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THE PROTON PUMP INHIBITOR, E3810, BINDS TO THE N-TERMINAL HALF OF THE α-SUBUNIT OF GASTRIC H^+,K^+-ATPase

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Abstract—E3810 (2-{[4-(3-methoxypropoxy)-3-methylpyridine-2-yl]methylsulphinyl}-1*H*-benzimidazole sodium salt), an inhibitor of gastric proton pump (gastric H^+,K^+-ATPase), is activated in a luminal acidic environment of gastric glands and binds to a Cys residue of H^+,K^+-ATPase on its luminal side. It was found that bound E3810 is transformed into a strongly fluorescent compound by UV-light irradiation (excitation wavelength = 335 nm, emission wavelength = 470 nm). The location of Cys residue bound with E3810 in the α-subunit of hog gastric H^+,K^+-ATPase was estimated from the fluorescence labelling and limited tryptic digestion of the enzyme. Tryptic digestion in the presence of Mg-ATP produces N-terminal 67 kDa subfragment which contains the phosphorylation and fluorescein 5′-isothiocyanate binding sites and C-terminal 35 kDa subfragment. Trypsin digestion in the presence of KCl produces N-terminal 42 kDa and C-terminal 56 kDa subfragments. E3810 was found to bind to both N-terminal but not to any of two C-terminal subfragments. Taking the amino acid sequence and topology of this ATPase as well as the fact that the ratio of specific binding sites per α-subunit is one into consideration, the possibility that E3810 specifically binds to Cys^{322} residue of hog gastric H^+,K^+-ATPase is discussed.

Key words: E3810; gastric H^+,K^+-ATPase; proton pump inhibitor; omeprazole; limited tryptic digestion

It has been shown that E3810† is a potent gastric H^+,K^+-ATPase inhibitor [1, 2] similar to omeprazole [3–7], both of which are prodrugs and activated in the acidic lumen of the gastric parietal cell. The acid-activated E3810 modifies a Cys residue of gastric H^+,K^+-ATPase, resulting in inhibitions of K^+-simulated ATPase activity and proton transport across the apical membrane of the parietal cell or the gastric vesicle membrane [8–10]. E3810 was 6.5-times more potent than omeprazole, another H^+,K^+-ATPase inhibitor, in inhibiting the K^+-ATPase activity of isolated gastric vesicles [1]. The acid antisecretory effect of E3810 was slightly greater than that of omeprazole in histamine-stimulated fistula dogs *in vivo*, and the duration of the antisecretory activity of E3810 at doses of 2 and 4 mg/kg was significantly less than that of omeprazole at the same doses in pentagastrin-stimulated fistula dogs [2]. We previously reported that the biochemical inhibition mechanism of H^+,K^+-ATPase by E3810 was different from that by omeprazole; the partial reaction affected most differently by inhibitors was luminal K^+-dependent phosphorylation for E3810 and the conformational change from the E_2 to E_1 form for omeprazole [10].

In this study, we determined the E3810 binding domain in the α-subunit of gastric H^+,K^+-ATPase. Two facts are taken into consideration: (i) binding of E3810 to H^+,K^+-ATPase does not affect the conformation since the conformational transition from the E_1 ATP form to the E_2 K^+ form is not inhibited [10]; and (ii) Van Uem *et al.* [11] and the authors [12] have shown that limited tryptic digestion of intact membrane-bound H^+,K^+-ATPase produces N-terminal 67 kDa and C-terminal 35 kDa subfragments in the presence of Mg-ATP and N-terminal 42 kDa and C-terminal 56 kDa subfragments in the presence of K^+. These facts enabled us to determine the E3810 binding domain by using limited tryptic digestion together with a new fluorescence labelling technique developed in this study.

MATERIALS AND METHODS

Chemicals and drugs. E3810 was obtained from Eisai Co. (Tokyo, Japan). TPCK-treated trypsin and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Other chemicals used were of highest purity available.

Preparation of hog gastric vesicles. Tightly sealed membrane vesicles containing H^+,K^+-ATPase were prepared from hog stomachs as described previously [13]. Gastric vesicles in 250 mM sucrose solution were stored at −85° and used within 1 month. Protein concentration was determined by the method of Lowry *et al.* with BSA as a standard [14].

Specific binding of E3810 to H^+,K^+-ATPase. The intravesicular space of gastric vesicles mimics the acidic lumen of gastric gland and was acidified when Mg-ATP was added to the vesicle solution containing

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† Abbreviations: E3810, 2-{[4-(3-methoxypropoxy)-3-methylpyridine-2-yl]methylsulphinyl}-1*H*-benzimidazole sodium salt; FITC, fluorescein 5′-isothiocyanate; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

valinomycin and a high concentration of K^+. For this purpose, gastric vesicles (2 mg/mL) were incubated with 10 mM Mg-ATP in a solution containing 5 mM $MgCl_2$, 150 mM KCl, 50 μg/mL valinomycin, 40 mM Tris/HCl (pH 7.40) for 10 min. Then 100 μM E3810 was added. E3810 was accumulated in the acidic intravesicular space, activated by acid and bound to Cys residue of H^+,K^+-ATPase from the luminal side [10]. After 60 min incubation at room temperature, the sample solution (1 mL) was passed through a Sephadex G-50 column equilibrated with 40 mM Tris/HCl (pH 7.40) to remove KCl, $MgCl_2$, Mg-ATP and unreacted E3810. Under these conditions, K^+-ATPase activity was inhibited by 49%, indicating that labelling was achieved under half-maximal inhibitory conditions. When E3810 was added before the addition of Mg-ATP, a much greater inhibition of enzyme activity was obtained, but this method was not employed in this study. As shown later, the acid-activated E3810 has strong absorbance at 335 nm. By measuring the absorbance, we found that E3810 incubated in a vesicle-free buffer solution at pH 7.40 did not produce the acid-activated form. This result indicates that acid activation of E3810 occurred only in the intravesicular space under the above conditions and the inhibition of the pump was due to the binding of E3810 from the luminal side of the pump.

Limited tryptic digestion of H^+,K^+*-ATPase.* E3810-labelled H^+,K^+-ATPase was digested with TPCK-treated trypsin following the method of Helmich-de Jong *et al.* [15]. A sample solution containing gastric vesicle (1.2–1.5 mg of protein/mL), 1 mM EDTA, 1 M glycerin, 40 mM Tris/HCl (pH 7.40) and 5 mM Mg-ATP (ATP form) or 100 mM KCl (K^+ form) was pre-incubated for 10 min at 37°. Then, 20–31 μg/mL of TPCK-treated trypsin was added. An appropriate digestion time that gives significant N- and C-terminal subfragments was chosen from trial experiments by evaluating the SDS-PAGE cleavage pattern [12]. Digestion was stopped by addition of 200–310 μg/mL of soyabean trypsin inhibitor, and the digested enzyme was frozen by liquid nitrogen and stored at −25° until use.

Labelling of H^+,K^+*-ATPase with fluorescent E*3810. Transformation of Cys-bound E3810 into a fluorescent molecule was undertaken by irradiation with UV-light (335 nm) in a spectrofluorometer (Hitachi 650-10S: with 150 W xenon lamp) equipped with a magnetic stirrer at a maximum slit width of excitation light (20 nm) for 1 hr at 25°. Free or unbound activated E3810 did not change into the fluorescent compound upon UV irradiation.

Measurement of K^+*-ATPase activity.* K^+-activated ATPase activity was measured in a pyruvate kinase–lactate dehydrogenase linked reaction where hydrolysis of ATP is coupled with oxidation of NADH [10]. The reaction mixture contained 10 μg of protein/mL of gastric vesicles, 40 mM Tris/HCl (pH 7.40), 150 mM KCl, 10 μg/mL of valinomycin, 2 mM $MgCl_2$, 0.16 mM NADH, 0.8 mM phosphoenolpyruvate, 3 U/mL pyruvate kinase, 2.75 U/mL lactate dehydrogenase and various concentrations of ATP. The decrease in the amount of NADH was measured by an Aminco DW-2C UV–Vis spectrophotometer in a dual wavelength mode at 340 and 500 nm at 25°. ATPase activity was calibrated by the addition of a known amount of ADP to the reaction mixture. Mg^{2+}-ATPase activity has usually been measured in K^+-free solution, but K^+-free condition is not available in the coupled enzyme method because K^+ (at least 0.5 mM) is necessary for pyruvate kinase reaction. A K^+-competitive H^+,K^+-ATPase inhibitor, SCH 28080, completely inhibited K^+-ATPase activity [16] and 38% of Mg-ATPase activity [17]. On the basis of these facts, we measured the ATPase activity in the presence of 2 mM $MgCl_2$, 1 mM KCl, 10 μM SCH 28080, and 250 mM sucrose and in the absence of valinomycin; the remaining ATPase activity was assumed to be equal to 62% of the Mg^{2+}-ATPase activity. The Mg^{2+}-ATPase activity was comparable to the value measured by the method of Yoda–Hokin [18]. K^+-ATPase activity was defined as the difference between the Mg^{2+} plus K^+-ATPase activity and the Mg^{2+}-ATPase activity.

SDS-PAGE of H^+,K^+*-ATPase labelled with fluorescent E*3810. SDS-PAGE of the tryptic digested H^+,K^+-ATPase was performed using a Tris-glycine-SDS buffer and 10 or 7.5% polyacrylamide gel following the method of Laemmli [19]. For the sample digested with trypsin in the presence of K^+, the SDS-solubilized protein solution was warmed up to 40° for solubilization of precipitated potassiumdodecylsulphate. SDS-solubilized samples (50 μg) were applied to two wells in the gel. β-mercaptoethanol was omitted during electrophoresis to avoid dissociation of bound E3810 from the Cys residue of H^+,K^+-ATPase. The absence of β-mercaptoethanol did not alter R_f-values of tryptic subfragments, but the bands diffused considerably. One lane of the gel was stained with Coomassie brilliant blue R-250, and the peptide distribution was assayed in a densitometer. The other lane was fixed with destaining solution containing 25% ethanol and 7% acetic acid and sliced into 3 mm wide pieces. The fluorescence intensity of each sliced gel in distilled water was directly measured by a spectrofluorometer (Ex = 335 nm, Em = 470 nm). The net fluorescence intensity of the labelled tryptic subfragments was obtained by subtracting the basal level from the apparent fluorescence intensity.

RESULTS

Transformation of SH*-bound E*3810 *to a fluorescent E*3810 *by UV-light irradiation*

We have found that omeprazole and E3810 transform into fluorescent compounds under several different conditions [8, 20, 21]. For example, acid-activation of omeprazole produces the cyclic sulphenamide derivative [3–6], which is a fluorescent compound (Ex = 370 nm, Em = 560 nm) [20]. Because this fluorescence disappears when it reacts with a sulphydryl group, we cannot use this fluorescence property for fluorescence labelling of gastric H^+,K^+-ATPase. Free and SH-bound omeprazole are transformed into a strongly fluorescent compound by irradiation with UV-light (270 nm) (Ex = 270 nm, Ex = 370 nm) [21]. This

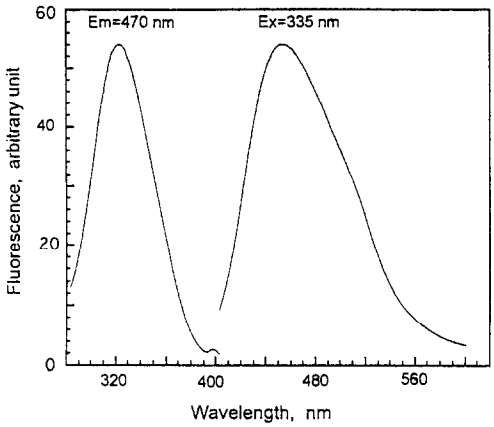

Fig. 1. The excitation (left) and emission spectra (right) of SH-bound E3810 irradiated with UV-light. Under UV-light irradiation (335 nm at slit width = 20 nm; 150 W xenon lamp), 10 μM E3810 was incubated in a solution containing 0.1 M HCl and 100 μM GSH for 60 min at 25°. Then, the excitation spectrum at Em = 470 nm and the emission spectrum at Ex = 335 nm were measured.

property was used for fluorescence labelling of gastric H^+,K^+-ATPase by omeprazole, and the binding site of omeprazole in hog gastric H^+,K^+-ATPase was estimated [21]. In the experiment, since the emission wavelength is near the intrinsic fluorescence of tryptophan (340 nm), an additional process to dissociate fluorescent omeprazole from tryptic subfragments of H^+,K^+-ATPase was necessary. Although E3810 also converted to a fluorescent molecule under the same conditions, we did not use this method in this study.

We found that SH-bound E3810 was converted to another strong fluorescent compound by irradiation with a different wavelength of UV-light (335 nm). This wavelength is the same as that of the acid-activated cyclic sulphenamide derivative of E3810 (335 nm). In the experiment shown in Fig. 1, E3810 (10 μM) was activated in 0.1 M HCl and reacted with 100 μM GSH under irradiation of UV-light (335 nm) by using a spectrofluorometer at 25° for 60 min; excitation and emission spectra were then measured. In the absence of GSH, acid activation and UV irradiation of E3810 did not produce a fluorescent compound (data not shown). In contrast, acid-activated, SH-bound omeprazole did not convert to a fluorescent compound under the present conditions.

Figure 2 shows the time course of increase in the fluorescence of the enzyme-bound E3810 upon UV-light irradiation. Before irradiation, the vesicles had been incubated with 100 μM E3810 under the specific labelling conditions as described in the Experimental section and then incubated with trypsin in the presence of Mg-ATP or K^+, followed by the addition of trypsin inhibitor. UV-light irradiation increased emission at 470 nm with time both in ATP and K^+ form preparations. The increase of fluorescence of undigested control vesicles that had not been treated with E3810 was very small compared with those of E3810-labelled vesicles (Fig. 2).

Identification of the E3810-bound tryptic subfragments

The E3810-bound subfragment of H^+,K^+-ATPase

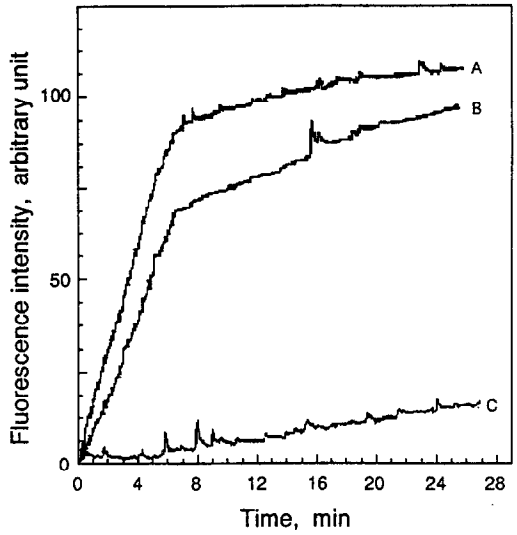

Fig. 2. Conversion to a fluorescent E3810 by UV-light irradiation. E3810-bound gastric vesicles (1.3 mg/mL) were digested with 20 μg/mL trypsin for 60 min at 37° in E_2 (A) or E_1 conformation (B). Digestion was then stopped. UV-light (335 nm) was irradiated and emitted light (470 nm) was measured. Undigested control gastric vesicles were irradiated (C).

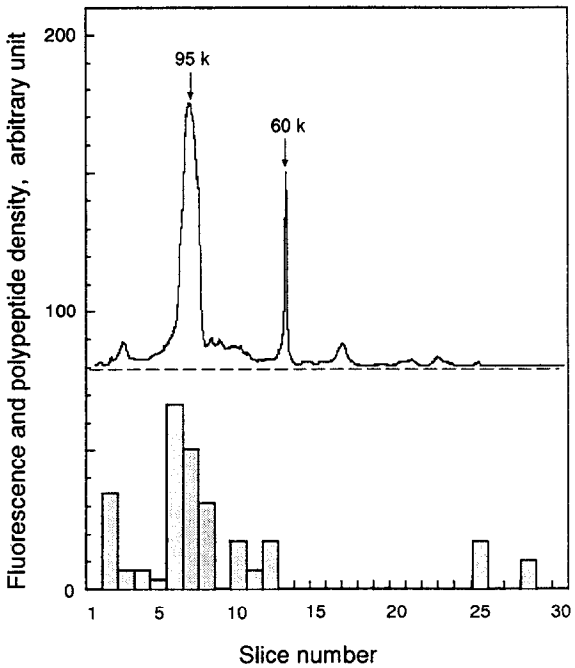

Fig. 3. Electrophoretic patterns (10% gel) of E3810-bound undigested hog gastric vesicles. The 10% SDS-PAGE gel of E3810-bound undigested gastric vesicles was stained with Coomassie brilliant blue R-250 and scanned with a densitometer (upper). Distribution of the irradiation-induced fluorescence of Cys-bound E3810 in 3 mm gel slices was measured at an excitation wavelength = 335 nm and an emission wavelength = 470 nm, respectively (lower).

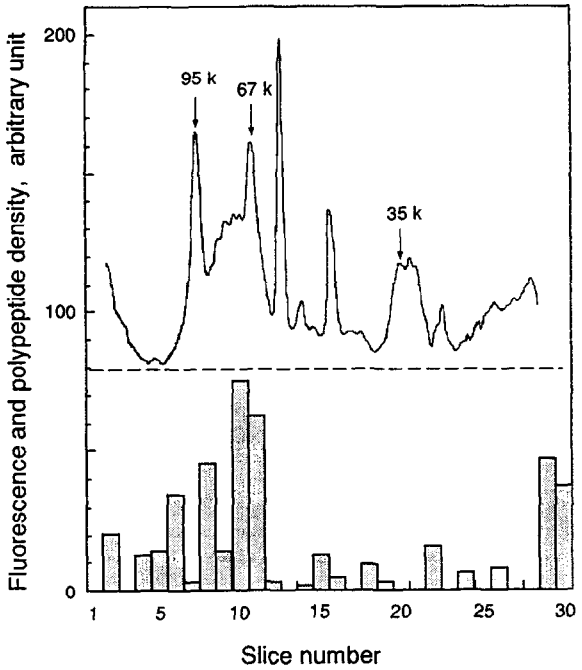

Fig. 4. Electrophoretic patterns (10% gel) of E3810-bound hog gastric vesicles produced by limited tryptic digestion in the presence of Mg-ATP. E3810-bound enzyme (1.5 mg/mL) was digested with 31 μg/mL trypsin in the presence of 5 mM Mg-ATP for 60 min at 37°. The 10% SDS-PAGE gel was stained with Coomassie brilliant blue and scanned with a densitometer (upper). Distribution of the irradiation-induced fluorescence of Cys-bound E3810 in 3 mm gel slices was measured (lower).

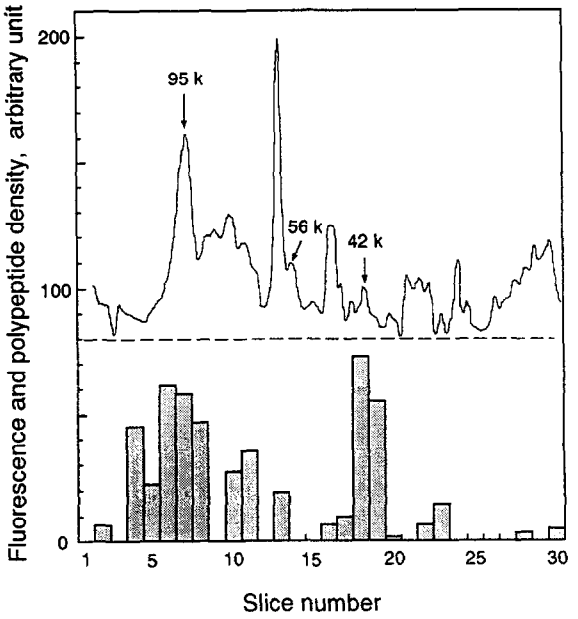

Fig. 6. Electrophoretic patterns (10% gel) of hog gastric vesicles produced by limited tryptic digestion in the presence of K^+. E3810-bound enzyme (1.2 mg/mL) was digested with 23 μg/mL trypsin in the presence of 100 mM KCl for 90 min at 37°. Other conditions were the same as those for Fig. 4.

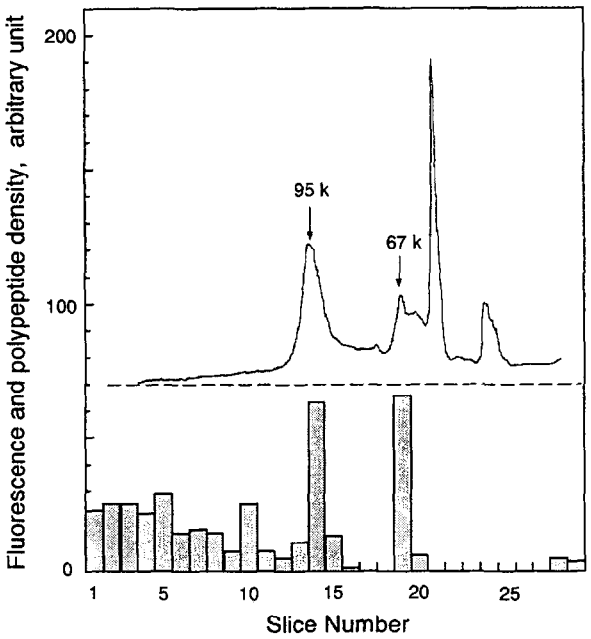

Fig. 5. Electrophoretic patterns (7.5% gel) of hog gastric vesicles produced by limited tryptic digestion in the presence of Mg-ATP. The experimental conditions were the same as those for Fig. 4 except that 7.5% gel was used.

was identified by using the specific fluorescence labelling and limited tryptic digestion of the E3810-bound H^+,K^+-ATPase. After hog gastric H^+,K^+-ATPase was specifically labelled with E3810, the enzyme was limitedly digested with TPCK-treated trypsin. Then digested subfragments were separated by SDS-PAGE. The electrophoretic polypeptide pattern was not altered by the absence of β-mercaptoethanol. Figure 3 shows the distributions of proteins and E3810 fluorescence before digestion in 10% gel. The 95 kDa α-subunit was strongly labelled by E3810. Figure 4 shows the distributions of polypeptide and fluorescence in 10% gel digested in the presence of 5 mM Mg-ATP. Tryptic digestion produced well-defined 67 and 35 kDa subfragments in the presence of 5 mM Mg-ATP. Undigested 95 kDa H^+,K^+-ATPase and the 67 kDa subfragment were fluorescence labelled with E3810. In contrast, the 35 kDa subfragment was not labelled. The fluorescence peak of 67 kDa did not completely align with the 67 kDa peptide, but this was due to the 3 mm wide cutting of the gel (if cut at 2 or 1 mm wide, more complete alignment was obtained but with less fluorescence intensity). In 7.5% gel, the 35 kDa subfragment was located in the electrophoretic front band, and the undigested 95 kDa H^+,K^+-ATPase and the 67 kDa subfragment were fluorescence labelled with E3810 (Fig. 5). Tryptic digestion in the presence of 100 mM KCl produced well-defined 56 and 42 kDa subfragments, and the undigested 95 kDa H^+,K^+-ATPase and the 42 kDa subfragment were greatly fluorescence labelled, whereas the fluorescence intensity of the

56 kDa subfragment was very weak compared with that of the 42 kDa subfragment (Fig. 6).

DISCUSSION

E3810 and omeprazole are very specific and potent inhibitors of gastric H^+,K^+-ATPase. Both are activated in the acidic lumen of parietal cells and modify a Cys residue of H^+,K^+-ATPase. Fryklund *et al.* [7] obtained a stoichiometry of 2.0 nmol of bound omeprazole/mg of α-subunit at 50% inhibition of enzyme activity, and a value of 4.0 nmol/mg of α-subunit they extrapolated to 100% inhibition. They obtained the second stoichiometry of 1.9 nmol of phosphate bound per 1 mg of α-subunit (0.47 nmol/mg of rabbit vesicle protein; i.e. the content of α-subunit was 25% of total protein). From these, the third stoichiometry of 2.1 nmol of omeprazole per 1 nmol of phosphorylated α-subunit was obtained (also reported to be 1.8 in Ref. 6). The first stoichiometry is equivalent to 0.46 mol of omeprazole per 1 mol of 114 kDa α-subunit. This fact indicates that (i) when omeprazole binds to one of two α-subunits, both subunits lose catalytic activity through the dimeric interaction; and (ii) that the ratio of specific binding sites of omeprazole/α-subunit should be one as previously discussed elsewhere [10]. The second stoichiometry is equivalent to 0.22 mol phosphate/mol of α-subunit; i.e. only 22% of α-subunit of rabbit H^+,K^+-ATPase was phosphorylated under these conditions. A similar phosphorylation level (22% in hog H^+,K^+-ATPase) has also been reported [22]. The third stoichiometry was frequently miscited as 2 mol of omeprazole per 1 mol of α-subunit, leading to a false stoichiometry of two omeprazole binding sites/α-subunit.

In the present experiment, the inhibition of enzyme activity was also approx. 49%, which excludes the possibility of non-specific labelling of the enzyme that occurs after full inhibition is achieved. Furthermore, the relation between the amount of bound omeprazole and the extent of H^+,K^+-ATPase inhibition was linear up to 100% inhibition [6, 7], suggesting that the number of the specific binding site of omeprazole per α-subunit is below 1, because the relation would have been nonlinear if there were two or three functional binding sites of omeprazole in one α-subunit [10]. We obtained similar results with E3810 (Morii, M., Hayata, Y. and Takeguchi, N., in preparation). That is, the ratio of specific binding sites of E3810 per the α-subunit of H^+,K^+-ATPase is one.

The α-subunit of hog H^+,K^+-ATPase consists of 1034 amino acid residues including 28 Cys residues [23]. The cleavage sites of the α-subunit of H^+,K^+-ATPase by limited tryptic digestion depend on the conformation of the enzyme. ATP stabilizes the conformation of the α-subunit of gastric H^+,K^+-ATPase at the E_1 form (ATP form), whereas K^+ stabilizes at the E_2 form (K^+ form). In the K^+ form, the tryptic split site is between Arg^{455} and Ile^{456}, and 42 and 56 kDa main subfragments are produced [11]. The N-terminal 42 kDa subfragment contains the phosphorylation site (Asp^{386}) and the transmembrane domains H1–H4. The C-terminal 56 kDa subfragment contains the binding site of FITC (Lys^{518}). In the ATP form, the split sites are between Lys^{47} and Glu^{48} and between Arg^{667} and Lys^{668}, and 67 and 35 kDa subfragments are produced [11]. These cleavage patterns in the ATP form are different from that of the α-subunit of Na^+,K^+-ATPase [24]. The 67 kDa subfragment of H^+,K^+-ATPase contains the phosphorylation (Asp^{386}) and FITC binding sites (Lys^{518}), and the 35 kDa subfragment contains the C-terminal region of the α-subunit. We also found that an antibody specific to a peptide corresponding to residues 1024–1034 bound to 56 and 35 kDa subfragments and not to 42 and 67 kDa subfragments [12]. Although omeprazole stabilizes enzyme conformation at the E_2 form, we have found that E3810 does not affect the conformational state of the enzyme [10] and the cleavage patterns. Therefore, E3810-bound enzyme could be digested both at the E_1 and E_2 forms distinguishably.

Among four main tryptic cleavage subfragments, the N-terminal 42 and 67 kDa subfragments were fluorescence labelled with E3810. The C-terminal 56 and 35 kDa subfragments were not fluorescence labelled. These results suggest that the Cys residue in an overlapped domain of the N-terminal 42 and 67 kDa subfragments (Glu^{48}–Arg^{455}) is modified with E3810. The transmembrane topology in the N-terminal half of the α-subunit is well established [25], but that in the C-terminal half is ambiguous and four or six transmembrane domains have been considered. There are 16 Cys residues in this overlapped N-terminal domain. Among them, 11 Cys residues locate in the cytoplasmic domains of the α-subunit and are excluded from the candidate because the inhibitor does not bind to Cys residues from the cytosolic side under the present conditions. There is no Cys residue in acidic luminal domains. Five Cys residues locate in the transmembrane domains from H1 to H4. One Cys residue, Cys^{322}, locates in the luminal end of the H3 transmembrane domain near the boundary between the lumen and the H3 domain where the pH would be more neutral than in the lumen. The reactivity of the sulphydryl group of the Cys residue is high at neutral pH and low at acidic pH [1], because the pK_a value of $-SH/-S^-$ dissociation of the Cys residue is approx. 8. The remaining four Cys residues locate in the centre or the cytosolic end of transmembrane domains and may be excluded from the candidate because the activated inhibitor is a permanent cation and would experience greater difficulty in reaching these Cys residues than Cys^{322}. Therefore, the location of Cys^{322} is the most reasonable because the acid-activated inhibitor can easily arrive there from the acidic luminal space. Based on the above consideration, we suggest that the candidate for modified Cys residue is Cys^{322}, the same Cys residue reported to be a candidate modified with omeprazole [21], although the final conclusion needs further study. Besancon *et al.* [26] and Shin *et al.* [27] found three omeprazole binding sites and two pantoprazole binding sites in the C-terminal domains. Their data showed that the N-terminal 68 kDa subfragment was also labelled by omeprazole (Fig. 3 in Ref. 26), and Cys^{322} was also included as one possible binding site of substituted benzimidazoles in a recent paper [28]. Shin *et al.* [27] reported that 94% inhibition was

obtained at a stoichiometry of 3 nmol pantoprazole bound per mg protein. Under these conditions, non-specific binding could have occurred as described above, which may explain the difference between their results and ours.

The structure of E3810 is similar to that of omeprazole except for the substituents of the pyridine and benzimidazole rings. One prominent difference is that E3810 has a long 3-methoxypropoxy group on the 4th position of the pyridine ring as opposed to a methoxy group in omeprazole [1]. Although both inhibitors are activated by protonation in the same way and form co-planer tetracyclic sulphenamide derivatives, the inhibition mechanisms are quite different: the partial reaction that is the most differently affected is the conformational change from the E_2 to E_1 form for omeprazole and the K^+-dependent dephosphorylation for E3810 [10]. Even if the binding site of E3810 is the same as that of omeprazole, the structural difference between these inhibitors may cause different steric interference on the conformation of the α-subunit, resulting in different inhibitory effects.

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