Oligomeric Regulation of Gastric $\text{H}^+\text{,K}^+\text{-ATPase}$*

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The $\text{H}^+\text{,K}^+\text{-ATPase}$ of intact gastric vesicles has two $K_m$ values for ATP hydrolysis, 7 and 80 $\mu M$. Irradiation of vesicles with ultraviolet light in the presence of $1 \text{mM}$ ATP resulted in $\text{K}^+\text{-ATPase}$ activity that shows only the low affinity ATP binding. The irradiation stimulated or inhibited proton uptake rate compared with control vesicles at high or low ATP concentrations, respectively. The relation between proton uptake rate and $\text{K}^+\text{-ATPase}$ activity at different ATP concentrations was linear with irradiated vesicles and nonlinear with control vesicles. These results indicate that irradiation of the high affinity ATP binding site regulates the energy-transport coupling in negative and positive manners at high and low ATP concentrations, respectively. The complete inhibition of $\text{K}^+\text{-ATPase}$ by a specific proton pump inhibitor E3810 (rabeprazole) (2–[4-(3-methoxypropoxy)-3-methylpyridin-2-yl]methylsulfinyl]-1H-benimidazole sodium salt) occurred when E3810 bound to half of the $\alpha$-subunit of $\text{H}^+\text{,K}^+\text{-ATPase}$ in unirradiated vesicles at both 200 and 10 $\mu M$ ATP, whereas the complete inhibition of proton uptake occurred when E3810 bound to half or a quarter of the $\alpha$-subunit at 200 or 10 $\mu M$ ATP, respectively. These results suggest that dimeric interaction between the $\alpha$-subunits is necessary for the enzyme activity at all ATP concentrations and that dimeric or tetrameric interaction is necessary for proton transport at high or low ATP concentrations, respectively.

The $\text{H}^+\text{,K}^+\text{-ATPase}$ is involved in the final step of gastric acid secretion and secretes $\text{H}^+$ in exchange for $\text{K}^+$ (1–6). The gastric $\text{H}^+\text{,K}^+\text{-ATPase}$ is classified as a P-type ATPase, closely related to the $\text{Na}^+-\text{K}^+\text{-ATPase}$ (7). The $\text{H}^+\text{,K}^+\text{-ATPase}$ consists of $\alpha$- and $\beta$-subunits. The $\alpha$-subunit is a catalytic subunit, and the $\beta$-subunit is suggested to maintain integrity of the enzyme structure and affect ATP hydrolyzing activity (8). The $\text{H}^+\text{,K}^+\text{-ATPase}$ has high and low $K_m$ values for ATP hydrolysis (3). Several different explanations are possible for the presence of high and low $K_m$ values (9). One possible explanation is that the oligomeric interaction of $\alpha(\beta)$-protomer results in two different affinities for ATP. Diffraction pattern analysis of the electron microscopic image of two-dimensional crystals of the enzyme provided evidence that the $\text{H}^+\text{,K}^+\text{-ATPase}$ exists as a dimer (10) or tetramer (11) of the $\alpha$-subunit. So far, there is no information as to whether close $\alpha\alpha$-subunit contact and interaction are necessary for the enzyme activity and active transport of proton and potassium. In $\text{Na}^+-\text{K}^+\text{-ATPase}$, specific cytoplasmic regions of the $\alpha$-subunit have been shown to be necessary for $\alpha\alpha$ contact (12–14), and the $\alpha$-subunit does not associate with $\text{H}^+\text{,K}^+\text{-ATPase}$ $\alpha$-subunit (12, 13).

In this paper, we studied the biochemical basis of two $K_m$ values for ATP hydrolysis by gastric $\text{H}^+\text{,K}^+\text{-ATPase}$ and found that ATP hydrolysis at the high affinity site regulated proton uptake with negative and positive cooperativity at high and low ATP concentrations, respectively. Furthermore, from measurements of the relations between ATP hydrolysis, proton transport rate and the amount of specific binding of a proton pump inhibitor E3810, we found that at high ATP concentrations the functions of ATP hydrolysis and proton transport require the dimeric interaction between the $\alpha$-subunits, but at low ATP concentrations, the function of ATP hydrolysis requires the dimeric interaction, whereas that of proton uptake requires the tetrameric interaction.

MATERIALS AND METHODS

Chemicals and Drugs—E3810 (rabeprazole), [a-methylene-14C]E3810 and [benzimidazole-2,4-C]E3810 were obtained from Eisai Co. (Tokyo, Japan). The specific activity and the radiochemical purity of [a-methylene-14C]E3810 were 4.26 MBq/mg and 97.3%, respectively, and those of [benzimidazole-2,4-C]E3810 were 3.89 MBq/mg and 98.6%, respectively. [8-14C]ATP was obtained from Du Pont NEN. Pyruvate kinase (200 units/mg at 25°C, solution in 50% glycerol) and lactate dehydrogenase (550 units/mg at 25°C, solution in 50% glycerol) were obtained from Boehringer Mannheim-Yamanouchi Co. (Tokyo, Japan); phosphoenolpyruvate, ATP, and AMP were from Oriental Yeast Co. (Tokyo, Japan); AMP-PNP and NADH from Sigma; and SCH 28080 was from Schering-Plough Co. (Bloomfield, NJ). Other chemicals used were of the highest purity available.

Preparation of Hog Gastric Vesicles—Tightly sealed membrane vesicles that contain $\text{H}^+\text{,K}^+\text{-ATPase}$ were prepared from hog stomachs as described previously (15). Gastric vesicles in 250 mM sucrose solution were stored at −85°C and used within 1 month. Protein concentration was determined by the method of Lowry et al. (16) with bovine serum albumin as a standard.

Measurement of Mg$^{2+}$-Activated ADP Hydrolyzing Activity—In preliminary experiments, we determined that Mg$^{2+}$-activated ADP hydrolyzing activity of gastric vesicles, which was previously unknown. This activity was measured in a pyruvinate-ketolactate dehydrogenase-adenylate kinase-linked reaction. In the reaction, 2 mol of ATP are regeneratated from 2 mol of ADP that are produced from 1 mol of AMP and 1 mol of ATP by adenylate kinase, and these reactions are coupled with oxidation of 2 mol of NADH (17, 18). The reaction mixture containing 10 $\mu$g/ml of gastric vesicles, 40 $\mu$M Tris/HCl (pH 7.40), 250 mM sucrose, 2 $\mu$M MgCl$_2$, 100 $\mu$M NADH, and 10 $\mu$M ADP was incubated for various time at 25°C. Then, 0.8 $\mu$M phosphoenolpyruvate, 4 units/ml pyruvate kinase, 10 units/ml lactate dehydrogenase, and then 15 $\mu$M KCl were added. The decrease in the amount of NADH was measured with an Aminco DW-2C UV-visible spectrophotometer in a dual wavelength mode at 340 and 500 nm at 25°C. Then, 0.8 units/ml of adenylate kinase was added, and the decrease in the amount of NADH was measured. The initial decrease after the addition of KCl corresponds to

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the amount of remaining ADP, and the subsequent decrease after the addition of adenylate kinase corresponds to the amount of hydrolyzed AMP. ADP hydrolyzing activity was calibrated by the addition of a known amount of AMP to a reaction mixture. Mg$^{2+}$-activated ADP hydrolyzing activity was expressed as µmol of liberated inorganic phosphate per mg of protein.

K+-ATPase Activity—The K$^+$-activated ATPase activity was measured in a pyruvate kinase-lactate dehydrogenase-linked reaction where hydrolysis of ATP is coupled with oxidation of NADH (19, 20). The reaction mixture contained 10 µg/ml of gastric vesicles, 40 mM Tris/HCl (pH 7.40), 150 mM KCl, 10 µg/ml of valinomycin, 2 mM MgCl$_2$, 160 M M NADH, 0.8 mM phosphoenolpyruvate, 100 µM AMP, 3 units of pyruvate kinase, 2.75 units of lactate dehydrogenase, and 1 mM ATP in various concentrations of ATP. When indicated, 2 µg/ml of nigericin and 15 mM KCl plus 135 mM choline chloride were used in place of 10 µg/ml of valinomycin and 150 mM KCl. The decrease in the amount of NADH was measured spectrophotometrically at 25°C. ATPase activity was calibrated by the addition of a known amount of ADP to a reaction mixture. Since the presence of 100 µM AMP was found to prevent hydrolysis of ADP to AMP, 100 µM AMP was added in the reaction mixture. When AMP was absent, ATP concentration decreased with time even in the presence of the ATP regenerating system. Mg$^{2+}$-ATPase activity has been measured usually in a K$^+$-free solution, but K$^+$-free condition is not available in the coupled enzyme method because K$^+$ is necessary for pyruvate kinase reaction. A K$^+$-competitive K$^+$-ATPase activity was measured in the presence of 100 µM AMP. Although this method to evaluate Mg$^{2+}$-ATPase activity employs an ATP regenerating system (phosphoenolpyruvate and pyruvate kinase), we found that the ATP concentration was not constant in the gastric vesicle suspension. The Mg$^{2+}$-dependent and K$^+$-independent man- ner of Mg$^{2+}$-ATPase comprised 48.3% of the total vesicle protein determined by densitometry of SDS-PAGE gels stained with Coomassie Brilliant Blue R-250.

RESULTS

Mg$^{2+}$-activated ADP-hydrolyzing Activity of Gastric Vesi-
cles—To obtain the exact relation between the ATP concentration and K$^+$-ATPase activity of gastric vesicles, it is necessary to maintain constant ATP concentrations during the measurement of the enzyme activity. Although the coupled enzyme method to measure ATPase activity employs an ATP regenerating system (phosphoenolpyruvate and pyruvate kinase), we found that the ATP concentration was not constant in the gastric vesicle suspension. The Mg$^{2+}$-dependent and K$^+$-ATPase activity at 10 µM ATP gradually decreased (data not shown). Prèincubation of vesicles in the absence of 2 mM Mg$^{2+}$ for 1 h did not decrease the Mg$^{2+}$- plus K$^+$-ATPase activity at 10 µM ATP, but prèincubation in the presence of 2 mM Mg$^{2+}$ and absence of K$^+$ for 1 h induced the same extent of decrease in the ATPase activity as that observed in the presence of Mg$^{2+}$ plus K$^+$. These results suggest the possibility that ADP was not completely converted to ATP, and part of ADP was further hydro-
lyzed to AMP in a Mg$^{2+}$-dependent and K$^+$-independent man-
ner. The hydrolysis of ADP to AMP can be detected by an
adenylate kinase-catalyzed reaction linked to the coupled en-
zyme method where 2 mol of ADP is produced from 1 mol of AMP and 1 mol of ATP. The hydrolyzing activity of ADP to AMP by gastric vesicles was 1.45 ± 0.58 µmol of P$_i$/mg (mean ± S.E., n = 3), which was about half of the Mg$^{2+}$-ATPase activity of gastric vesicles measured at 10 µM ATP. To keep the Mg$^{2+}$-plus K$^+$-ATPase activity constant over a long time, it was found that the addition of 100 µM AMP was effective, possibly because the high concentration of AMP shifts an enzymatic reaction equilibrium between ADP and AMP toward ADP.

[ATP] Dependence of K$^+$-ATPase Activity—K$^+$-ATPase activ-
ity was measured at various ATP concentrations (2–1,000 µM) in the presence of 100 µM AMP, 10 µg/ml gastric vesicles, 2 mM MgCl$_2$, 150 mM KCl, 10 µg/ml valinomycin, and 40 mM Tris/HCl (pH 7.40). The ATP concentration was kept constant during the reaction by inclusion of phosphoenolpyruvate, pyruvate kinase, and 100 µM AMP. The double-reciprocal plot between K$^+$-
ATPase activity and ATP concentration yielded two K$\text{m}$ values, 7 and 80 µM ATP in the presence of proton and potassium gradients and a membrane potential (Fig. 1). However, in the
Gastric vesicles (1 mg of protein/ml) were irradiated with UV light for 1 h at 25°C in the presence of 1 mM ATP in the presence of valinomycin. K⁺-ATPase activities were measured at various ATP concentrations as described under "Materials and Methods." For the control experiment, gastric vesicles were incubated without UV light irradiation for 1 h at 25°C. Then, K⁺-ATPase activity of unirradiated vesicles was measured in the presence of 15 mM KCl plus 135 mM choline chloride and nigericin in replacement of 150 mM KCl and valinomycin.

Values are means ± S.E. from three experiments. Straight lines were drawn using the Vₘₐₓ and Kₘ values obtained by nonlinear least-square curve-fitting to the Michaelis-Menten equation.

**Fig. 1.** Double-reciprocal plots between [ATP] and K⁺-ATPase activity of the control and UV light-irradiated vesicles. Gastric vesicles (1 mg of protein/ml) were irradiated with UV light for 1 h at 25°C in the presence of 1 mM ATP in the presence of valinomycin. K⁺-ATPase activities were measured at various ATP concentrations as described under "Materials and Methods." For the control experiment, gastric vesicles were incubated without UV light irradiation for 1 h at 25°C. Then, K⁺-ATPase activity of unirradiated vesicles was measured in the presence of 15 mM KCl plus 135 mM choline chloride and nigericin in replacement of 150 mM KCl and valinomycin. Values are means ± S.E. from three experiments. Straight lines were drawn using the Vₘₐₓ and Kₘ values obtained by nonlinear least-square curve-fitting to the Michaelis-Menten equation.

**Fig. 2.** Effects of UV light irradiation on K⁺-ATPase activity in the presence and absence of ATP. Gastric vesicles (1 mg of protein/ml) were irradiated with UV light at 260 nm for 1 h in the presence (C) and absence (D) of 1 mM ATP. For the control experiment (D), gastric vesicles were incubated without UV light irradiation for 1 h at 25°C. Then, K⁺-ATPase was measured as a function of the ATP concentration. Values are means ± S.E. from three experiments.

The presence of 2 μg/ml of nigericin, which dissipated K⁺ and H⁺ gradients across the vesicle membrane, only one Kₘ value (5 μM) was observed (Fig. 1). We confirmed the existence of a potassium gradient across the vesicle membrane, in the presence of valinomycin, by inhibition of K⁺-ATPase with SCH 28080 at 20 μM, a specific reversible inhibitor that competes with K⁺ at the luminal high affinity binding site, this inhibition being reversed by the addition of 2 μg/ml of nigericin (data not shown).

Specific Inhibition of the High Affinity ATP Binding by UV Light Irradiation—Irradiation of vesicles with UV light (260 nm) in the absence of ATP induced almost complete inhibition of the K⁺-ATPase activity of gastric vesicles, whereas irradiation in the presence of 1 mM ATP produced only minor inhibition (Fig. 2). Fig. 1 shows that the high affinity ATP binding is inhibited and the low affinity ATP binding is completely protected from UV light irradiation in the presence of 1 mM ATP. The values of Kₘ for the low affinity ATP binding and Vₘₐₓ did not change from control values. When UV light-irradiated vesicles were used, the binding of [8-³²P]ATP to the enzyme was not detected, indicating that UV light irradiation in the presence of 1 mM ATP did not induce the irreversible binding of ATP to the high affinity ATP binding site of the enzyme. We also examined the effect of UV light irradiation on proton uptake rate into gastric vesicles. The rate of proton uptake into the UV light-irradiated vesicles was enhanced at high ATP concentrations (>100 μM) but inhibited at low ATP concentrations (<20 μM) when compared with control vesicles (Fig. 3).
Oligomeric Regulation of Gastric Proton Pump

Effects of AMP-PNP on K⁺-ATPase activity
UV light irradiation (260 nm) was carried out in a solution containing 1 mg/ml of gastric vesicles, 1 mM ATP, 2 mM MgCl₂, 250 mM sucrose, and 40 mM Tris/HCl (pH 7.40) for 1 h at 25 °C. Then, K⁺-ATPase activity (μmol of Pi/mg·h) was measured in a solution containing 10 μM of gastric vesicle, 2 mM MgCl₂, 100 μM AMP, 40 mM Tris/HCl (pH 7.40), 200 μM ATP, the coupled enzyme system, 10 μg/ml of valinomycin, 150 mM KCl in the presence or absence of 10 μM AMP-PNP at 25 °C. Values are means ± S.E. from four experiments.

| AMP-PNP | +AMP-PNP | Change |
|---------|----------|--------|
| Control | 20.9 ± 0.5 | 24.8 ± 0.7 | +18.7a |
| UV-light-irradiated | 23.9 ± 0.1 | 20.7 ± 0.2 | -12.1b |

a p < 0.05 by paired Student’s t test.
b p < 0.01 by paired Student’s t test.

Effects of AMP-PNP—We studied the effect of a nonhydrolyzing ATP analog AMP-PNP on K⁺-ATPase activity at 200 μM ATP. The presence of 10 μM AMP-PNP enhanced K⁺-ATPase activity in control vesicles but decreased it in UV light-irradiated vesicles (Table I). We discuss this AMP-PNP effect later.

The relationship between K⁺-ATPase activity and proton uptake rate was not linear in control vesicles, but it was linear in UV light-irradiated vesicles (Fig. 4), suggesting that ATP hydrolysis at the high affinity ATP binding site regulates the coupling between K⁺-ATPase activity and proton uptake rate in negative and positive cooperative manners at high and low ATP concentrations, respectively.

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The relationship between the Amount of Bound E3810 and K⁺-ATPase Activity and between the Amount of Bound E3810 and the Rate of Proton Uptake (A) of gastric vesicles measured at 200 μM ATP. Gastric vesicles (1 mg of protein/ml) were incubated with various concentrations of radioactive E3810 in the presence of 150 mM KCl, 40 mM Tris/HCl (pH 7.40), 2 mM MgCl₂, 10 μg/ml valinomycin, and 2 mM ATP for 20 min at room temperature. The reaction was terminated by gel filtration through Sephadex G-50 column equilibrated with 150 mM KCl and 40 mM Tris/HCl (pH 7.40). The amount of bound E3810 was measured with a liquid scintillation counter. K⁺-ATPase activity was measured at 200 μM ATP. The rate of proton uptake was determined by the initial slope of fluorescence quench of acridine orange after the addition of 200 μM ATP in a solution containing 10 μg/ml of vesicles, 150 mM KCl, 40 mM Tris/HCl (pH 7.40), 2 mM MgCl₂, 10 μg/ml valinomycin, 100 μM AMP, 5 μM acridine orange, 0.8 mM phosphoenolpyruvate, and 3 units/ml pyruvate kinase at 25 °C. The experimental values less than 10% of the control were omitted for the linear least-square fits, which gave the intercepts of 2.6 (A) and 2.1 nmol/mg of protein (B) at the complete inhibition of the enzyme activity and proton uptake, respectively.

FIG. 5. Specificity of E3810 binding to α-subunit of H⁺,K⁺-ATPase. Gastric vesicles (1 mg of protein/ml) were incubated with 5 μM of radioactive E3810 in the presence of 150 mM KCl, 40 mM Tris/HCl (pH 7.40), 2 mM MgCl₂, 10 μg/ml valinomycin, and 2 mM ATP for 20 min at room temperature. Immediately, the sample solution was applied to SDS-PAGE (top). The radioactivities of sliced gel pieces were measured as described under “Materials and Methods” (bottom).

FIG. 6. Relationship between the amount of bound E3810 and the K⁺-ATPase activity (A) and between the amount of bound E3810 and the rate of proton uptake (B) of gastric vesicles measured at 200 μM ATP. Gastric vesicles (1 mg of protein/ml) were incubated with various concentrations of radioactive E3810 in the presence of 150 mM KCl, 40 mM Tris/HCl (pH 7.40), 2 mM MgCl₂, 10 μg/ml valinomycin, and 2 mM ATP for 20 min at room temperature. The reaction was terminated by gel filtration through Sephadex G-50 column equilibrated with 150 mM KCl and 40 mM Tris/HCl (pH 7.40). The amount of bound E3810 was measured with a liquid scintillation counter. K⁺-ATPase activity was measured at 200 μM ATP. The rate of proton uptake was determined by the initial slope of fluorescence quench of acridine orange after the addition of 200 μM ATP in a solution containing 10 μg/ml of vesicles, 150 mM KCl, 40 mM Tris/HCl (pH 7.40), 2 mM MgCl₂, 10 μg/ml valinomycin, 100 μM AMP, 5 μM acridine orange, 0.8 mM phosphoenolpyruvate, and 3 units/ml pyruvate kinase at 25 °C. The experimental values less than 10% of the control were omitted for the linear least-square fits, which gave the intercepts of 2.6 (A) and 2.1 nmol/mg of protein (B) at the complete inhibition of the enzyme activity and proton uptake, respectively.

FIG. 7. Relationship between the amount of bound E3810 and the K⁺-ATPase activity (A) and between the amount of bound E3810 and the rate of proton uptake (B) of gastric vesicles measured at 10 μM ATP. These experiments were carried out as described in the legend to Fig. 6. K⁺-ATPase activity and proton uptake were measured at 10 μM ATP. The experimental values less than 10% of the control were omitted for the linear fits, which gave the intercepts of 2.5 (A) and 1.0 nmol/mg of protein (B) at the complete inhibition of the enzyme activity and proton uptake, respectively.

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the differences in proton uptake at 5 and 10 μM ATP were significant, and those at 200-1000 μM ATP were not significant (three observations). Increasing the number of observations at 500 μM ATP to nine resulted in the difference becoming significant.

The relationship between K⁺-ATPase activity and proton uptake rate was not linear in control vesicles, but it was linear in UV light-irradiated vesicles (Fig. 4), suggesting that ATP hydrolysis at the high affinity ATP binding site regulates the coupling between K⁺-ATPase activity and proton uptake rate in negative and positive cooperative manners at high and low ATP concentrations, respectively.

TABLE I
Effects of AMP-PNP on K⁺-ATPase activity
UV light irradiation (260 nm) was carried out in a solution containing 1 mg/ml of gastric vesicles, 1 mM ATP, 2 mM MgCl₂, 250 mM sucrose, and 40 mM Tris/HCl (pH 7.40) for 1 h at 25 °C. Then, K⁺-ATPase activity (μmol of Pi/mg·h) was measured in a solution containing 10 μM of gastric vesicle, 2 mM MgCl₂, 100 μM AMP, 40 mM Tris/HCl (pH 7.40), 200 μM ATP, the coupled enzyme system, 10 μg/ml of valinomycin, 150 mM KCl in the presence or absence of 10 μM AMP-PNP at 25 °C. Values are means ± S.E. from four experiments.

| AMP-PNP | +AMP-PNP | Change |
|---------|----------|--------|
| Control | 20.9 ± 0.5 | 24.8 ± 0.7 | +18.7a |
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Effects of AMP-PNP—We studied the effect of a nonhydrolyzing ATP analog AMP-PNP on K⁺-ATPase activity at 200 μM ATP. The presence of 10 μM AMP-PNP enhanced K⁺-ATPase activity in control vesicles but decreased it in UV light-irradiated vesicles (Table I). We discuss this AMP-PNP effect later.

The relationship between the Amount of Bound E3810 and K⁺-ATPase Activity and between the Amount of Bound E3810 and the Rate of Proton Uptake (A) of gastric vesicles measured at 200 μM ATP. The presence of 10 μM AMP-PNP enhanced K⁺-ATPase activity in control vesicles but decreased it in UV light-irradiated vesicles (Table I). We discuss this AMP-PNP effect later.

Distribution of gastric vesicle proteins and ¹⁴C-counts in sliced gels, indicating that E3810 bound only to the α-subunit of H⁺,K⁺-ATPase (98.7%). The relations between the amounts of bound E3810, K⁺-ATPase, and proton uptake rate at 200 and 10 μM ATP were measured. At 200 μM ATP, K⁺-ATPase activity was completely inhibited when 2.6 nmol of E3810 bound per mg of vesicle protein, and proton uptake was completely inhibited when 2.1 nmol of E3810 bound per mg of vesicle proteins (Fig. 6). E3810 binding increased further to 8.6 nmol/mg of vesicle proteins, more than required for complete inhibition of proton uptake, probably due to nonspecific binding. At 10 μM ATP, K⁺-ATPase activity was completely inhibited when 2.5
nmol of E3810 bound per mg of vesicle proteins, whereas proton uptake was completely inhibited when 1.0 nmol of E3810 bound per mg of vesicle proteins (Fig. 7). The ratio of bound E3810/α-subunit was calculated taking into consideration that E3810 binds only to the α-subunit of H⁺/K⁺-ATPase, the α-subunit comprised 48.3% vesicle proteins and the mass of the α-subunit is 114 kDa; for example, 2.1 nmol/mg of proteins gives the ratio of 0.50 (= 2.1 × 10⁻⁹ × 1.14 × 10⁻³/10⁻⁸ × 0.483). Table II shows the ratios necessary for complete inhibition of K⁺-ATPase activity and proton uptake at 200 and 10 μM ATP. These results suggest that binding of one molecule of E3810 can arrest the K⁺-ATPase activity of two molecules of the α-subunit at the high and low ATP concentrations, and it can arrest protein uptake of two or four molecules of the α-subunit at the high or low ATP concentration, respectively.

### DISCUSSION

The gastric H⁺/K⁺-ATPase consists of α- and β-subunits. Crystallization of H⁺/K⁺-ATPase has shown that two (9) or four (10) monomers comprised the crystalline unit, which may suggest the presence of α-α-subunit contact and interaction, as shown in Na⁺/K⁺-ATPase (12-14). So far there has been no report whether the α-α-subunit interaction of H⁺/K⁺-ATPase is involved in the function of the enzyme activity and the active ion transport. The present study using a specific proton pump inhibitor E3810 has demonstrated that the ratio of bound E3810/α-subunit necessary for the complete inhibition of K⁺-ATPase activity is about 0.6 at both high and low ATP concentrations, and the ratio necessary for the complete inhibition of proton uptake is 0.5 or 0.24 at high or low ATP concentrations, respectively (Table II). That is, when E3810 binds to one of two α-subunits, both subunits lose the catalytic activity at every ATP concentration. When E3810 binds to one of two α-subunits, both subunits lose the proton transporting activity at high ATP concentration, and when E3810 binds to one of four α-subunits, all four subunits lose the proton transporting activity at low ATP concentrations. These results strongly suggest that the functional unit for K⁺-ATPase activity is the (αβ)₂-diprotomer at every ATP concentration, whereas the functional unit for proton transport is (αβ)₂₄-tetraprotomer at high ATP concentrations and the (αβ)₄₂-diprotomer at low ATP concentrations.

When H⁺/K⁺-ATPase was irradiated by UV light in the presence of ATP, the high affinity ATP binding was inhibited, whereas the low affinity ATP binding remained intact (Fig. 1). In these irradiated vesicles, proton uptake rate was stimulated at high ATP concentrations and inhibited at low ATP concentrations compared with those of control vesicles (Fig. 3). Furthermore, control vesicles show negative or positive cooperative coupling between the K⁺-ATPase activity and proton uptake rate at high or low ATP concentrations, respectively, whereas the UV light-irradiated vesicles show no cooperativity (Fig. 4). These results suggest that the high affinity ATP binding site has a regulatory role in addition to that of ATP hydrolysis.

The presence of AMP-PNP enhanced K⁺-ATPase activity in control vesicles but decreased it in UV light-irradiated vesicles. We propose the following explanation. Although AMP-PNP competes with ATP and inhibits ATP hydrolysis at both ATP binding sites in control vesicles, competition at the high affinity site diminishes the negative cooperativity, resulting in a net increase of K⁺-ATPase activity. In UV light-irradiated vesicles, which have only the low affinity ATP binding site, AMP-PNP competition with ATP inhibits K⁺-ATPase activity.

We suggest that the presence of high and low affinity ATP binding sites in H⁺/K⁺-ATPase is due to the dimeric α-α interaction shown in this study. Other explanations, however, are also possible as previously discussed for Na⁺/K⁺-ATPase (9, 27). For example, subutilized μg Na⁺, K⁺-ATPase protomer had two different affinities for ATP (27). We have shown that H⁺/K⁺-ATPase has only one ATP binding affinity (Kₘ = 5 μM) when measured in the presence of nigericin, which dissipates the proton and potassium ion gradients across the vesicle membrane. This result shows that each α-subunit has a single ATP binding site that is involved in hydrolysis. Our explanation clearly differs from a previous one that the nucleotide binds to H⁺/K⁺-ATPase with different affinities in the sequential reaction cycle, E-ATP-H⁺ and EP-H⁺-ADP (or EP-H⁺-ATP) (28).

In conclusion, we propose that the dimeric or tetrameric subunit interaction is necessary for the function of H⁺/K⁺-ATPase, and two different affinities for ATP of this enzyme are due to the dimeric subunit interaction, which is sensitive to UV light irradiation and pH and/or K⁺ gradient across the vesicle membrane.

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