J. Biochem. 127, 993–1000 (2000)

Mutational Analysis of the Putative K^+-Binding Site on the Fourth Transmembrane Segment of the Gastric H^+,K^+-ATPase[1]

Shinji Asano,*[2] Ryoko Furumoto,† Yasuhiro Tega,† Saiko Matsuda,† and Noriaki Takeguchi†

Molecular Genetics Research Center and †Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194

Received December 7, 1999; accepted March 13, 2000

By means of a functional expression system and site-directed mutagenesis, we analyzed the role of the putative K^+-binding site, Glu-345, located in the fourth transmembrane segment of the gastric H^+,K^+-ATPase α-subunit. In the present study, we used several mutants, with alanine, isoleucine, leucine, glutamine, valine, lysine, and aspartic acid instead of Glu-345, and analyzed the H^+,K^+-ATPase partial reactions of the mutants to determine the precise role of this residue. All the mutants except E345Q exhibited no H^+,K^+-ATPase activity. The E345Q mutant showed 3-times higher affinity for ATP. This mutation shifted the optimum pH toward a more alkaline one. The E345A, E345I, E345L, E345V as well as E345Q mutants were phosphorylated with ATP as in the case of the wild-type H^+,K^+-ATPase, whereas the E345K mutant was not phosphorylated. The E345Q mutant was dephosphorylated in the presence of K^+, but its affinity for K^+ was significantly lower than that of the wild type. The E345A, E345I, E345L, and E345V mutants did not exhibit sensitivity to K^+ in the dephosphorylation step below 3 mM K^+. Therefore, Glu-345 is important for the conformational change induced by K^+, especially in the dephosphorylation step in which K^+ reacts with the enzyme from the luminal side with high affinity and accelerates the release of inorganic phosphate. The glutamic acid in the fourth transmembrane segment is conserved, and was found to be involved in the cation-induced conformational change in H^+,K^+-ATPase as well as Na^+,K^+-ATPase and Ca^{2+}-ATPase, however, the precise roles of the side chain in the function were different.

Key words: gastric acid secretion, H^+, K^+-ATPase, ion-selectivity, proton pump, site-directed mutagenesis.

H^+,K^+-ATPase is the proton pump responsible for gastric acid secretion (*1*). This pump belongs to the family of P-type ATPases including Na^+,K^+-ATPase, and plasma membrane and sarco(endoplasmic) reticulum Ca^{2+}-ATPases (*2*). One of the major questions regarding the reaction mechanisms of ion pumps is where the channel-like structure and the binding sites for translocated ions are located in the pump molecules. Recently, the structure-function relationship of these ion pumps was extensively studied by means of the site-directed mutagenesis technique. In sarcoplasmic Ca^{2+}-ATPase, several acidic or polar amino acid residues in the fourth (M4), fifth (M5), sixth (M6), and eighth (M8) transmembrane segments (Glu-309, Glu-771, Asn-796, Thr-799, Asp-800, and Glu-908 in the Ca^{2+}-ATPase) were reported to be involved in the high affinity binding with Ca^{2+} (*3*, *4*). In Na^+,K^+-ATPase, several acidic amino acid residues located in the M4, M5, M6, and M8 transmembrane segments (Glu-327, Glu-778, Asp-803, Asp-807, and Asp-925 in rat α2) were studied as candidates for the sites recognizing or interacting with Na^+ and K^+ (*5*). These amino acid residues in Na^+,K^+-ATPase can be divided into two groups; one is critical for the enzyme activity and not able to be replaced with other amino acids (Asp-803 and Asp-807, or the corresponding residues in other α-isoforms), and the other tolerates isosteric or isocharge replacements (Glu-327, Glu-778, and Asp-925, or the corresponding residues in other α-isoforms) (*5–8*). Recently, we reported that Glu-345 in the M4 segment, and Glu-822 in the M6 segment of gastric H^+,K^+-ATPase were responsible for determining the affinity for K^+ (*9*, *10*). These residues belong to the latter category. Replacement of Glu-345 by glutamine (E345Q mutant) reduced the enzyme activity by 50%, while the mutations with aspartic acid, valine, or lysine abolished the activity. The E345Q mutant showed 10-times lower affinity for K^+ than the wild type (*9*). In the present study, we precisely investigated (i) properties such as partial reactions of several mutants as to Glu-345 of H^+,K^+-ATPase (phosphorylation and dephosphorylation), and (ii) the affinity for ATP and the pH dependence of the partially active mutant E345Q. The glutamic acid in the M4 segment is well conserved in P-type ATPases such as Na^+,K^+-ATPase and Ca^{2+}-ATPase. We compared the roles of Glu-345 in the cat-

[1]This study was supported in part by Grants-in-Aid for Scientific Research (to S.A. and N.T.) from the Ministry of Education, Science, Sports and Culture of Japan, and a fellowship from the Fujisawa Research Foundation (to S.A.).

[2]To whom correspondence should be addressed. Tel: +81-76-434-7187, Fax: +81-76-434-5176, E-mail: shinji@ms.toyama-mpu.ac.jp

Abbreviation: SCH 28080, 2-methyl-8-(phenylmethoxy)imidazo[1,2-α]pyridine-3-acetonitrile.

ion recognition and conformational change with those of the corresponding glutamic acid in Na^+,K^+-ATPase and Ca^{2+}-ATPase.

EXPERIMENTAL PROCEDURES

Materials—HEK-293 cells (human embryonic kidney cell line) were a kind gift from Dr. Jonathan Lytton (University of Calgary, Calgary, Canada). The pcDNA3 vector was obtained from Invitrogen (San Diego, CA). The MutanK kit was from Takara Shuzo (Ohtsu). Vent DNA polymerase was obtained from New England Biolabs (Beverly, MA). Restriction enzymes and other DNA modifying enzymes were from Toyobo (Osaka), New England Biolabs., Life Technologies, or Amersham Pharmacia-Biotech. (Tokyo). 2-Methyl-8-(phenylmethoxy)imidazo[1,2-α]pyridine-3-acetonitrile (SCH 28080) was obtained from Schering-Plough (Kenilworth, NJ). All other reagents were of molecular biology grade or the highest grade of purity available.

cDNAs of α- and β-Subunits of H^+,K^+-ATPase—cDNAs of the α- and β-subunits of H^+,K^+-ATPase were prepared from rabbit gastric mucosae as described elsewhere (*9*). The α- and β-subunit cDNAs were digested with *Eco*RI and *Xho*I. The obtained fragments were each ligated with the pcDNA3 vector treated with *Eco*RI and *Xho*I.

DNA Sequencing and Site-Directed Mutagenesis—DNA sequencing was performed by the dideoxy chain termination method using an Autocycle DNA sequencing kit and an ALFexpress DNA sequencer (Amersham Pharmacia-Biotech). The introduction of site-directed mutations in the H^+,K^+-ATPase α-subunit was carried out as described elsewhere using the MutanK kit (*9*, *11*).

Cell Culture, Transfection, and Preparation of Membrane Fractions—Cell culture of HEK-293 cells was carried out as described previously (*9*). α- and β-subunit cDNA transfection was performed by the calcium phosphate method with 10 μg of cesium chloride-purified DNA (molar ratio of α- and β-subunit cDNA constructs, 1:1.3) per 10 cm dish. The resulting transfected cells comprised a mixture of α-expressing, β-expressing, and α,β-expressing cells. Cells were harvested 2 days after the cDNA transfection. Membrane fractions of HEK cells were prepared as described previously (*9*). Briefly, cells in a 10 cm Petri dish were washed with PBS, and then incubated with 2 ml of an extremely hypotonic buffer [0.5 mM $MgCl_2$, 10 mM Tris/HCl (pH 7.4)] at 0°C for 10 min. After the addition of phenylmethylsulfonyl fluoride (1 mM) and aprotinin (0.09 unit/ml), the cells were homogenized in a Dounce homogenizer, and then the homogenate was diluted with an equal volume of a solution comprising 500 mM sucrose and 10 mM Tris/HCl (pH 7.4). The homogenized suspension was centrifuged at 800 ×*g* for 10 min. The supernatant was centrifuged at 100,000 ×*g* for 90 min, and the resulting pellet was suspended in a solution comprising 250 mM sucrose and 5 mM Tris/HCl (pH 7.4).

SDS–Polyacrylamide Gel Electrophoresis and Immunoblotting—SDS–polyacrylamide gel electrophoresis was carried out as described elsewhere (*12*). Membrane preparations (30 μg protein) were incubated in a sample buffer comprising 2% SDS, 2% β-mercaptoethanol, 10% glycerol, and 10 mM Tris/HCl (pH 6.8) at room temperature for 2 min, and then applied to an SDS–polyacrylamide gel. Immunoblotting was carried out as described previously (*9*).

Antibodies—Ab1024 was previously raised against the carboxy terminal peptide (residues 1024–1034) of the H^+,K^+-ATPase α-subunit (PGSWWDEELYY) (*13*). Monoclonal antibody 2B6, derived from the splenocytes of mice with autoimmune gastritis, was purchased from Molecular Biological Laboratories (Nagoya).

Assaying of H^+,K^+-ATPase Activity and Protein—H^+,K^+-ATPase activity was assayed as the decrease in the amount of NADH coupled with regeneration of ATP from ADP ("coupled-enzyme assay") in 1.2 ml of a reaction mixture comprising 50 μg membrane protein, 3 mM $MgCl_2$, 800 μM ATP (sodium salt), 160 μM NADH, 0.8 mM phosphoenolpyruvate, 3 units/ml pyruvate kinase, 2.75 units/ml lactate dehydrogenase, 5 mM NaN_3, 1 mM ouabain, 15 mM KCl, and 40 mM Tris/HCl (pH 6.8). The decrease in the amount of NADH was measured at 37°C as the absorbance at 340 nm with a Beckman spectrophotometer as described elsewhere (*14*). H^+,K^+-ATPase activity, defined as that of the SCH 28080-sensitive K^+-ATPase, was calculated as the difference between the K^+-ATPase activities in the presence and absence of 50 μM SCH 28080.

When the K^+-ATPase activity was measured as a function of pH, the ATPase activity was assayed by measurement of inorganic phosphate released from ATP. K^+-ATPase activity was measured in 1 ml of a solution comprising 50 μg membrane protein, 3 mM $MgCl_2$, 1 mM ATP (Tris salt), 5 mM NaN_3, 2 mM ouabain, 15 mM KCl, and 40 mM MES/Tris (pH 5.5–7.0) or Tris/HCl (pH 7.0–8.5) in the presence and absence of 50 μM SCH 28080. After incubation at 37°C for 30 min, the inorganic phosphate released was determined from the absorbance at the wavelength at 320 nm as described elsewhere (*15*). The K^+-ATPase activity was calculated as the difference between the activities in the presence and absence of 50 μM SCH 28080. Inorganic phosphate released in the enzyme reaction with the wild-type H^+,K^+-ATPase was 5–6 times higher than the background level of inorganic phosphate released in the absence of the enzyme at pH 6.8. The K^+-ATPase activity values determined on colorimetric assaying of released inorganic phosphate were comparable to those obtained with the coupled enzyme assay at neutral pH. When the K^+-ATPase activity was measured in the presence of ATP (Tris salt) and in the absence of Na^+, 5 μM oligomycin was added to the medium instead of NaN_3.

Phosphorylation and Dephosphorylation—Fifty microgram portions of membrane proteins were phosphorylated in 110 μl of a solution comprising 1 μM ATP ([γ-^{32}P]ATP 4 × 10^6 cpm), 2 mM $MgCl_2$, 1 mM EGTA, 3 mM ouabain, and 40 mM Tris/HCl (pH 6.8 or 8.0) at 0°C for 10 s. The reaction was quenched by the addition of 590 μl of an ice-cold stop solution containing 10% trichloroacetic acid and 10 mM inorganic phosphate. The protein was collected by centrifugation at 13,000 ×*g* at 4°C for 3 min, and the pellet was washed with 500 μl of the ice-cold stop solution and 30% sucrose successively, and then solubilized in a sample buffer comprising 2% SDS, 2.5% dithiothreitol, 10% glycerol, and 50 mM Tris/HCl (pH 6.8), and subjected to SDS–polyacrylamide gel electrophoresis under acidic conditions at pH 6.5 (*16*). The radioactivity associated with the separated H^+,K^+-ATPase α-subunit was visualized and quantified by digital autoradiography of the dried gels using a Bio-Imaging Analyzer BAS2000 (Fuji Photo Film, Tokyo).

Membrane protein was phosphorylated as mentioned

above, followed by incubation with various concentrations of KCl or ADP and 1 mM non-radioactive ATP at 0°C for 10 s. Portions of the phosphorylated proteins were dephosphorylated in the presence of K^+ or ADP. The reaction was stopped as described above, and the samples were run on an SDS–polyacrylamide gel. The radioactivity associated with the H^+,K^+-ATPase α-subunit was visualized as described above.

RESULTS

Mutation of Glu-345 to Hydrophobic Amino Acids—Glutamic acid-345 (Glu-345) in the M4 segment of the rabbit gastric H^+,K^+-ATPase α-subunit is involved in determination of the affinity for K^+ (*9*). In the previous study, we observed that on the mutation of Glu-345 of gastric H^+,K^+-ATPase to glutamine (isosteric mutant, E345Q) 50% of the K^+-ATPase activity was retained, whereas other mutants, E345V, E345D, and E345K, lost the activity (*9*). However, it is not clear whether Glu-345 can be replaced by hydrophobic amino acids with different sizes of side chain other than valine.

In the present study, we newly prepared alanine (E345A), isoleucine (E345I), and leucine (E345L) mutants as to Glu-345, and examined their functions. The expression patterns and levels of all the mutant α-subunits and the co-expressed β-subunits were not significantly different from in the case of the wild-type enzyme, as judged from the results of immunoblotting with anti–α and anti–β-subunit antibodies (data not shown). However, the H^+,K^+-ATPase activities (both K^+-stimulated ATPase activity and SCH 28080–sensitive K^+-ATPase activity) of all the mutants except E345Q were judged to be completely lost because the enzyme activities of the mutants were not significantly different from the activity in the membrane preparation of cells transfected only with β-subunit cDNA (data not shown).

Phosphorylation Capacity of Glu-345 Mutants—Membrane fractions of cells expressing the wild-type α- and β-subunits were phosphorylated with [γ-^{32}P]ATP in the presence of ouabain (to inhibit endogenous Na^+,K^+-ATPase present in the membrane fraction), separated on an SDS–polyacrylamide gel under weakly acidic conditions, and then the patterns of the phosphorylated proteins were observed. With this membrane fraction, several radioactive bands were observed; doublet bands corresponding to a molecular mass of 100 kDa and a band corresponding to a molecular mass of 60 kDa (Fig. 1A). With the membrane fraction of mock-transfected cells, the lower doublet band corresponding to a molecular mass of 100 kDa was hardly observed, whereas the upper one was significantly observed. A hog gastric vesicle preparation rich in gastric H^+,K^+-ATPase was also phosphorylated with ATP and the phosphorylated proteins on the gel were examined. This H^+,K^+-ATPase α-subunit moved to the same position as the lower doublet band observed for the membrane fraction of

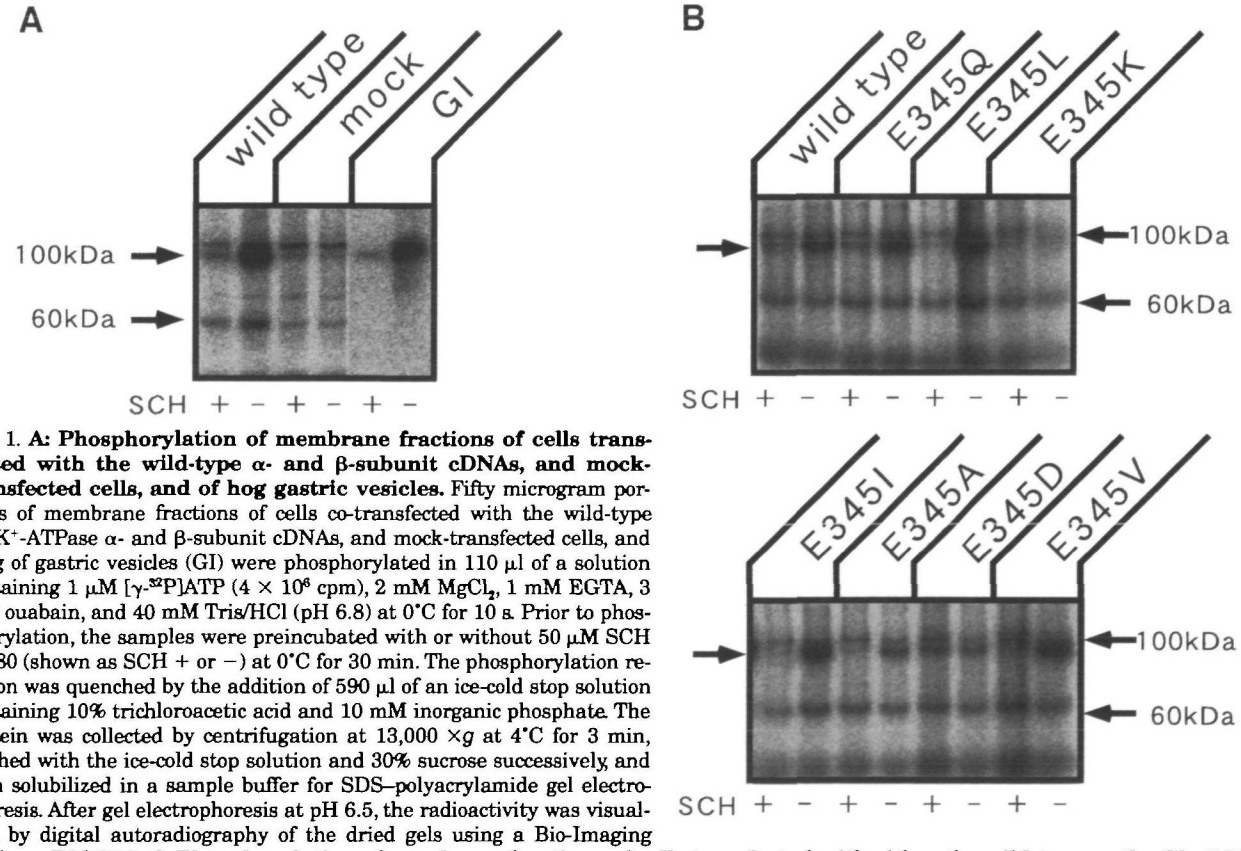

Fig. 1. **A: Phosphorylation of membrane fractions of cells transfected with the wild-type α- and β-subunit cDNAs, and mock-transfected cells, and of hog gastric vesicles.** Fifty microgram portions of membrane fractions of cells co-transfected with the wild-type H^+,K^+-ATPase α- and β-subunit cDNAs, and mock-transfected cells, and 1 μg of gastric vesicles (GI) were phosphorylated in 110 μl of a solution containing 1 μM [γ-^{32}P]ATP (4×10^6 cpm), 2 mM $MgCl_2$, 1 mM EGTA, 3 mM ouabain, and 40 mM Tris/HCl (pH 6.8) at 0°C for 10 s. Prior to phosphorylation, the samples were preincubated with or without 50 μM SCH 28080 (shown as SCH + or −) at 0°C for 30 min. The phosphorylation reaction was quenched by the addition of 590 μl of an ice-cold stop solution containing 10% trichloroacetic acid and 10 mM inorganic phosphate. The protein was collected by centrifugation at 13,000 ×*g* at 4°C for 3 min, washed with the ice-cold stop solution and 30% sucrose successively, and then solubilized in a sample buffer for SDS–polyacrylamide gel electrophoresis. After gel electrophoresis at pH 6.5, the radioactivity was visualized by digital autoradiography of the dried gels using a Bio-Imaging Analyzer BAS2000. **B: Phosphorylation of membrane fractions of cells transfected with either the wild-type or the Glu-345 mutant cDNAs.** Fifty microgram portions of membrane fractions of cells co-transfected with the H^+,K^+-ATPase β-subunit cDNA plus wild-type H^+,K^+-ATPase α-subunit, E345Q, E345L, E345K, E345I, E345A, E345D, or E345V mutant cDNA were phosphorylated. Bands representing the H^+,K^+-ATPase α-subunit are indicated by the arrow.

cells expressing the wild-type α- and β-subunits (Fig. 1A). The phosphorylation of these 100-kDa bands was inhibited by 50 μM SCH 28080 (Fig. 1A) or 1 mM sodium vanadate (data not shown). From these findings, it is concluded that the lower 100-kDa bands represent the phosphorylated H^+,K^+-ATPase α-subunit.

Membrane fractions of cells expressing the Glu-345 mutants (E345Q, E345L, E345K, E345I, E345A, E345D, and E345V) were also phosphorylated with [γ-^{32}P]ATP and the patterns of the phosphorylated proteins on the gel were examined (Fig. 1B). Mutants E345Q, E345L, E345I, E345A, E345D, and E345V as well as the wild-type enzyme were also phosphorylated with ATP, whereas mutant E345K was not phosphorylated. The phosphorylation of the mutant α-subunits was inhibited by preincubation with 50 μM SCH 28080 in the cases of the E345Q, E345L, E345I, E345A, and E345V mutants (Fig. 1B). The E345D mutant was phosphorylated even after preincubation with SCH 28080 (Fig. 1B), suggesting that the mutation of Glu-345 to Asp decreased the sensitivity to SCH 28080.

Dephosphorylation Capacity of Glu-345 Mutants—Next, we studied the dephosphorylation capacity of the wild-type H^+,K^+-ATPase and the Glu-345 mutants. Membrane fractions of cells expressing the wild type and Glu-345 mutants were phosphorylated with [γ-^{32}P]ATP followed by incubation with KCl or ADP in the presence of 1 mM non-radioactive ATP at 0°C. In the absence of KCl or ADP, the upper 100-kDa band observed for HEK cells disappeared on incubation with cold ATP. In the case of the wild-type H^+,K^+-ATPase, the phosphorylated α-subunit was almost insensitive to ADP (Fig. 2), however, it was dephoshorylated with the addition of K^+ in a concentration-dependent manner (Fig. 3), indicating that the major form of phosphorylated H^+,K^+-ATPase in the steady state was E_2P (K^+-sensitive phosphorylated intermediate). The half-maximal effective concentration of K^+ for the dephosphorylation was about 0.2 mM (Fig. 3). The phosphorylated α-subunit of the E345Q mutant was also dephosphorylated with the addition of K^+ (Figs. 2 and 3). However, the sensitivity of the phosphorylated enzyme to K^+ in the E345Q mutant was lower than that of the wild type (Fig. 3). The E345L, E345I, E345A, and E345V mutants were dephosphorylated by only 10–20% in the presence of 50 mM K^+ (Fig. 3). The phosphorylated α-subunits of all these mutants were insensitive to 1 mM ADP, and unstable under alkaline conditions (data not shown). Therefore, it is unlikely that E_1P (ADP-sensitive phosphorylated intermediate) accumulated in the reaction cycle due to the mutations introduced at Glu-345. These results suggest that the $E_1P \rightarrow E_2P$ step was not inhibited in these mutants. From these findings, it was concluded that these mutants have some defects in the dephosphorylation step, resulting in the loss of overall H^+,K^+-ATPase activity.

ATP Dependence of the E345Q Mutant—In order to further study the roles of Glu-345 in the reaction cycle and conformational change, we compared the properties of the

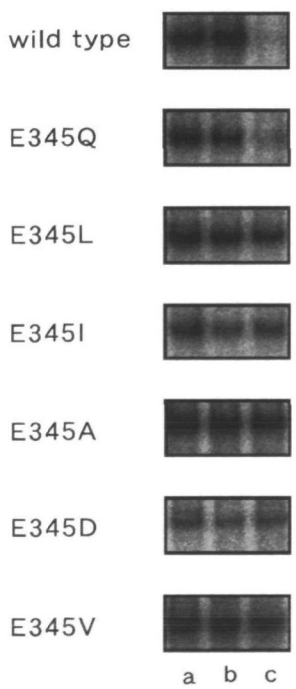

Fig. 2. **ADP and K^+ sensitivity of phosphorylated intermediates of the wild-type H^+,K^+-ATPase and the Glu-345 mutants.** Fifty microgram portions of membrane proteins (wild-type H^+,K^+-ATPase, and E345Q, E345L, E345I, E345A, E345D, E345V mutants) were phosphorylated with 1 μM [γ-^{32}P]ATP at 0°C for 10 s (lane a), followed by incubation with 1 mM non-radioactive ATP with 1 mM ADP (lane b) or 10 mM KCl (lane c) at 0°C for 10 s. The reaction was quenched by the addition of the ice-cold stop solution. The precipitated proteins were separated on an SDS–polyacrylamide gel, and then the radioactivity associated with the H^+,K^+-ATPase α-subunit was visualized by digital autoradiography.

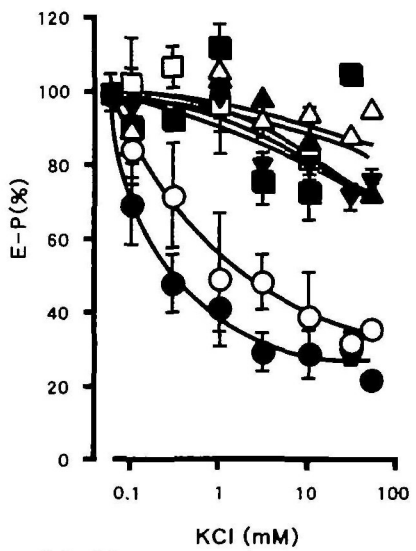

Fig. 3. **Effects of the K^+ concentration on the dephosphorylation reaction of the phosphorylated intermediates of the wild-type H^+,K^+-ATPase and the Glu-345 mutants.** Fifty microgram portions of membrane proteins; the wild-type H^+,K^+-ATPase (●), and E345Q (○), E345L (△), E345I (□), E345A (■), and E345V (▲) mutants, were phosphorylated with 1 μM [γ-^{32}P]ATP at 0°C for 10 s as described in the legend to Fig. 1, followed by incubation with 1 mM non-radioactive ATP with various concentrations of KCl at 0°C for 10 s. The reaction was quenched by the addition of the ice-cold stop solution. The precipitated proteins were separated on an SDS–polyacrylamide gel, and then the radioactivity associated with the H^+,K^+-ATPase α-subunit was visualized by digital autoradiography and expressed as the percentage of the control values measured in the absence of KCl.

partially active mutant, E345Q, with those of the wild-type H⁺,K⁺-ATPase. The E345Q mutant showed similar sensitivity to SCH 28080 to that of the wild-type; the IC_{50} values for SCH 28080 were 1.6 and 2.1 μM at pH 6.8 for the E345Q mutant and wild type, respectively. Figure 4 shows the effects of the ATP concentration on the expressed H⁺,K⁺-ATPase activity of the E345Q mutant and wild type. The affinity of the E345Q mutant for ATP was about 3-times higher than that of the wild-type enzyme ($K_{1/2}$ values, 7.9 and 25 μM, respectively). It is interesting that the affinity for ATP on the cytoplasmic side of the enzyme changed when the amino acid in the transmembrane segment was mutated. It is likely that the conformational change was conducted through the M4 segment. This property of H⁺,K⁺-ATPase is similar to that of Na⁺,K⁺-ATPase. That is, several Na⁺,K⁺-ATPase mutants, of which the affinity for K⁺ was decreased by a mutation in the K⁺-recognition (or K⁺-binding) sites (Glu-329, Glu-781, and Thr-809 in rat kidney α1, and Ser-775 in sheep kidney α1), showed higher affinities for ATP than the wild-type enzyme (*6–8*, *17*).

pH Dependence of the E345Q Mutant—We compared the pH dependency of the expressed H⁺,K⁺-ATPase activity between the E345Q mutant and the wild-type enzyme (Fig. 5). The mutation shifted the pH-dependence curve in the alkaline direction; the pH dependence of the wild-type enzyme showed a bell-shaped curve with maximum activity at pH 6.5 to 7.0, whereas that of E345Q mutant showed a peak at around pH 7.5. At pH 8.0, the mutant showed 60% of the maximal ATPase activity, whereas the wild type showed less than 20% of the maximal ATPase activity. Therefore, the E345Q mutant has a conformation that hydrolyzes ATP more efficiently than in the case of the wild-type enzyme in the presence of a low concentration of protons.

Next, we compared the phosphorylation and dephosphorylation capacities of the wild type and E345Q mutant at pH 6.8 and 8.0 (Fig. 6). At pH 8.0, the wild-type H⁺,K⁺-ATPase α-subunit was phosphorylated with ATP, however, the phosphorylation level was lower than that observed at pH 6.8. It is interesting that the phosphorylation was not inhibited by SCH 28080 at pH 8.0. It is known that SCH 28080 is a potent inhibitor under acidic conditions. The phosphorylated band of the wild-type disappeared on incubation with 1 mM cold ATP in the absence of KCl. The phosphorylation level of the E345Q mutant at pH 8.0 was also slightly lower than that observed at pH 6.8. The phosphorylated E345Q mutant was stable in the presence of cold ATP, and was dephosphorylated in the presence of KCl. Therefore, both the wild-type H⁺,K⁺-ATPase and the E345Q mutant retained their phosphorylation capacity. However, their phosphorylation was insensitive to SCH 28080 at pH 8.0. The phosphoenzyme of the wild type at pH 8.0 was unstable in the absence of KCl compared with that at pH 6.8 (compare lanes 3 and 8 in Fig. 6A), resulting in low K⁺-ATPase activity, as shown in Fig. 5.

It cannot be completely excluded that these changes in pH dependence represent a non-specific effect found in mutants. However, it should be noted that when the seven lysine residues located in the amino-terminal lysine/glycine cluster of the H⁺,K⁺-ATPase α-subunit were mutated to alanine residues, the mutants showed pH dependence similar to that observed for the wild-type H⁺,K⁺-ATPase (Asano *et al.*, submitted). This finding suggests that the differences in pH dependence between the wild type and E345Q mutant presented here are not due to the non-specific effect

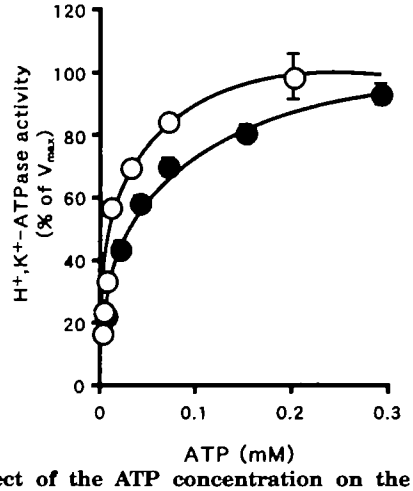

Fig. 4. **Effect of the ATP concentration on the expressed H⁺,K⁺-ATPase activity.** The H⁺,K⁺-ATPase activities of the wild-type (●) and E345Q mutant (○) were measured as a function of the ATP concentration by the coupled enzyme method. The H⁺,K⁺-ATPase activity was expressed as the percentage of the V_{max} value obtained on least-square curve fitting. The V_{max} value is 0.94 ± 0.03 μmol/mg/h for the wild-type enzyme, and 0.56 ± 0.03 μmol/mg/h for the E345Q mutant. The $K_{1/2}$ values are 25 and 7.9 μM for the wild-type enzyme and the E345Q mutant, respectively. The values are the means ± SE for three observations. Several error bars are not visible because of their small size.

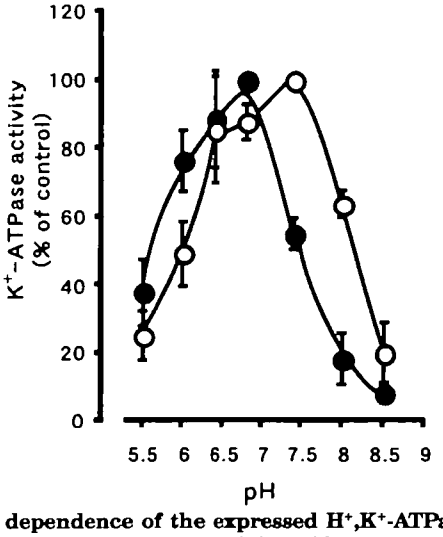

Fig. 5. **pH dependence of the expressed H⁺,K⁺-ATPase activity.** The H⁺,K⁺-ATPase activities of the wild-type enzyme (●) and E345Q mutant (○) were assayed in 1 ml of a solution containing 50 μg membrane protein, 3 mM $MgCl_2$, 1 mM ATP, 5 mM NaN_3, 1 mM ouabain, 15 mM KCl, and 40 mM MES/Tris (pH 5.5–7.0) or Tris/HCl (pH 7.0–8.5) in the presence and absence of 50 μM SCH 28080. Inorganic phosphate released from ATP was measured. H⁺,K⁺-ATPase activity was calculated as the difference between the K⁺-ATPase activities measured in the presence and absence of 50 μM SCH 28080, and expressed as a percentage of the control value measured at pH 6.8 and 7.4 for the wild-type enzyme and E345Q mutant, respectively. The control value is 1.00 ± 0.06 μmol/mg/h for the wild-type enzyme, and 0.54 ± 0.02 μmol/mg/h for the E345Q mutant. The values are the means ± SE for three observations.

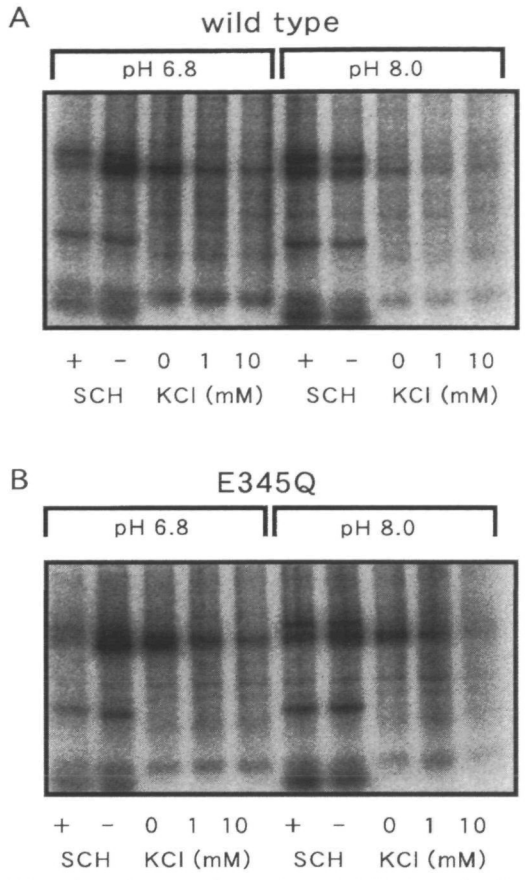

Fig. 6. **Phosphorylation of membrane fraction of cells transfected with either the wild-type or E345Q mutant cDNA at pH 8.0.** Fifty microgram portions of membrane fractions of cells co-transfected with the H^+,K^+-ATPase β-subunit cDNA plus wild-type H^+,K^+-ATPase α-subunit (A) or E345Q mutant cDNA (B) were phosphorylated in 110 μl of a solution containing 1 μM [γ-^{32}P]ATP (4×10^6 cpm), 2 mM $MgCl_2$, 1 mM EGTA, 3 mM ouabain, and 40 mM Tris/HCl (pH 6.8 [lanes 1–5] or 8.0 [lanes 6–10]) at 0°C for 10 s. Prior to phosphorylation, the samples were preincubated with (lanes 1 and 6) or without 50 μM SCH 28080 (lanes 2-5 and 7–10) at 0°C for 30 min. For lanes 1, 2, 6, and 7, the phosphorylation reaction was immediately terminated. For lanes 3–5 and 8–10, the phosphorylation reaction was followed by incubation with 1 mM non-radioactive ATP with 0 (lanes 3 and 7), 1 (lanes 4 and 8), or 10 mM KCl (lanes 5 and 10) at 0°C for 10 s, and then terminated. The phosphorylation reaction was quenched, and the radioactivity of the protein was visualized as shown in Fig. 1.

found after manipulating positively or negatively charged residues.

The K^+-ATPase activity (measured in the presence of oligomycin instead of NaN_3 to remove Na^+ from the standard incubation medium) found for the E345Q mutant was not affected by 30 mM NaCl (data not shown). This result suggests that the E→Q mutation of Glu-345 did not confer the Na^+-sensitivity to the mutant.

DISCUSSION

H^+,K^+-ATPase is a member of the P-type ATPase family which actively transports ions coupled with the hydrolysis of ATP by forming an acid-stable acylphosphate compound as a high-energy intermediate. It has been considered that the catalytic centers of P-type ATPases generally have common structures including the phosphorylation site and the ATP-binding site. The locations of the domains containing their cation recognition sites and transport pathways are also common to some extent (*18*). However, the roles of individual amino acid residues comprising cation recognition sites are supposed to differ with the species of transported cations. In fact, several acidic or polar amino acid residues in the M4, M5, and M6 segments of the catalytic (α) subunits were identified as the binding sites or the sites involved in determination of the affinity for cations in Ca^{2+}-ATPase (*3*, *4*), Na^+,K^+-ATPase (*5–8*), and H^+,K^+-ATPase (*9*, *10*, *19*). Many of these amino acid residues are considerably well conserved among these P-type ATPases (*3*). Among them, we studied the roles of the glutamic acid residue (Glu-345) in the M4 transmembrane segment of gastric H^+,K^+-ATPase.

Glu-345 is well conserved in sarcoplasmic and endoplasmic Ca^{2+}-ATPases, and Na^+,K^+-ATPases (*3*). The corresponding glutamic acid residues in Ca^{2+}-ATPases and Na^+,K^+-ATPases are involved in Ca^{2+}-binding, and in determination of the affinity for Na^+ and K^+, respectively (*3*, *5*, *6*, *20*). However, the detailed roles of these glutamic acid residues are quite different between Na^+,K^+-ATPases and Ca^{2+}-ATPases.

In Ca^{2+}-ATPase, the carboxylate side chain of this residue was indispensable for Ca^{2+} transport. When this residue was replaced by glutamine and aspartic acid, Ca^{2+} transport activity was abolished (*3*, *4*). Several mutational studies have indicated that this glutamic acid provides ligands for one of the two high affinity Ca^{2+}-binding sites in Ca^{2+}-ATPase, which transports 2 mol Ca^{2+} for each mol of ATP hydrolyzed, and contains two Ca^{2+}-binding sites in the membrane (*20–22*). The mutation of this residue to glutamine altered one cytoplasmic Ca^{2+}-binding site in Ca^{2+}-ATPase, leaving the other luminal Ca^{2+}-binding apparently intact.

In Na^+,K^+-ATPase, on the other hand, the carboxylate side chain of the corresponding glutamic acid residue was not essential for Na^+ and K^+ transport, or Na^+,K^+-ATPase activity, because this residue can be replaced by glutamine or even leucine with retention of the Na^+,K^+-ATPase activity (and enough transport activity to maintain cell growth). The glutamine and leucine mutants showed lower affinity for Na^+ and K^+ than the wild-type Na^+,K^+-ATPase (*5*). However, the aspartic acid and alanine mutations abolished the ATPase activity (*5*, *23*). Therefore, neither the negative charge nor the hydrophilic property of the side chain is indispensable for the Na^+,K^+-ATPase function, but rather a proper size of side chain at this position is important for the function of Na^+,K^+-ATPase. The glutamic acid residue was reported to be involved in cation (Na^+ and K^+)-induced conformational changes rather than to provide ligands for cation binding site(s) (*24–27*).

In the present rabbit gastric H^+,K^+-ATPase, Glu-345 is also involved in determination of the affinity for K^+. The glutamine mutant (E345Q) partly retained the H^+,K^+-ATPase activity, whereas the alanine, aspartic acid, isoleucine, leucine, lysine, and valine mutants showed no H^+,K^+-ATPase activity at all. Thus, both the hydrophilic property (not the negative charge) and the proper size of side chain of the glutamic acid at this position are important for the ATPase activity, which is different from in the cases of

Ca^{2+}-ATPase and Na^+,K^+-ATPase.

Among the Glu-345 mutants, many (E345Q, E345L, E345I, E345A, E345D, and E345V) were phosphorylated with ATP. The phosphorylation was inhibited by preincubation with SCH 28080 except in the case of the E345D mutant. These results indicate that these mutants retained the phosphorylation capacity although they (except the E345Q mutant) lost the overall H^+,K^+-ATPase activity. The phosphoenzymes of these mutants (E345L, E345I, E345A, E345D, and E345V) were not effectively dephosphorylated by 10 mM K^+. Therefore, these mutants have some defect in the dephosphorylation step of H^+,K^+-ATPase ($E_2P \rightarrow E_2K$). The properties of these Glu-345 mutants are similar to those of the E822Q mutant of rabbit H^+,K^+-ATPase (E820Q mutant of rat H^+,K^+-ATPase) (*10*, *19*). This E822Q mutant showed no H^+,K^+-ATPase activity and was phosphorylated with ATP, whereas the phosphoenzyme was not dephosphorylated by K^+ (*19*). Both Glu-345 and Glu-822 are important for determination of the affinity for K^+. At Glu-345, an E→Q mutation is tolerated but E→A, D, and L mutations are not, whereas at Glu-822, E→A, and D mutations are tolerated but E→Q and L mutations are not. This opposite toleration except for leucine mutations at Glu-345 and Glu-822 suggest that these glutamic acids are involved in the formation of different parts of a K^+-binding pocket, possibly the entrance and end of the pocket, respectively, as previously suggested for Ca^{2+}-ATPase (*28*).

The E345Q mutant showed apparently lower affinity for K^+ and higher affinity for ATP together with a larger optimal pH value. At present, it is difficult to conclude whether Glu-345 is a part of binding sites for K^+ and protons or not. However, the properties of the E345Q mutant presented here are in good agreement with the view that the mutation shifted the conformational equilibrium toward the E_1 form. These findings are also in agreement with the idea that the M4 segment is involved in the conformational change in the energy transduction between cation transport and ATP hydrolysis, as reported for Ca^{2+}-ATPase and Na^+,K^+-ATPase (*29*, *30*).

There are several mutants of Na^+,K^+-ATPase which modify the affinity for Na^+ and K^+ (*5–7*, *17*). Many mutants show lower affinity for both Na^+ and K^+ (Glu-327 in sheep α1, and Glu-781 and Thr-809 in rat α1), whereas a few of them show lower affinity for K^+ without any effect on the affinity for Na^+ (Ser-775 in sheep α1) (*17*). These results suggest that many of these residues involved in K^+ recognition also play roles in Na^+ recognition. In fact, the mutation of Glu-327 in sheep Na^+,K^+-ATPase with glutamine and leucine decreased the affinity for both Na^+ and K^+ (*5*). In H^+,K^+-ATPase, mutation of Glu-345 to glutamine decreased the affinity for K^+ and shifted the optimal pH toward a more alkaline one (apparently increasing the affinity for proton) compared with the wild-type enzyme.

In conclusion, mutation of Glu-345 with glutamine decreased the affinities for K^+, increased the affinities for ATP, and shifted the pH-dependence curve toward a more alkaline pH. Glu-345 plays a role in the cation (K^+)-induced conformational change, which is common to H^+,K^+-ATPase, Na^+,K^+-ATPase, and Ca^{2+}-ATPase, however, the precise role of the side chain of this residue differs among them.

We wish to thank Dr. Hiroshi Suzuki in Asahikawa Medical College for the technical advice. We also thank Toshiro Io for his technical assistance. We are also grateful to Dr. Michael Caplan for careful reviewing of the manuscript.

REFERENCES

1. Wallmark, B., Larsson, H., and Humble, L. (1985) The relationship between gastric acid secretion and gastric H^+,K^+-ATPase activity. *J. Biol. Chem.* **260**, 13681–13684
2. Møller, J.V., Juul, B., and le Maire, M. (1996) Structural organization, ion transport, and energy transduction of P-type ATPases. *Biochim. Biophys. Acta* **1286**, 1–51
3. Clarke, D.M., Loo, T.W., Inesi, G, and MacLennan, D.H. (1989) Location of high affinity Ca^{2+}-binding sites within the predicted transmembrane domain of the sarcoplasmic reticulum Ca^{2+}-ATPase. *Nature* **339**, 476–478
4. Clarke, D.M., Loo, T.W., and MacLennan, D.H. (1990) Functional consequences of alterations to polar amino acids located in the transmembrane domain of the Ca^{2+}- ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* **265**, 6262–6267
5. Jewell-Motz, E.A. and Lingrel, J.B. (1993) Site-directed mutagenesis of the Na,K-ATPase: consequences of substitutions of negatively-charged amino acids localized in the transmembrane domains. *Biochemistry* **32**, 13523–13530
6. Vilsen, B. (1993) Glutamate 329 located in the fourth transmembrane segment of the α-subunit of the rat kidney Na^+,K^+-ATPase is not an essential residue for active transport of sodium and potassium ions. *Biochemistry* **32**, 13340–13349
7. Vilsen, B. (1995) Mutant Glu781→Ala of the rat kidney Na^+,K^+-ATPase displays low cation affinity and catalyzes ATP hydrolysis at a high rate in the absence of potassium ion. *Biochemistry* **34**, 1455–1463
8. Koster, J.C., Blanco, G., Mills, P.B., and Mercer, R.W. (1996) Substitutions of glutamate 781 in the Na,K-ATPase α subunit demonstrate reduced cation selectivity and an increased affinity for ATP. *J. Biol. Chem.* **271**, 2413–2421
9. Asano, S., Tega, Y., Konishi, K., Fujioka, M., and Takeguchi, N. (1996) Functional expression of gastric H^+,K^+-ATPase and site-directed mutagenesis of the putative cation binding site and the catalytic center. *J. Biol. Chem.* **271**, 2740–2745
10. Asano, S., Matsuda, S., Tega, Y., Shimizu, K., Sakamoto, S., and Takeguchi, N. (1997) Mutational analysis of putative SCH 28080 binding sites of the gastric H^+,K^+-ATPase. *J. Biol. Chem.* **272**, 17668–17674
11. Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**, 488–492
12. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
13. Asano, S., Arakawa, S., Hirasawa, M., Sakai, H., Ohta, M., Ohta, K., and Takeguchi, N. (1994) C-terminal topology of gastric H^+,K^+-ATPase. *Biochem. J.* **299**, 59–64
14. Morii, M., Hayata, Y., Mizoguchi, K., and Takeguchi, N. (1996) Oligomeric regulation of gastric H^+,K^+-ATPase. *J. Biol. Chem.* **271**, 4068–4072
15. Yoda, A. and Hokin, L.E. (1970) On the reversibility of binding of cardiotonic steroids to a partially purified (Na + K)-activated adenosinetriphosphatase from beef brain. *Biochem. Biophys. Res. Commun.* **40**, 880–886
16. Weber, K. and Osborn, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**, 4406–4412
17. Argüello, J.M. and Lingrel, J.B. (1995) Substitutions of serine 775 in the α subunit of the Na,K-ATPase selectively disrupt K^+ high affinity activation without affecting Na^+ interaction. *J. Biol. Chem.* **270**, 22764–22771
18. Luckie, D.B., Lemas, V., Boyd, K.L., Fambrough, D.M., and Takeyasu, K. (1992) Molecular dissection of functional domains of the E1E2-ATPase using sodium and calcium pump chimeric molecules. *Biophys. J.* **62**, 220–227
19. Swarts, H.G.P., Klaassen, C.H.W., De Boer, M., Fransen, J.A.M., and De Pont, J.J. (1996) Role of negatively charged residues in

the fifth and sixth transmembrane domains of the catalytic subunit of gastric H^+,K^+-ATPase. *J. Biol. Chem.* **271**, 29764–29772
20. Skerjanc, I.S., Toyofuku, T., Richardson, C., and MacLennan, D.H. (1993) Mutation of glutamate 309 to glutamine alters one Ca^{2+}-binding site in the Ca^{2+}-ATPase of sarcoplasmic reticulum expressed in Sf9 cells. *J. Biol. Chem.* **268**, 15944–15950
21. Chen, L., Sumbilla, C., Lewis, D., Zhong, L., Strock, C., Kirtley, M.E., and Inesi, G. (1996) Short and long range functions of amino acids in the transmembrane region of the sarcoplasmic reticulum ATPase. *J. Biol. Chem.* **271**, 10745–10752
22. Rice, W.J., Green, N.M., and MacLennan, D.H. (1997) Site-directed disulfide mapping of helices M4 and M6 in the Ca^{2+} binding domain of SERCA1a, the Ca^{2+}- ATPase of fast twitch skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **272**, 31412–31419
23. Feng, J. and Lingrel, J.B. (1994) Analysis of amino acid residues in the H5–H6 transmembrane and extracellular domains of Na,K-ATPase α subunit identifies threonine 797 as a determinant of ouabain sensitivity. *Biochemistry* **33**, 4218–4224
24. Kuntzweiler, T.A., Wallick, E.T., Johnson, C.L., and Jingrel, J.B. (1995) Glutamic acid 327 in the sheep α1 isoform of Na^+,K^+-ATPase stabilizes a K^+-induced conformational change. *J. Biol. Chem.* **270**, 2993–3000
25. Yamamoto, S., Kuntzweiler, T.A., Wallick, E.T., Sperelakis, N., and Yatani, A. (1996) Amino acid substitutions in the rat Na^+,K^+-ATPase α2-subunit alter the cation regulation of pump current expressed in HeLa cells. *J. Physiol.* **495**, 733–742
26. Vilsen, B. and Andersen, J.P. (1998) Mutation to the glutamate in the fourth membrane segment of Na^+,K^+-ATPase and Ca^{2+}-ATPase affects cation binding from both sides of the membrane and destabilizes the occluded enzyme forms. *Biochemistry* **37**, 10961–10971
27. Nielsen, J.M., Pedersen, P.A., Karlish, S.J.D., and Jorgensen, P.L. (1998) Importance of intramembrane carboxylic acids for occlusion of K^+ ions at equilibrium in renal Na,K-ATPase. *Biochemistry* **37**, 1961–1968
28. Andersen, J.P. and Vilsen, B. (1995) Structure-function relationship of cation translocation by Ca^{2+}- and Na^+,K^+-ATPases studied by site-directed mutagenesis. *FEBS Lett.* **359**, 101–106
29. Clarke, D.M., Loo, T.W., Rice, W.J., Andersen, J.P., Vilsen, B., and MacLennan, D.H. (1993) Functional consequences of alterations to hydrophobic amino acids located in the M_4 transmembrane sector of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* **268**, 18359–18364
30. Vilsen, B. (1997) Leucine 332 at the boundary between the fourth transmembrane segment and the cytoplasmic domain of Na^+,K^+-ATPase plays a pivotal role in the ion translocating conformational changes. *Biochemistry* **36**, 13312–13324