Involvement of the H$_3$O$^+$–Lys-164–Gln-161–Glu-345 Charge Transfer Pathway in Proton Transport of Gastric H$^+$,K$^+$-ATPase$^{*3}$

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Gastric H$^+$,K$^+$-ATPase is shown to transport 2 mol of H$^+$/mol of ATP hydrolysis in isolated hog gastric vesicles. We studied whether the H$^+$ transport mechanism is due to charge transfer and/or transfer of hydronium ion (H$_3$O$^+$). From transport of [$^{18}$O]H$_2$O, 1.8 mol of water molecule/mol of ATP hydrolysis was found to be transported. We performed a molecular dynamics simulation of the three-dimensional structure model of the H$^+$,K$^+$-ATPase α-subunit at E1 conformation. It predicts the presence of a charge transfer pathway from hydronium ion in cytosolic medium to Glu-345 in cation binding site 2 (H$_3$O$^+$–Lys-164–Gln-161–Glu-345). No charge transport pathway was formed in mutant Q161L, E345L, and E345D. Alternative pathways (H$_3$O$^+$–Gln-161–Glu-345) in mutant K164L and (H$_3$O$^+$–Arg-105–Gln-161–Gln-345) in mutant E345Q were formed. The H$^+$,K$^+$-ATPase activity in these mutants reflected the presence and absence of charge transfer pathways. We also found charge transfer from sites 2 to 1 via a water wire and a charge transfer pathway (H$_2$O–Asn-794–Glu-797). These results suggest that protons are charge-transferred from the cytosolic side to H$_2$O in sites 2 and 1, the H$_2$O comes from cytosolic medium, and H$_3$O$^+$ in the sites are transported into lumen during the conformational transition from E1P to E2P.

In P-type ATPases such as Na$^+$,K$^+$-ATPase, Ca$^{2+}$-ATPase, and H$^+$,K$^+$-ATPase, at least two conformation states exist, E1 and E2, with conformational changes being accompanied by ion transportation (1). Phosphorylation forces the enzyme into the E2 conformation and, after dephosphorylation, returns the enzyme to the E1 conformation. The cation binding sites of these pumps have high affinity for cytosolic transporting cation (H$^+$ in the case of H$^+$,K$^+$-ATPase) in the E1 and low affinity in the E2 conformation, and they face the cytoplasm in the E1 and lumen in the E2 conformation. Furthermore, cation binding to the sites transiently induces the cation-occluded conformation (1).

Recent reports have described precise three-dimensional structures of rabbit SR Ca$^{2+}$-ATPase at several different conformations (2, 3). E2P structure of hog renal Na$^+$,K$^+$-ATPase (4) and E1ATP structure of plasma membrane AHA2 H$^+$-ATPase (5), using x-ray diffraction crystallography analysis. The crystal structure of gastric H$^+$,K$^+$-ATPase has not yet determined. However, previous and recent many studies (6–17) show that reaction steps of gastric H$^+$,K$^+$-ATPase are similar to those of Ca$^{2+}$-ATPase and Na$^+$,K$^+$-ATPase and that the α-subunit contains two cation binding sites. The molecular K$^+$ pathway from the lumen to the cytoplasm via the cation binding site has been studied based on the homology modeling with site-directed mutagenesis (17). However, the molecular mechanism of proton transport, especially transport from the cytosolic medium to the cation binding sites, has not been clarified yet. Here, we studied whether the H$^+$ transport mechanism is due to charge transfer and/or movement of hydronium ion (H$_3$O$^+$). For this, we measured water transport activity of gastric H$^+$,K$^+$-ATPase using [$^{18}$O]H$_2$O and found H$_2$O and/or H$_3$O$^+$ transport is coupled with the ATP hydrolysis. We also constructed three-dimensional structure models of the gastric H$^+$,K$^+$-ATPase α-subunit based on the homology modeling of the E1 conformation of the Ca$^{2+}$-ATPase and performed molecular dynamics simulations. These simulations predicted the presence of charge transfer pathways from the cytosolic medium to cation binding site 2 and the presence of another charge transfer pathway from site 2 to cation binding site 1. We also simulated these pathways in several mutated H$^+$,K$^+$-ATPase α-subunits and compared the results of simulation with those of mutant K$^+$-ATPase activity.

EXPERIMENTAL PROCEDURES

Materials—pcDNA3.1/ZEO(+) and pcDNA3 vectors, Lipo-lectamine 2000 reagent, Alexa Fluor 546-conjugated anti-mouse IgG antibody, and Alexa Fluor 488-conjugated anti-rabbit IgG antibody were obtained from Invitrogen. Anti-gastric H$^+$,K$^+$-ATPase β-subunit mouse monoclonal antibody, 2B6,
was obtained from Medical and Biological Laboratories (Nagoya, Japan). The EndoFree Plasmid Maxi kit was obtained from Qiagen GmbH (Hilden, Germany). The QuikChange II site-directed mutagenesis kit was obtained from Stratagene Corp. (La Jolla, CA). Collagen type I-coated tissue culture wares were obtained from Asahi Technoglass Co., Ltd. (Tokyo, Japan). Avidin-agarose and SCH28080, a specific H^+,K^+-ATPase inhibitor, were obtained from Sigma-Aldrich. [18O]H_2O was obtained from Taiyo Nippon Sanso Corp. (Tokyo, Japan). EZ Link Sulfo-HNS-SS-Biotin labeling reagent was obtained from Pierce. ^86Rb^+ was obtained from GE Healthcare and PerkinElmer Life Sciences. All other reagents were of molecular biology grade or the highest grade of purity available.

**Preparation of Hog Gastric Vesicles**—Tightly sealed membrane vesicles (GI gastric vesicles) which contain H^+,K^+-ATPase were prepared from hog stomachs as described previously (18). The content of tightly sealed inside-out vesicles evaluated from valinomycin-induced K^+-ATPase activity was 97% at 25 °C (19). Gastric vesicles in 250 mM sucrose solution were stored at −85 °C until use and thawed on ice before use.

**Assay of Water Transport in Gastric Vesicles**—ATP hydrolysis-dependent water transport activity in hog GI gastric vesicles was assayed using stable [18O]H_2O. One ml of gastric lysis-dependent water transport activity in hog GI gastric vesicle solution (GI fraction, 1 mg/ml) containing 130 mM KCl, 4 mM MgSO_4, 10 μg/ml valinomycin, 8 atom% [18O]H_2O, and 40 mM PIPES/Tris (pH 6.8) was incubated in the presence or absence of 4 mM ATP for 20 min at 25 °C. Then, sample solutions were filtered through a 0.45-μm membrane filter (Millipore, HAWP 02500).

Gastric vesicles on the filter were washed with 10 ml of washing solution containing 130 mM KCl and 40 mM PIPES/Tris (pH 6.8). The wet filter was sandwiched between two polyester sheets, heat-sealed, and preserved in a cooler with dry ice until measurement. Trapped [18O]H_2O on the filter was equilibrated with pure [16O]CO_2 in a closed chamber. Then, the content of [18O]CO_2 was quantified by a mass spectrum analysis. These analyses were performed at the stable isotope analytical service center of Iso-Analytical Ltd. (Sandbach, UK).

ATP hydrolysis-dependent proton accumulation into gastric vesicles was measured using acridine orange fluorescence under the same conditions as those employed for the [18O]H_2O transport experiments as described previously (20).

**Site-directed Mutagenesis**—cDNAs of α- and β-subunits were prepared from rabbit gastric mucosa as described elsewhere (9). These were cloned in pcDNA3.1/ZEO(+) and pcDNA3, respectively. For numbering the amino acid sequence of the gastric H^+,K^+-ATPase α-subunit, the cDNA sequence of the rabbit gastric H^+,K^+-ATPase α-subunit (GenBank access number P27112) was adopted. Site-directed mutagenesis was performed using the QuikChange II site-directed mutagenesis kit as described previously (21). The mutated cDNA sequences were verified using a Long-Read Tower DNA sequencer (GE Healthcare). HEK293 cell line stably expressing the wild type β-subunit was subjected to transfection with the α-subunit (either wild type or mutant) using Lipofectamine 2000 reagent, which resulted in stable co-expression of the α and β subunits (22).

**Characterizations of Transfected H^+,K^+-ATPase in HEK293 Cells**—Membrane fractions of HEK293 cells were prepared as described previously (7). Briefly, the samples (30 μg of protein) were incubated in a modified Laemmli sample buffer containing 2% SDS, 2% β-mercaptoethanol, 10% glycerol, and 65 mM Tris/HCl (pH 6.8) for 10 min at room temperature and applied to 7.5% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane. The membranes were blocked with a 4% Block Ace solution (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). The proteins were detected with an anti-gastric H^+,K^+-ATPase α-subunit antibody, Ab1024, which recognizes the carboxyl-terminal peptide (residues 1024–1034) (23). After incubation with a horseradish peroxidase-conjugated anti-rabbit IgG antibody, the bands were visualized with the ECLplus kit (GE Healthcare) and scanned using a luminescent image analyzer LAS-1000plus (FUJIFILM Corp., Tokyo, Japan).

The contents of α-subunit in membrane fractions prepared from HEK293 cells were estimated from the linear range of a standard curve. To obtain the standard calibration curve, the signal intensities of the α-subunit band of a series of diluted gastric vesicle preparations were plotted against the content of the α-subunit. About 60% of gastric vesicle proteins was H^+,K^+-ATPase α-subunit (22).

To confirm the expression of transfected protein to the plasma membrane, cell surface biotinylation was performed in a cold room (4 °C) as following. Transfected HEK293 cells (1 × 10^6 cells/well) were washed with phosphate-buffered saline containing 1 mM MgSO_4 and 2.5 mM CaCl_2 (PBS ++) and incubated with 1 ml of EZ Link Sulfo-HNS-SS-Biotin labeling reagent (0.5 mg/ml) for 15 min. The cells were washed with PBS + + and solubilized with 500 μl of lysis buffer solution (150 mM NaCl, 0.5 mM EDTA, 50 mM Tris/HCl (pH 7.4), 1% Triton X-100, 0.09 units/ml aprotinin, 10 μg/ml phenylmethysulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) and vortexed for 30 min. Lysed solution was centrifuged at 16,000 × g for 20 min, and the protein concentration of supernatant was adjusted to 1.5 mg/ml. Twenty μl of avidin-agarose solution (≥1.0 mg/ml) was added to 400 μl of the supernatant and rotated for 2 h, then centrifuged at 5000 × g for 7 min. This procedure was repeated one more time. The combined precipitates were washed with PBS solution containing 0.5% Triton X-100 and suspended in 100 μl of PBS solution. Isolated surface proteins were separated from bacteria-avidin complex by incubation with a modified Laemmli sample buffer solution for 15 min at 37 °C and separated with 7.5% SDS-polyacrylamide gel electrophoresis. The α- and β-subunits were detected with Ab1024 (for α-subunit) and 2B6 (for β-subunit) antibodies. Cell surface expression levels of the α-subunit were calculated as content percentage of the isolated surface α-subunit.

**Fluorescence Immunocytochemistry**—HEK293 cells were fixed with ice-cold methanol for 7 min and permeabilized with PBS containing 0.3% Triton X-100 and 0.1% bovine serum albumin for 15 min at room temperature. Nonspecific binding was
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blocked using 3% bovine serum albumin. The permeabilized cells were incubated with the Ab1024 (for α-subunit) and 2B6 (for β-subunit) antibodies (1:100 dilution) overnight at 4 °C and then with the Alexa Fluor 488-conjugated anti-rabbit IgG antibody (for α-subunit) and Alexa Fluor 546-conjugated antimouse IgG antibody (for β-subunit) (1:100 dilution) for 1 h at room temperature. Immunofluorescence images were visualized with a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Assay of SCH28080-sensitive K⁺-ATPase Activity—K⁺-stimulated ATPase activity (K⁺-ATPase activity) of the membrane fraction prepared from HEK293 cells was measured in the presence and absence of 50 μM SCH28080 in 1 ml of sample solution which contained 50 μg of membrane protein, 3 mM MgSO₄, 1 mM ATP, 5 mM NaN₃, 2 mM ouabain, 15 mM KCl, and 40 mM Tris/HCl (pH 6.8). After incubation for 30 min at 37 °C, the released inorganic phosphate was measured as described previously (24). The SCH28080-sensitive K⁺-ATPase activity was calculated as the difference between activities in the presence and absence of SCH28080. Usually, K⁺-ATPase activities of mutants and the wild type were measured in pairs where the wild type cells and mutant cells were cultured also at the same time. pH profile of the K⁺-ATPase activity was measured in a 1 ml of sample solution containing 50 μg of membrane protein, 3 mM MgSO₄, 1 mM ATP, 5 mM NaN₃, 2 mM ouabain, 15 mM KCl, and 40 mM Tris/HCl (pH 5.5–6.5), or 40 mM Tris/HCl (pH 6.8–8.0) in the presence and absence of 50 μM SCH28080.

Assay of Rb⁺ Transport Activity—Rb⁺ was used as a surrogate ion of K⁺. Rb⁺ transport assay was performed as described previously (22). One day before the transport assay, cells were seeded in two sets of 6-well collagen type I-coated plates; one set for the Rb⁺ transport assay and the other set for counting cell number. They were grown until confluence. Cells in each well were incubated in a 1 ml sample solution containing 144 mM NaCl, 5 mM HEPES/NaOH (pH 6.8), 0.5 mM MgSO₄, 0.5 mM CaCl₂, 1 mM RbCl (containing 3 × 10⁶ cpm Rb⁺), 500 μM ouabain, and 10 μM furosemide, an inhibitor of Na⁺-K⁺-2Cl⁻ symporter, for 10 min at 37 °C. Rb⁺ transport was measured in the presence and absence of 50 μM SCH28080. After a 10-min incubation, the medium was removed, and each well was washed 3 times with 2 ml of ice-cold wash solution containing 144 mM NaCl and 5 mM HEPES/NaOH (pH 7.4). Cells were solubilized with 2 ml of lysis buffer containing 1% Nonidet P40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris/HCl (pH 7.4). One ml of solution was mixed with 5 ml of aqueous counting scintillant ACS-II (GE Healthcare), and radioactivity was counted. The Rb⁺ transport activity was calculated as the difference between activities in the presence and absence of SCH28080 and expressed in μmol of Rb⁺/10⁶ cells/min. After subtraction of mock activity, the Rb⁺ transport activities were normalized, dividing by the number of the cells on the each well and also dividing by the cell surface expression level of the α-subunit.

Homology Modeling of the Gastric H⁺,K⁺-ATPase α-Subunit and Molecular Dynamics Simulation—Amino acid sequences of the transmembrane helices and lumenal loops of gastric H⁺,K⁺-ATPase (from six different animal species) and Na⁺,K⁺-ATPase α-subunit were aligned with that of rabbit SR Ca²⁺ pump using the gapped BLAST program (25) with default parameters. Then, multiple alignments were obtained by ClustalW program (26) to reduce the possibility of different alignments under the assumption that lipid-facing residues tend to be variable, whereas those involved in helix-helix contacts tend to be conserved (26, 27). Aligned sequences were inspected and adjusted manually to avoid gaps within the transmembrane helices. Three-dimensional structure models of the gastric H⁺,K⁺-ATPase α-subunit were constructed using the MODELLER program (28) based on the atomic model of the Ca²⁺-bound form (E₁, conformation, Protein Data Bank code 1SU4) of rabbit SR Ca²⁺-ATPase with restriction of partial structures. Structures of long insertion of the H⁺,K⁺-ATPase α-subunit (Gly-2–Met-50 and Arg-658–Asn-677) were restricted to the predicted structures obtained by PredictProtein (29).

Next, we performed energy minimization and molecular dynamics simulation to obtain the dynamic structure of the E₁ conformation in the presence of 14 neutralizing Na⁺ ions and about 5000 water molecules in both the cytosolic and luminal medium. Energy minimization and molecular dynamics calculations at constant temperature 300 K were carried out using the AMBER 8 program (30). Calculations were performed using a Linux high performance cluster computer system (32 CPUs). Models were visualized by the VMD program (31). Side chain hydrogen bonds and hydrogen bonds between water molecules (–NH–O=C–, –NH–O<, –OH–O=C–, –OH–O<) were surveyed with criteria of a 3.0-Å bond length and a 160–180° bond angle in VMD program.

Results

Statistics—Results are shown as mean ± S.E. Comparison between the two groups was made using Student’s t test. Statistically significant differences were assumed at p < 0.05 versus control.

RESULTS

Water Transport Activity of Hog Gastric Vesicles—If the pump transports hydronium ions (H₃O⁺) from the cytosolic medium into gastric vesicles or if charge transfer couples with water transport (H₂O), [¹⁸O]H₂O in the cytosolic medium will be transported into the vesicles. And if only the charge transfer is involved in H⁺ pumping, [¹⁸O]H₂O in the cytosolic medium will not be transported.

We measured water transport using stable [¹⁸O]H₂O from the cytosolic medium into the vesicles. The naturally occurring (environmental) content of ¹⁸O is about 2000 ppm (32). In fact, our experimental value was 1998.69 ± 0.23 ppm (n = 3). Taking this background ¹⁸O content into consideration, we added 8 atom % of [¹⁸O]H₂O to a sample solution which contained 1 mg of protein/ml of GI vesicles. After incubating the vesicle solution in the presence and absence of ATP for 20 min, the solution was filtered. The incubation time (20 min) was chosen because protons that had accumulated in the intravesicular space began to leak due to exhaustion of added ATP as determined from the measurement of change in acridine orange fluorescence (supplemental Fig. 1). Trapped [¹⁸O]H₂O on the filter was equilibrated with pure [¹⁶O]CO₂ in a closed chamber. Then the content of [¹⁸O]CO₂ was quantified with a mass spectrometer analysis. The averaged weight of trapped water on the filter was measured using a drying method and was 55.4 ± 0.78 mg (n = 18). Therefore, 123 μg ((55.4 × 10⁻³)/(18 × 20 × 1998.69 ×
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FIGURE 1. $[^{18}O]$H$_2$O transport by gastric H\(^+\),K\(^-\)-ATPase. ATP hydrolysis-dependent water transport in hog GI gastric vesicles was assayed using $[^{18}O]$H$_2$O. One ml of gastric vesicle solution containing 1 mg/ml proteins, 130 mM KCl, 4 mM MgCl$_2$, 10 $\mu$g/ml valinomycin, 8 atom% $[^{18}O]$H$_2$O, and 40 mM PIPES/Tris (pH 6.8) was incubated in the presence (+ATP) or absence (−ATP) of 4 mM ATP for 20 min at 25 °C. Where indicated, SCH28080 was added in the solution (+ATP, +SCH28080). Where indicated, $[^{18}O]$H$_2$O and ATP were not added (−ATP, −$[^{18}O]$H$_2$O). Then sample solutions were filtrated. Content of $[^{18}O]$H$_2$O on the filter was analyzed by a mass spectrum analysis (n = 4). * * p < 0.05.

$10^{-6}) \times 1998.69 \times 10^{-6} \times 20$ of environmental $[^{18}O]$H$_2$O was found on the filter without the addition of $[^{18}O]$H$_2$O (Fig. 1). In a sample solution containing 8 atom% of $[^{18}O]$H$_2$O in the absence of ATP, the content of trapped $[^{18}O]$H$_2$O was higher than that of the above background. This may be due to absorption of $[^{18}O]$H$_2$O to the surface of gastric vesicles and filter. In a sample solution incubated in the presence of 4 mM ATP for 20 min, the trapped $[^{18}O]$H$_2$O was significantly increased by 7.25 $\mu$g per filter, and this increment was completely diminished by the addition of 50 $\mu$m SCH28080 (Fig. 1). Taking a separate experimental result into consideration that ~80% of GI vesicle proteins was trapped on the membrane filter, 9 $\mu$g of $[^{18}O]$H$_2$O was calculated to be trapped per 1 mg of protein of GI vesicles. This trapped $[^{18}O]$H$_2$O was equivalent to 0.45 $\mu$mol (9 × 10^{-6} / 20.0) of $[^{18}O]$H$_2$O. That is, 5.8 $\mu$mol (0.45 × 10^{-6} × 100/8)/0.97) of water molecules were transported per tightly sealed 1 mg of protein of GI vesicles during the ATP hydrolysis reaction. The K\(^-\)-ATPase activity of GI vesicles was 44.2 ± 1.7 $\mu$mol of P$_i$/mg of protein/h (n = 3) and accounted for about 80% of the total ATPase activity in the present vesicle preparation (20% of the total enzyme activity was due to Mg\(^2+\)-ATPase activity). Assuming that 4 mM ATP (4 $\mu$mol ATP/ml sample solution) was almost completely hydrolyzed during the 20-min incubation (for transport, 3.2 $\mu$mol of ATP/ml sample solution), the present experimental results showed that 5.8 $\mu$mol of the cytosolic water was transported during the hydrolysis of 3.2 $\mu$mol of ATP. The coupling ratio between the water transport and ATP hydrolysis was 1.8 (5.8/3.2). This value is close to the theoretical (2.0) and experimental coupling ratios (1.74–2.79) obtained from the change in the extravesicular pH induced by proton uptake into the vesicles in a weak buffer (33). The present results indicate that H\(^+\) transport by gastric H\(^+\),K\(^-\)-ATPase is due to H$_2$O movement or to charge transfer, which is coupled with H$_2$O movement. Effects of site-directed mutagenesis in H\(^+\),K\(^-\)-ATPase on the water transport activity could not be evaluated because the membrane preparations from HEK293 cells were not tightly sealed as described previously (7).

Proton Transport Pathway of the Gastric H\(^+\),K\(^-\)-ATPase α-Subunit from the Cytosolic Side to Cation Binding Site 2—Here we explored the possibility that charge transfer pathway is involved in the H\(^+\) transport. The molecular dynamics simulation in the presence of 14 neutralizing Na\(^+\) ions and about 5000 water molecules showed the presence of side-chain hydrogen bonds that are necessary for charge transfer. We found that a charge transfer pathway (H$_2$O\(^-\)-Lys-164—Gln-161—Glu-345) toward site 2 is possible, where Lys-164 on the M2 stalk is involved in the charge transfer (blue dotted lines). Red dotted lines show the hydrogen bonds between O– and HO– of the water molecules.

FIGURE 2. Charge transfer pathway found in the wild type α-subunit of gastric H\(^+\),K\(^-\)-ATPase. This three-dimensional structure model was constructed based on the E$_2$ conformation of Ca\(^2+\)-ATPase. The charge transfer pathway is H$_2$O\(^-\)—Lys-164—Gln-161—Glu-345, which is marked with orange arrows. One hydrogen bond between –O of cytosolic water molecule and HN of Lys-164 and two side chain hydrogen bonds between –NH and O=C are involved in the charge transfer (blue dotted lines). Red dotted lines show the hydrogen bonds between O– and HO– of the water molecules.
Glu-345 is relayed to the water molecule in site 2, which results in formation of H$_3$O$^+$. Next, we simulated charge transfer pathway in mutant K164L and K164A and found that hydrogen bonds between Leu-164 and Gln-161 and between Ala-164 and Gln-161 are not formed, but an alternative shorter charge transfer pathway (H$_3$O$^+$$\rightarrow$Gln-161$\rightarrow$Glu-345) is possible (Fig. 3). Also, we simulated charge transfer pathways in mutant Q161L (Fig. 4A) and E345L (Fig. 4B), but no charge transfer pathway was found.

The SCH28080-sensitive K$^+$/ATPase Activity—The H$^+$/K$^+$/ATPase \(\alpha\)-subunit of the wild type and single site mutants K164A, K164L, Q161L, and E345L were stably expressed in HEK293 cells together with the wild type \(\beta\)-subunit. Fig. 5 (A and B) shows the expression level of the \(\alpha\)-subunit of mutants and the wild type in the membrane preparations from these cells. Fig. 5C shows the SCH28080-sensitive K$^+$/ATPase activity in these preparations. The mutant expression levels were 75–122% that of the wild type level. Cell surface biotinylation experiments showed that there is no significant difference between cell surface expression levels of the wild type and these mutants (Fig. 6, A and C). Sugar chains of the \(\beta\)-subunit in the cell surface were of only a complex type, whereas the cell lysate contained both high mannose and complex types (Fig. 6B) (22). Cell surface expressions of the \(\alpha\)- and \(\beta\)- subunits were also confirmed by fluorescence immunocytochemistry. The \(\alpha\)-subunit in the wild type and mutant K164L, K164A, Q161L, and E345L was co-localized with \(\beta\)-subunit in the plasma membrane (supplemental Fig. 2).

The K$^+$/ATPase activity of the wild type was 0.56 ± 0.02 \(\mu\)mol of Pi/mg protein/h \((n = 5)\). Fig. 5C shows that the K$^+$/ATPase activity of mutant K164L and K164A are about half and one-third of the wild type level, respectively. Mutant Q161L and E345L showed no K$^+$/ATPase activity. Fig. 7 shows the pH profiles of the SCH28080-sensitive K$^+$/ATPase activities of the wild type, mutant K164L, and K164A. The pH profiles of the K$^+$/ATPase activity showed that the enzyme activities of mutant K164L and K164A were smaller than the wild type level, especially in the alkaline pH range. \(K_m\) values for K$^+$ were 0.73 ± 0.04 mM \((n = 4)\) for the wild type and 0.67 ± 0.06 mM \((n = 5)\) for mutant K164L, respectively (supplemental Fig.

**FIGURE 3.** A shorter charge transfer pathway found in mutant K164L of gastric H$^+$/K$^+$/ATPase. Leu-164 is not involved in the proton charge transfer. The initial proton transfer starts from the H$_3$O$^+$ molecule to Gln-161 via one hydrogen bond between –OH and O=C– (red dotted line). Then, charge is transferred to Glu-345 via the hydrogen bonds between –NH and O=C– (blue dotted line).

**FIGURE 4.** Molecular dynamics simulation of mutant Q161L (A) and E345L (B) in the E$_l$ conformation. No charge transfer to Glu-345 (A) or to Leu-345 (B) in binding site 2 occurred in both mutants. Orange arrows in B indicate charge movement until Gln-161.
shows that the hydrogen bond is not formed between Lys-164 and Gln-161, but an alternative transfer pathway (H_3O^+—Arg-105—Gln-161—Glu-345) is possible (Fig. 8). This mutation from Glu to Gln results in no formation of H_3O^+ in site because site loses the cation binding property. But the charge on Gln-345 will be transferred finally to Glu-797 in cation binding site 1, as shown in the next section. These simulation results are in parallel with the results of the K^+–ATPase activity of these mutants.

The Charge Transfer from H_3O^+ in Site 2 to H_2O in Site 1 —The present three-dimensional structure model of H^+–K^+–ATPase shows that the distance between two cation binding sites (1.8 nm) is greater than that of Ca_2^+–ATPase (0.6 nm). In site 2, one water molecule resides near Glu-345. In site 1, a water molecule resides near Asp-794, and another water molecule resides near Glu-797.

**DISCUSSION**

As a likely mechanism for proton transport through biomembranes, charge transfer is well known (36), for an example, in bacteriorhodopsin (37). Charge transfer involves water molecules and side chains of acidic (electron acceptor), basic (electron donor), and polar amino acid residues. Main chain amide —NH—CO— can form hydrogen bonds for formation of α-helix or β-sheet structures, but charges cannot transfer along the amide bond under normal conditions. Exceptionally, a main chain amide is involved in the charge transfer of cytochrome c oxidase (38).

In gastric H^+–K^+–ATPase, our molecular dynamics simulation has shown the presence of the charge transfer pathway from the cytosolic space to cation binding site 2 (H_3O^+—Lys-164—Gln-161—Glu-345). It includes typical K and D paths; that is, Lys-164 (electron donor) and Gln-161 because of the shorter length of the side chain of Asp compared with that of the Glu residue (supplemental Movie 2). On the other hand, the simulation of mutant E345Q

In addition, a third water molecule is trapped by hydrogen bond with either Asp-826 or Lys-793, as also found by others (35). Besides these four water molecules, several water molecules are present in the regions of these sites. Fig. 9 shows our simulation results for the wild type H^+–K^+–ATPase. The charge of Glu-345 is transferred to H_2O in site 2 and then to a water wire which is composed of two water molecules. The charge is transferred from the water wire to the trapped water, then to a water molecule that interacts with Asp-794. Finally, this charge is transferred to the water molecule in site 1 via a charge transfer pathway (H_3O^+—Asn-794—Glu-797). Hence, both cation binding sites 2 and 1 can house each one molecule of H_3O^+.
These three amino acids of Lys-164, Gln-161, and Glu-345 in gastric H⁺,K⁺-ATPase are conserved in non-gastric H⁺,K⁺-ATPase 2-subunit (human ATP1AL1; GenBank™ accession number P54707, Lys-171, Gln-168, and Glu-352), and the homology of amino acid sequence between rabbit H⁺,K⁺-ATPase α2-subunit and human non-gastric H⁺,K⁺-ATPase α2-subunit is 63.5%. To know whether a similar charge transfer pathway is present in non-gastric H⁺,K⁺-ATPase, we constructed the homology model at its E1 conformation and found that Lys-171, Gln-168, and Glu-352 forms a similar charge transfer pathway (data not shown). These three amino acids are also conserved in Na⁺,K⁺-ATPase of human (human ATP1B1; GenBank™ accession number P05025, Lys-153, Gln-150 and Glu-334), and the homology of amino acid sequence between rabbit gastric H⁺,K⁺-ATPase α1-subunit and human non-gastric H⁺,K⁺-ATPase α1-subunit is 60.3%. However, Lys-153, Gln-150, and Glu-334 did not form a charge transfer pathway (data not shown).

Next, we have found that the charge of H₃O⁺ in site 2 is able to be transported to H₂O in site 1 via the water wire and another charge transfer pathway (H₃O⁺–Asn-794–Glu-797). There is a hydrogen bond network (Asp-826, Lys-793, Gln-794, and Glu-797) in site 1. Lys-793 is unique to H⁺,K⁺-ATPase because Ser substitutes for Lys in Na⁺,K⁺-ATPase and Ca²⁺-ATPase. Asp-826 and Lys-793 are involved in trapping of a water molecule. Glu-797 interacts with a water molecule in site 1. The charge in site 2 is transferred to Glu-797 via the water wire and the charge transfer pathway, and the charge is transferred from Glu-797 to a water molecule in site 1 forming H₃O⁺, which will be transported into lumen together with another H₃O⁺ in site 2 during the conformational transition from E₁P to E₂P. We simulated charge transfer in mutant K164L and K164A. No hydrogen bond formation was formed between Leu/Ala-164 and Gln-161, but an alternative charge transfer pathway (H₃O⁺–Gln-161–Glu-345) was formed (Fig. 4). Corresponding to these simulations, mutant K164L and K164A retained half and one-third of the K⁺-ATPase activity of the wild type, respectively. These mutants also showed lower Rb⁺ transport activities compared with the wild type activity. Affinity for K⁺ was not altered by mutation in mutant K164L. pH profiles of the activity of the mutants show that K⁺-ATPase activities of the mutants are smaller than the wild type level especially in alkaline pH. In the wild type and mutant K164L and K164A, the side chain of Gln-161 is crucial and serves as a relay point of charge transfer. In fact, mutant Q161L lost both the K⁺-ATPase and Rb⁺ transport activities.
Mutations at the position of Glu-345 of the H⁺,K⁺-ATPase α-subunit have shown interesting results (7, 12, 34). Mutant E345A, E345I, E345L, E345V, and E345K lost the K⁺-ATPase activity reflecting the absence of charge acceptor in site 2. Mutant E345D also lost the K⁺-ATPase activity. Our simulation of mutant E345D shows inability of hydrogen bond formation between Gln-161 and Asp-345 because length of the Asp side chain is too short to form the hydrogen bond. On the other hand, mutant E345Q retained about half of the K⁺-ATPase activity. Our simulation of mutant E345Q has shown that a long charge transfer pathway (H₃O⁺–Arg-105–Gln-161–Gln-345–H₂O) is possible.

Previously, it was reported that Glu-797 might play a role in H⁺ binding in E₁ conformation, and the K⁺-ATPase activity in mutant E797Q was similar to the wild type level (14). Although our
simulation of mutant E797Q has shown that the charge transfer from the water wire to Gln-797 is possible, H$_3$O$^+$ will not be formed in site 1 because of loss of the cation binding property. However, Glu-822 in this mutant can interact newly with a water molecule near cation binding site 2 in addition to the Glu-345 interaction with another water molecule, forming two H$_2$O$^+$ in site 2 (only one H$_2$O$^+$ in site 2 of the wild type) (data not shown), and these two H$_2$O$^+$ molecules will be transported into lumen. Glu-822 (aspartate residue) of H$^+$/K$^+$-ATPase is not conserved in rabbit Na$^+$/K$^+$-ATPase α-subunit (aspartate residue) or rabbit SR Ca$^{2+}$/ATPase (aspartate residue). These simulation results are in parallel with those of K$^+$-ATPase activity. The mutations described in this paper have revealed interesting roles of charge transfer pathways in proton transport, but it is noted that these mutations also induce changes in K$^+$ binding properties at these sites in the E$_2$ conformation.

From $^{18}$O/H$_2$O transport experiments in gastric vesicles, we have found that the coupling ratio between water transport and ATP hydrolysis is almost equivalent to the coupling ratio between proton transport and ATP hydrolysis reported previously (33). This indicates that H$^+$ transport involves H$_2$O$^+$ transport and/or charge transfer, which is coupled with H$_2$O transport. Because the present mutant studies of the α-subunit have clearly shown involvement of the charge transfer in the H$^+$/K$^+$-ATPase reaction, a plausible mechanism is that H$_2$O molecules move from the cytosolic medium into cation binding sites 2 and 1, whereas the charge transfer pathways supply charges into these sites, which results in formation of H$_2$O$_3^-$.

Hence, the H$^+$/K$^+$-ATPase α-subunit in the E$_1$ conformation can accept two charged H$_2$O$^+$ molecules. The coupling of the charge flow with the H$_2$O flow at the ratio of 1 to 1 and the acceptance of each one H$_2$O molecule in the sites per one cycle of the enzyme reaction would function as a rate-limiting step. Then the H$_2$O$^+$ in the sites would be transported into the lumen during the conformational transition from E$_3$P to E$_2$P because we found no charge transfer pathway between the cation binding sites and the lumen (simulated both at E$_1$ and E$_2$ conformations, data not shown). Fig. 10 shows the present model for proton transport in the gastric H$^+$/K$^+$-ATPase α-subunit.

Here, we did not pursue the H$_2$O pathway from the cytosolic medium to the cation binding sites in the E$_1$ conformation and the H$_2$O$^+$ pathway from the cation binding sites to the lumen. Molecular dynamics simulation including lipid bilayer molecules would be necessary especially for the survey of H$_2$O pathway.

In conclusion, we have found that charge on H$_2$O$^+$ in cytosolic medium is transferred to two H$_2$O in cation binding sites via charge transfer pathways and a water wire. The H$_2$O molecules in the sites are separately transported from the cytosolic medium. Then the H$_2$O$^+$ in the sites is transported to the lumen during the conformational transition from E$_3$P to E$_2$P.

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