutants Y801A, Y801F, Y801L and Y801S, respectively. The values are mean ± S.E. for 4-11 experiments.](image1)

![Effects of Rb<sup>+</sup> concentrations on the <sup>86</sup>Rb transport activities of the wild-type and the Y801A mutant. The <sup>86</sup>Rb transport activities were corrected for specific activity of <sup>86</sup>Rb transport by subtracting the <sup>86</sup>Rb transport of the mock cells from that of each cell. The results are shown as the double reciprocal plots between the <sup>86</sup>Rb transport and Rb<sup>+</sup> concentrations. The line for the wild type was almost identical with that for the Y801A mutant.](image2)

![Effects of SPI-447 concentrations on the <sup>86</sup>Rb transport activities of the wild-type gastric proton pump (●) and the Y801A (●) and Y801F (●) mutants. Specific <sup>86</sup>Rb transport activity was calculated by subtracting the <sup>86</sup>Rb transport activity observed in mock cells and expressed as the percentage of the control values measured in the absence of SPI-447. The values are mean ± S.E. for four experiments.](image3)

with the <i>K</i><sub>i</sub> value of 42 µM (25). Therefore, the sensitivity of the Y801A and Y801S mutants of the gastric proton pump to SCH 28080 is almost comparable to that found in the ATP1AI gene product. This tyrosine residue, Tyr<sup>801</sup>, in the M5 segment is well conserved in the gastric proton pump among different animal species. In the Na<sup>+</sup>-K<sup>+</sup>-ATPases (<i>α</i>1, <i>α</i>2, and <i>α</i>3) and the non-gastric isoforms of H<sup>+</sup>-K<sup>+</sup>-ATPase, the corresponding amino acid is a phenylalanine. However, the present results indicate that the presence of hydroxyl moiety in the side chain of Tyr<sup>801</sup> is not critical for the interaction with SCH 28080; rather the bulkiness of the side chain at this position seems to be important for the interaction.

The present results suggest that the side chain of Tyr<sup>801</sup> forms a major part of the binding site of SCH 28080. However, it cannot be completely excluded that the mutation from tyrosine to alanine shifted the conformational equilibrium between the E<sub>1</sub> and E<sub>2</sub> forms toward the E<sub>1</sub> form. In the E<sub>1</sub> form, the enzyme shows high affinity for proton, whereas in the E<sub>2</sub> form, the cation binding site faces the luminal bulk solution, and the enzyme shows high affinity for K<sup>+</sup> and its analogous ion, Rb<sup>+</sup>. SCH 28080 specifically binds to the E<sub>0</sub> and E<sub>2</sub>P forms in a K<sup>+</sup>-competitive manner (42). To check the possibility mentioned above, we compared the affinity of the wild type and the Y801A mutant for Rb<sup>+</sup> in the <sup>86</sup>Rb transport assay as shown in Fig. 5. The <i>K</i><sub>a</sub> value for Rb<sup>+</sup> of the Y801A mutant was 0.85 mM, which was almost comparable to that found in the wild type (0.76 mM). Therefore, the mutation of Tyr<sup>801</sup> to alanine does not shift the conformational equilibrium of the gastric proton pump; rather it is more likely that the side chain of Tyr<sup>801</sup> is a part of the binding site of SCH 28080.

**Tyr<sup>801</sup> as the Binding Site of SPI-447**—SPI-447 is another member of the APAs, and its manner of inhibition is also K<sup>+</sup>-competitive. The inhibitory effect of SPI-447 on the K<sup>+</sup>-ATPase activity found in native gastric enzyme is almost comparable to that of SCH 28080 (16). Here we studied whether Tyr<sup>801</sup> is also involved in determining the sensitivity to SPI-447. The inhibitory effect of SPI-447 on the <sup>86</sup>Rb transport activity found in the cell line expressing the wild-type proton pump was slightly weaker than that of SCH 28080. The IC<sub>50</sub>
Fig. 7. Effects of omeprazole concentrations on the \(^{86}\)Rb transport activities of stable cell lines expressing the wild-type gastric proton pump (●) and the Y801A (△) and Y801F (□) mutants. The cell lines grown to confluence in 6-well collagen type I-coated plates were preincubated with various concentrations of omeprazole in 1 ml of a solution containing 144 mM NaCl, 0.5 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 5 mM glucose, and 2 mM HEPES-NaOH (pH 5.8) at 37°C for 20 min. After removal of this solution, \(^{86}\)Rb transport activities of the cells were studied.

Fig. 8. Side view of the gastric proton pump α-subunit in the E\(_1\) (left) and E\(_2\) forms (right) by ribbon representation. Red and blue ribbons show α-helices and β-sheets, respectively.

value of SPI-447 was 2.7 \(\mu\)M. The cell line expressing the Y801F mutant showed slightly lower sensitivity to SPI-447 than the cell line expressing the wild type with the IC\(_{50}\) value of 6.1 \(\mu\)M. On the other hand, the cell line expressing the Y801A mutant showed much lower sensitivity to SPI-447 with the IC\(_{50}\) value of 47 \(\mu\)M (Fig. 6). These results suggest that Tyr\(^{801}\) is also important for the binding to SPI-447. It is also noteworthy that the contribution of Tyr\(^{801}\) to the interaction with SCH 28080 is larger than that to the interaction with SPI-447 because the ratio of the IC\(_{50}\) value of SCH 28080 between the wild type and mutant Y801A was higher than that of SPI-447 (58 and 17, respectively).

Tyr\(^{801}\) is Not Involved in the Binding of Omeprazole—Cys\(^{815}\) is the binding site of omeprazole (17) and it is reported to be involved in the interaction with SCH 28080 also. The C815T mutant showed a 5-fold higher \(K_i\) value for SCH 28080 in the NH\(_4\)\(_+_\)-stimulated ATPase activity (22). SCH 28080 was reported to prevent the irreversible inhibition of acid transport by omeprazole (43). Therefore, the binding site of SCH 28080 seems to be partially overlapped with that of omeprazole. Here we studied the role of Tyr\(^{801}\) on the inhibitory effect of omeprazole. As shown in Fig. 7, \(^{86}\)Rb transport activity of the stable cell line expressing the wild-type proton pump was inhibited by omeprazole treatment in a concentration-dependent manner with an IC\(_{50}\) value of 8.3 \(\mu\)M. Omeprazole also inhibited \(^{86}\)Rb transport activity of the stable cell lines expressing the Y801A and Y801F mutants with IC\(_{50}\) values of 7.2 and 7.2 \(\mu\)M, respectively. That is, these IC\(_{50}\) values of the mutants are not significantly different from that of the wild type. These results indicate that the side chain of Tyr\(^{801}\) is not directly involved in the binding to omeprazole and its acid-activated form.

DISCUSSION

In this study, we showed that the side chain of Tyr\(^{801}\) is very important for determining the sensitivity to SCH 28080 as well as SPI-447. The mutations of Tyr\(^{801}\) to alanine and serine significantly reduced the sensitivity to these APAs. Here we will discuss the structure of the binding site(s) of these inhibitors in the putative three-dimensional structure of the gastric proton pump α-subunit based on the present as well as the previous mutational results.

Recent reports have described precise three-dimensional structures of the E\(_1\) (in the presence of Ca\(^{2+}\)) (Protein Data Bank code 1EUL) and the E\(_2\) (in the absence of Ca\(^{2+}\) and presence of thapsigargin) (Protein Data Bank code 1WDO) forms of rabbit SR Ca\(^{2+}\) pump using x-ray crystallography analysis at 2.6- and 3.1-Å resolutions, respectively (44, 45). The Ca\(^{2+}\) pump contains 10 transmembrane segments. The Ca\(^{2+}\) binding sites (sites I and II) are located in these transmembrane segments. The cytoplasmic part is composed of three separate domains designated the actuator (A), nucleotide binding (N), and phosphorylation (P) domains. The cytoplasmic domains as well as 6 of 10 transmembrane segments (M1–M6) undergo drastic rearrangements in the conformational change from the E\(_1\) to E\(_2\) forms. The A, N, and P domains are well separated in the E\(_1\) form, whereas they form a compact headpiece in the E\(_2\) form as a result of their rotation and inclination. The inclination of the P domain is directly related to tilting of the transmembrane segments, especially of the M5 segment. Thus, the conformational change in the cytoplasmic domains is conducted or linked to the movements of the transmembrane segments, resulting in the conformational change in the luminal domains.

On the other hand, unfortunately, no high resolution crystal structure of gastric proton pump has been presented yet. Therefore, here we constructed three-dimensional structure models of the E\(_1\) and E\(_2\) forms of the gastric proton pump α-subunit based on the present as well as the previous mutational results.

SCH 28080 specifically binds to the E\(_2\) or E\(_2P\) forms of the gastric proton pump α-subunit by homology modeling based on the three-dimensional structures of SR Ca\(^{2+}\) pump (Fig. 8). The overall amino acid identity between the rabbit gastric proton pump α-subunit and rabbit SR Ca\(^{2+}\) pump is 29%. The constructed models of the E\(_1\) and E\(_2\) forms of the gastric proton pump α-subunit were similar to those of the SR Ca\(^{2+}\) pump (data not shown). The model of the E\(_1\) form of the gastric proton pump α-subunit was apparently comparable to the model previously presented by Sweadner and Donnet (32). We studied the binding site of the APAs on these models.

SCH 28080 specifically binds to the E\(_2\) or E\(_2P\) forms of the gastric proton pump as reported from the kinetic study (42). Fig. 9 shows that, in the E\(_2\) form of the gastric proton pump, Tyr\(^{801}\) in the luminal half of the M5 segment is located at the upper surface of a cavity surrounded by the transmembrane segments M1, M4, M5, M6, and M8 and the extracellular M5/M6, M7/M8, and M9/M10 loops. The cavity is separately located from the cation binding sites. Both SCH 28080 and SPI-447 can dock in this cavity as shown in Fig. 9. This cavity was surrounded by several amino acid residues, which were reported to be involved in determining the sensitivity to SCH 28080 so far. Leu\(^{813}\) at the edge of the M5/M6 loop is located at the surface of this cavity. The L811F and L811V substitutions...
resulted in a 90- and 4.5-fold decrease in the affinity for SCH 28080, respectively (24). According to this result, a bulky side chain at this residue seems to hinder the interaction with SCH 28080, which is well explained in our three-dimensional model. Met336 in the M4 segment and Cys815 in the M6 segment are located at the top surface of this cavity. Both the M336I and C815T substitutions resulted in only a 9-fold decrease in the affinity for SCH 28080 (21, 23). These substitutions do not significantly change the bulkiness of the side chain. Therefore, they have a small influence on the structure of the cavity. This explains well why the 10-fold reductions in the SCH 28080 sensitivity of the C815T and M336I mutants were smaller than those of mutants Y801A, Y801S, and L811F. Although the K793S and E797D mutants showed a 12–20-fold higher $K_i$ value for SCH 28080 (22), Lys793 and Glu797 in the M5 segment do not face this cavity. These two amino acids seem to be involved in the interaction with the transporting ions ($K^+$) rather than the binding to SCH 28080.

Farley et al. (46) reported that the Gln907–Gln912 peptide in the M7/M8 loop is involved in the interaction with SCH 28080. This loop is not close to the cavity reported in the present study. The M7/M8 loop is involved in the association with the $\beta$-subunit. Therefore, it cannot be excluded that the structure of this loop changed by the interaction with the $\beta$-subunit. However, at present, it is difficult to explain their finding in our model.

In this study, we replaced Tyr801 with several amino acid residues containing different side chains: alanine, serine, leucine, and phenylalanine. It is very interesting that the Y801F and Y801L mutants showed the sensitivity to SCH 28080, which was slightly lower than that of the wild type, whereas the Y801A and Y801S mutants showed much lower sensitivity. These results indicate that an aromatic side chain at this position is not essential for the interaction with SCH 28080; rather a bulky side chain at this position seems to support the cavity structure itself. Therefore, the behavior of the Tyr801 mutants is opposite to that of the Leu811 mutants.

In the conformational change from the $E_1$ to $E_2$ forms, the tilt of the M3/M4 loop is expected. Docking of SCH 28080 or SPI-447 seems to inhibit this process, resulting in the stabilization of the $E_2$ form as reported in the binding and kinetics analysis (42). It is also very interesting that the tilting of the M3 and M4 segments resulted in the change of the shape of the cavity when the conformation was changed from the $E_2$ to $E_1$ forms. Therefore, in the $E_1$ form, the inhibitors cannot dock in the same location as in the $E_2$ form (Fig. 9).

Cys125 in the M6 segment was reported to be the binding site of pump inhibitors as well as a part of the binding site of SCH 28080. In fact, omeprazole inhibition of gastric proton pump was partly prevented by SCH 28080 in the gastric microsomal preparation (43). On the other hand, as reported in the present study, the side chain of Tyr801 is not involved in the interaction with omeprazole. These results suggest that the binding site of SCH 28080 is partially but not completely overlapped with that of omeprazole.

In conclusion, we identified Tyr801 as a major part of the binding site of APAs especially for SCH 28080. The Y801A and Y801S mutants retained the activity and showed much lower sensitivity to SCH 28080 compared with the wild type. The sensitivity to SCH 28080 was dependent on the bulkiness of the side chain of the amino acid at this position.

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Fig. 9. Side view of the luminal half of the gastric proton pump $a$-subunit in the $E_1$ form (left) and the $E_2$ forms docked with SCH 28080 (center) and SPI-447 molecules (right), respectively. Docking models were constructed using the AutoDock3.0 program. Tyr801, Leu811, Cys815, and Met336 are located at the top surface of the luminal cavity found in the $E_2$ form.
Cavity Structure of the Docking Site in Gastric Proton Pump

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The Cavity Structure for Docking the K⁺-competitive Inhibitors in the Gastric Proton Pump
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