Alanine-scanning Mutagenesis of the Sixth Transmembrane Segment of Gastric H⁺,K⁺-ATPase α-Subunit*

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The sixth transmembrane (M6) segment of the catalytic subunit plays an important role in the ion recognition and transport in the type II P-type ATPase families. In this study, we singly mutated all amino acid residues in the M6 segment of gastric H⁺,K⁺-ATPase α-subunit with alanine, expressed the mutants in HEK-293 cells, and studied the effects of the mutation on the functions of H⁺,K⁺-ATPase; overall K⁺-stimulated ATPase, phosphorylation, and dephosphorylation. Four mutants, L819A, D826A, I827A, and L833A, completely lost the K⁺-ATPase activity. Mutant L819A was phosphorylated but hardly dephosphorylated in the presence of K⁺, whereas mutants D826A, I827A, and L833A were not phosphorylated from ATP. We found that almost all of these amino acid residues, which are important for the function, are located on the same side of the α-helix of the M6 segment. In addition, we found that amino acids involved in the phosphorylation are located exclusively in the cytoplasmic half of the M6 segment and those involved in the K⁺-dependent dephosphorylation are in the luminal half. Several mutants such as I821A, L823A, T825A, and P829A partly retained the K⁺-ATPase activity accompanying the decrease in the rate of phosphorylation.

H⁺,K⁺-ATPase is the proton pump responsible for gastric acid secretion (1). This pump belongs to the family of type-II P-type ATPases, including Na⁺,K⁺-ATPase, sarco(endo)plasmic reticulum, and plasma membrane Ca²⁺-ATPase (2). One of the major themes for the reaction mechanisms of these ion pumps is to answer the question where the channel-like structure and the binding sites of cations are located in the pump molecules. The structure-function relationship for these ion pumps has been extensively studied by the site-directed mutagenesis technique. In the sarcoplasmic Ca²⁺-ATPase, several acidic or polar amino acid residues in the fourth (M4), fifth (M5), sixth (M6), and eighth (M8) transmembrane segments (Glu⁹⁰⁹, Glu⁷⁷⁷, Asn⁸⁹⁶, Thr⁹⁷⁹, Asp⁵⁰⁰, and Glu⁶⁰⁸ in the Ca²⁺-ATPase) are involved in high affinity binding with Ca²⁺ (3, 4). In the Na⁺,K⁺-ATPase, several acidic amino acid residues located in the M4, M5, M6, and M8 transmembrane segments of the α-subunit (Glu³⁵⁷, Glu⁷⁷⁸, Asp⁸⁰⁳, Asp⁸⁰⁷, and Asp⁹²⁵ in rat α₂) were demonstrated as the candidates for the sites recognizing or interacting with Na⁺ and K⁺ (5). In gastric H⁺,K⁺-ATPase, Glu⁴⁴⁵ in the M4 segment, Glu⁷⁹⁷ in the M5 segment, and Glu⁸²⁵ in the M6 segment of the α-subunit were responsible for determining the affinity for K⁺ (6–10). Glu⁴⁴⁵ is also involved in determining the pH sensitivity of gastric H⁺,K⁺-ATPase (10).

In conjunction with the cation binding and transport, the M5/M6 hairpins (i.e., the M5 and M6 transmembrane segments, and the extracellular loop between them) of Na⁺,K⁺-ATPase and H⁺,K⁺-ATPase α-subunits gain attention on account of their very unique characters: (i) They do not by themselves traverse the membrane in translation experiments in vitro, and any sequence in the M5/M6 domains of H⁺,K⁺-ATPase does not act as either signal anchor or stop transfer sequences (11). In the carboxy-terminal third of Na⁺,K⁺-ATPase α-subunit, which contains the putative M5, M6, M7, M8, and M9 segments, only M5 and M9 have topogenic function, whereas the other three segments do not have this function in translation/insertion experiments in vitro (12). (ii) The M5/M6 hairpins are released from the membrane into the luminal side after trypsin digestion of Na⁺,K⁺-ATPase and H⁺,K⁺-ATPase in the absence of K⁺ (13, 14). (iii) The M5/M6 hairpins are stabilized in the membrane in the presence of K⁺ (13, 14). These results altogether suggest that the M5/M6 hairpins of the Na⁺,K⁺-ATPase and H⁺,K⁺-ATPase α-subunits are surrounded with other transmembrane segments and are intimately associated with K⁺. It is proposed that these amphipathic characters of the M5/M6 hairpins are important for the transport of ions.

In gastric H⁺,K⁺-ATPase, the M6 segment contains cysteine residues reported to be the major binding sites of the benzimidazole derivatives, which are irreversible inhibitors of gastric H⁺,K⁺-ATPase (15, 16). These cysteine residues are themselves not involved in the enzyme function (16). Rather, the covalent binding of the inhibitor with these cysteines interferes with the conformational changes of the ATPase. Therefore, it is likely that the M6 segment is close to the structural center important for the function of gastric H⁺,K⁺-ATPase.

In the present study, we focused on the M6 segment of gastric H⁺,K⁺-ATPase α-subunit and studied the role of the side chain of each amino acid residue from Cys¹⁵ to Lys⁸⁴⁵ in the M6 segment in the partial reactions of H⁺,K⁺-ATPase to determine the affinity for cations. For this purpose, we performed alanine-scanning mutagenesis, which had been used previously to study the structure-function relationships of specific domain(s) of membrane proteins, including ion pumps and ion channels, especially for the binding site of drugs, ligands, and proteins (17–21).
Site-directed Mutagenesis of Gastric H⁺,K⁺-ATPase

EXPERIMENTAL PROCEDURES

Materials—HEK-293 cells (human embryonic kidney cell line) were a kind gift from Prof. Jonathan Lyttton (University of Calgary, Calgary, Canada). The pcDNA3 vector was obtained from Invitrogen Co. (San Diego, CA). A MutanK kit was from Takara Shuzo Co. (Kyoto, Japan). Ffu DNA polymerase was obtained from Stratagene (La Jolla, CA). Restriction enzymes and other DNA- and RNA-modifying enzymes were from Toyobo (Osaka, Japan), New England BioLabs (Beverly, MA), or Amersham Pharmacia Biotech Inc. (Tokyo, Japan). 2-Methyl-8-(phenylmethylximido)imidazol[1,2-a] pyridine-3-acetonitrile (SCH28080) was obtained from Schering-Plough Co. (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 mucosa as described elsewhere (6). The α- and β-subunit cDNAs were digested with EcoRI and XhoI. The obtained fragments were each ligated into the pcDNA3 vector treated with EcoRI and XhoI.

DNA Sequencing—DNA sequencing was performed by the dideoxy chain termination method using an Autocycle DNA sequencing kit and an ALFExpress DNA sequencer (Amersham Pharmacia Biotech). Site-directed Mutagenesis—A plasmid containing cDNA for rabbit gastric H⁺,K⁺-ATPase α-subunit was subjected to site-directed mutagenesis to create a unique restriction site of AvrII at the nucleotide position of 2436 (corresponding to Leu813). The sequence of CCTGGG was mutated to CCTAGG without amino acid replacement. The following introduction of site-directed mutations in the M6 segment of the H⁺,K⁺-ATPase α-subunit was carried out by sequential polymerase chain reaction (PCR) steps (7), in which appropriately mutated subunit cDNAs (segments between the AvrII site (nucleotide 2436) and the AatII site (nucleotide 2830)) were prepared. Two kinds of flanking sequence primers were prepared, one is the 5'-flanking sense primer, 5'-TGCACCTGAAAGATCCATC-3' (nucleotide 2340 to 2361), and the other is the 3'-flanking antisense primer, 5'-CGATCCACCTG- GAACACGAT-3' (nucleotide 2906 to 2929). In addition, sense and antisense nucleotides, each containing long enough oligo or two mutated bases near the center, were designed (referred as the sense-mutating primer and the antisense-mutating primer). In the first PCR amplification step, the H⁺,K⁺-ATPase α-subunit cDNA subcloned in pBluescript SK(-) was used as a DNA template (6). Two fragments were prepared in this step: one between the 5'-flanking sense primer and the antisense mutating primer, and the other between the sense mutating primer and the 3'-flanking antisense primer. Each amplified fragment was purified by gel electrophoresis, combined, and incubated with the 5'-flanking sense primer and the 3'-flanking antisense primer in the second PCR amplification. The amplified fragment was purified by gel electrophoresis, subcloned in pCR-Script Amp SK(-) (Stratagene), and sequenced. PCR was routinely carried out in the presence of 200 μM of each dNTP, 6 μM primers, 10 mM KCl, 20 mM Tris- HCl (pH 8.8), 1.5 mM MgCl₂, 0.1% Triton X-100, 100 μM DEF, bovine serum albumin, and 2 units of Ffu DNA polymerase for 25 cycles. After sequencing, the amplified fragment in the second PCR was digested with AvrII and AatII, and ligated back into the relevant position of the wild-type construct of the α-subunit. Site-directed mutagenesis in DNA polymerase for 25 cycles.

Cell Culture, Transfection, and Preparation of Membrane Fractions—Cell culture of HEK-293 was carried out as described previously (6, 10). The α- and β-subunit cdNA transfection was performed by the calcium phosphate method with 10 μg of DNA per 10-cm dish. DNA was purified by cesium chloride density gradient centrifugation or with an EndoFree Plasmid Maxi kit (Qiagen, Tokyo). Cells were harvested 2 days after the DNA transfection. Membrane fractions of HEK cells were prepared as described previously (6). Briefly, cells in a 10-cm Petri dish were washed with phosphate-buffered saline and incubated in 2 ml of low ionic salt buffer (0.5 mM MgCl₂ and 10 mM Tris-HCl, pH 7.4) at 0 °C for 10 min. After the addition of phenylmethysulfonyl fluoride (1 μM) and aprotinin (0.09 unit/ml), the cells were homogenized in a Dounce homogenizer, and the homogenate was diluted with an equal volume of a solution comprising 500 mM sucrose, 2 mM MgCl₂, and 5 mM Tris-HCl, pH 7.4. The homogenized suspension was centrifuged at 800 x g for 10 min. The supernatant was centrifuged at 100,000 x g for 90 min, and the pellet was suspended in a solution containing 250 mM sucrose and 5 mM Tris-HCl, pH 7.4.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot—SDS-polyacrylamide gel electrophoresis was carried out as described elsewhere (22). Membrane preparations (30 μg of 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 applied to the SDS-polyacrylamide gel. Immunoblot was carried out as described previously (6).

Assay of H⁺,K⁺-ATPase Activity—A hog gastric vesicle preparation, which contains H⁺,K⁺-ATPase, was prepared as described elsewhere (24). The membrane fractions of HEK cells in parallel with a series of diluted gastric vesicle preparations were run on the same SDS-polyacrylamide gel and blotted. The blot was scanned using a laser scanning image system. The content of H⁺,K⁺-ATPase in the HEK membrane fraction was estimated from the standard curve obtained using the gastric vesicle preparation.

RESULTS

Construction of M6 Mutants of Gastric H⁺,K⁺-ATPase α-Subunit—Fig. 1 shows a schematic model of the M5/M6 hairpin structure of rabbit gastric H⁺,K⁺-ATPase α-subunit. It was shown that rabbit gastric H⁺,K⁺-ATPase α-subunit spans the membrane between Lys793 and Cys815 (the M5 segment) and between Cys815 and Lys837 (the M6 segment), respectively. The following experimental results: (i) Trypsin cleaved peptide bonds of hog gastric H⁺,K⁺-ATPase α-subunit at Lys793 and Lys837 (which correspond to Lys793 and Lys837 in rabbit gastric H⁺,K⁺-ATPase, respectively) in the cytoplasmic space (Fig. 1) (27), and (ii) Cys815 and Cys822 of hog gastric H⁺,K⁺-ATPase α-subunit (which correspond to Cys815 and Cys824 in rabbit gastric H⁺,K⁺-ATPase, respectively) reacted...
with a cationic sulfenamide derived from omeprazole from the luminal space (15, 16). It was also demonstrated that Leu793 in sheep Na⁺,K⁺-ATPase α₁-subunit (which corresponds to Leu811 in rabbit gastric H⁺,K⁺-ATPase) is exposed to the extracellular phase from the finding that the cysteine residue introduced in this position was labeled with a membrane-impermeable cysteine-directed reagent, N-biotinylaminoethyl methanethiosulfonate (28). Amino acid residues around Leu793 (PLPLG) are well conserved between Na⁺,K⁺-ATPase and gastric H⁺,K⁺-ATPase α-subunits. Here, we replaced each of the amino acid residues between Cys815 and Leu831, in or close to the M6 segment, of rabbit H⁺,K⁺-ATPase and gastric H⁺,K⁺-ATPase α-subunits with alanine. These mutant α-subunit cDNAs were co-transfected with the gastric H⁺,K⁺-ATPase β-subunit cDNA in HEK-293 cells. The expression levels of each mutant α-subunit in the membrane fractions were compared with that of the wild-type α-subunit in the Western blot detected with an anti-α-subunit antibody. The expression levels ranged between 80% (C824A) and 125% (I818A) of that of the wild-type except for 31% for mutant L833A as shown in Table I.

**H⁺,K⁺-ATPase Activity of the Mutants**—Table I shows the K⁺-ATPase and H⁺,K⁺-ATPase activities of the M6 mutants. These two activities are comparable for each mutant, indicating that the K⁺-ATPase activity of all the mutants is sensitive to 50 μM SCH28080 and that this concentration is high enough to inhibit the K⁺-ATPase activity of all the M6 mutants. Mutants C815A, I816A, T817A, F820A, C824A, T825A, F828A, and S832A retained 110, 76, 67, 65, 137, 62, 90, and 88% of the K⁺-ATPase activity found in the wild-type, respectively. Mutants I818A, I821A, E822A, L823A, P829A, S830A, and V831A showed 31, 33, 15, 47, 34, 25, and 30% of the K⁺-ATPase activity found in the wild-type, respectively. Mutants L819A, D826A, I827A, and L833A almost lost the K⁺-ATPase activity.

H⁺,K⁺-ATPase consists of the α- and β-subunits. The region involved in association with the β-subunit on the α-subunit is localized in and close to the fourth extracellular loop (between Arg898 and Arg922), which is very close to the M6 segment (29). Thus, it is not completely excluded that introducing a mutation in the M6 segment abolishes the assembly between the α- and β-subunits, resulting in the loss of H⁺,K⁺-ATPase activity. However, judged from the immunoprecipitation experiments, all the mutant α-subunits prepared in this study assembled with the β-subunit (data not shown), indicating that inactivation of the K⁺-ATPase activity in mutants L819A, D826A, I827A, and L833A is not due to the abolishment of the αβ assembly.

**Phosphorylation Capacity of the Mutants**—Membrane fractions of the cells expressing the wild-type enzyme and each alanine mutant were phosphorylated from [γ-³²P]ATP in the presence of 3 mM ouabain (to inhibit endogenous Na⁺,K⁺-ATPase present in the membrane fraction) at 0 °C for 15 s, separated on an SDS-polyacrylamide gel under weakly acidic conditions, and the patterns of the phosphorylated proteins were observed as reported previously (10) (Fig. 2). In the membrane fraction expressing the wild-type α- and β-subunits, several radioactive bands were observed; doublet bands with molecular mass of about 100 kDa and a band with a molecular mass of 60 kDa (Fig. 2A). The phosphorylation of the lower 100-kDa band was inhibited by 50 μM SCH28080 (Fig. 2A) or 1 mM sodium vanadate (data not shown). The phosphorylation of this band was not observed in the membrane fraction of cells expressing the β-subunit alone (Fig. 2A) nor in mutant D387N, a phosphorylation site mutant (6), which cannot form an acyl-phosphate intermediate (data not shown). From these findings, it is concluded that the lower 100-kDa band represents the phosphorylated H⁺,K⁺-ATPase α-subunit.

Mutants C815A, I816A, T817A, I818A, L819A, F820A, E822A, C824A, F828A, and S832A retained the ATP-dependent phosphorylation capacity. The phosphorylation was inhibited by 50 μM SCH28080 (Fig. 2B). Mutants I821A, L823A, T825A, D826A, I827A, F829A, V831A, and L833A were hardly phosphorylated from ATP, because the phosphorylation levels of these mutants were much lower than that of the wild-type in the absence of SCH28080 (Fig. 2B). It should be noted that the phosphorylation levels of these mutants in the absence of SCH28080 were higher than those of the membrane fraction expressing the β-subunit alone or mutant D387N (data not shown) but comparable with the phosphorylation level of the wild-type in the presence of 50 μM SCH28080 (Fig. 2B). Therefore, it is not completely excluded that these mutants retained very low capacity for phosphorylation at 0 °C and that the sensitivity of the mutants to SCH28080 was lower than that of the wild-type. Mutant L819A retained the phosphorylation capacity, although this mutant lost the K⁺-ATPase activity (Table I). Mutants I821A, L823A, T825A, F829A, and V831A apparently lost the phosphorylation capacity, although these...
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Expression of K⁺-ATPase and H⁺,K⁺-ATPase activities of M6 Ala mutants

The expression levels of mutant H⁺,K⁺-ATPase α-subunits were estimated from the standard curve obtained using gastric vesicle preparation and expressed as percentages of the expression of the wild-type. All values of K⁺-ATPase and H⁺,K⁺-ATPase activities are given as means ± S.E. for more than three membrane fractions. K⁺-ATPase and H⁺,K⁺-ATPase activities of the mutants were expressed as percentages of those of wild-type and normalized on the same expression levels.

| Mutation | Expression % | K⁺-ATPase activity μmol Pi/mg/h | H⁺,K⁺-ATPase activity μmol Pi/mg/h |
|----------|--------------|---------------------------------|-----------------------------------|
| Wild-type| 100          | 1.43 ± 0.04                     | 1.41 ± 0.04                       |
| C815A    | 98           | 1.26 ± 0.03                     | 3.77 ± 0.06                       |
| I816A    | 116          | 1.35 ± 0.07                     | 3.02 ± 0.06                       |
| T817A    | 107          | 1.03 ± 0.19                     | 1.12 ± 0.16                       |
| I818A    | 125          | 0.91 ± 0.08                     | 0.86 ± 0.08                       |
| L819A    | 113          | 0.06 ± 0.07                     | 0.01 ± 0.01                       |
| F820A    | 109          | 0.02 ± 0.06                     | 0.01 ± 0.01                       |
| I821A    | 83           | 0.03 ± 0.07                     | 0.01 ± 0.01                       |
| E822A    | 106          | 0.29 ± 0.07                     | 0.30 ± 0.02                       |
| L823A    | 84           | 0.57 ± 0.02                     | 0.55 ± 0.02                       |
| C824A    | 80           | 1.57 ± 0.05                     | 1.26 ± 0.06                       |
| T825A    | 85           | 0.76 ± 0.09                     | 0.68 ± 0.06                       |
| D826A    | 96           | 0.01 ± 0.01                     | 0.01 ± 0.01                       |
| I827A    | 82           | 0.00 ± 0.02                     | 0.07 ± 0.01                       |
| F828A    | 82           | 0.16 ± 0.02                     | 0.13 ± 0.07                       |
| P829A    | 103          | 0.50 ± 0.05                     | 0.59 ± 0.03                       |
| S830A    | 101          | 0.36 ± 0.05                     | 0.51 ± 0.04                       |
| V831A    | 105          | 0.47 ± 0.07                     | 0.50 ± 0.05                       |
| S832A    | 124          | 1.05 ± 0.07                     | 0.98 ± 0.06                       |
| L833A    | 31           | 0.10 ± 0.03                     | 0.01 ± 0.01                       |

The phosphorylation of hog gastric vesicles, and the membrane fractions of the cells transfected with the wild-type α- and β-subunit cDNAs, β-subunit cDNA alone. One microgram of gastric vesicles (GI) (lanes 1 and 2), and each 50 μg of membrane fractions obtained from the cells co-transfected with the wild-type H⁺,K⁺-ATPase α- and β-subunit cDNAs (lanes 3 and 4) and from the cells transfected with the wild-type H⁺,K⁺-ATPase β-subunit cDNA alone (lanes 5 and 6) were phosphorylated in 110 μl of solution containing 1 μM [γ-32P]ATP (4 × 10⁶ cpm), 2 mM MgCl₂, 1 mM EGTA, 3 mM ouabain, and 40 mM Tris-HCl (pH 6.8) at 0 °C for 15 s. Prior to phosphorylation, the samples were preincubated with or without 50 μM SCH28080 (shown as SCH 28080 + or − at 0 °C for 30 min. The phosphorylation reaction was quenched by the addition of 590 μl of ice-cold stop solution containing 10% trichloracetic acid and 10 mM inorganic phosphate. The protein was collected at 13,000 g for 3 min, washed with ice-cold stop solution and 30% sucrose, successively, and 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 the membrane fractions of the cells transfected with either the wild-type or the M6 mutant α-subunit cDNA in combination with the wild-type β-subunit cDNA. Fifty micrograms of membrane fractions of the cells co-transfected with the cDNA of the H⁺,K⁺-ATPase β-subunit plus the cDNA of the wild-type H⁺,K⁺-ATPase α-subunit or the indicated alanine mutant α-subunit were phosphorylated in the presence or absence of 50 μM SCH28080 (shown as SCH 28080 + or −). Bands representing the H⁺,K⁺-ATPase α-subunit are shown by the arrow.

Mutants partly retained the K⁺-ATPase activity (Table I). Later, phosphorylation of these mutants will be studied precisely.

Dephosphorylation Capacity of the Mutants—Next, we studied the dephosphorylation capacity of the wild-type H⁺,K⁺-ATPase and the M6 mutants. Membrane fractions of the cells expressing the wild-type and the M6 mutants were dephosphorylated from [γ-32P]ATP followed by the incubation with KCl in the presence of 1 mM non-radioactive ATP at 0 °C for 15 s. Even in the absence of KCl, the upper 100-kDa band present in HEK cells disappeared during incubation with cold ATP. A portion of the phosphorylated α-subunit was dephosphorylated during incubation with cold ATP in the absence of KCl. The phosphorylated α-subunit of the wild-type H⁺,K⁺-ATPase was dephosphorylated by the addition of K⁺ in a concentration-dependent manner. The half-maximally effective concentration of K⁺ for the dephosphorylation was about 0.1 mM (Fig. 3, A and B). The phosphorylated α-subunits of mutants C815A and I818A were also dephosphorylated by the addition of K⁺, although the sensitivity of these phosphorylated mutants for K⁺ were slightly lower than that of the wild-type enzyme (Fig. 3B). On the other hand, mutants L819A and E822A were dephosphorylated by only 20% and 50% in the presence of 50 mM KCl, respectively (Fig. 3B), indicating the presence of defect in the K⁺-dependent dephosphorylation step, which resulted in complete or considerable loss of the overall H⁺,K⁺-ATPase activity, respectively (Table I).
The phosphorylated α-subunit of the wild-type H⁺,K⁺-ATPase was not dephosphorylated by the addition of ADP, indicating that the major phosphorylated intermediate is in the E₂ form (ADP-insensitive, K⁺-sensitive form). Mutants L819A and E822A were not dephosphorylated by the addition of ADP, indicating that these mutants have no defect in the process of conversion from E₃P to E₂P (Fig. 4).

Substitutions of Leu₈₁₉, Asp₈₂₆, Ile₈₂₇, and Leu₈₃₂ with Other Amino Acid Residues—Mutant L819A has a defect in the K⁺-dependent dephosphorylation step, whereas mutants D826A, I827A, and L833A have defects in the phosphorylation steps. Here, we replaced Leu₈₁₉ with Gly, Val, or Met; Ile₈₂₇ with Val
or Met; and Leu833 with Gly, Val, or Met to study the role of the size of the side chain on the K\(^{+}\)-ATPase activity and its partial reactions. No K\(^{+}\)-ATPase activity was observed in mutant L819G, whereas mutants L819V and L819M retained 14\% and 26\% of the K\(^{+}\)-ATPase activity found in the wild-type enzyme, respectively (Table II). These mutants retained the phosphorylation capacity (Fig. 4). Mutants L819M and L819V were dephosphorylated by the addition of K\(^{+}\), however, their sensitivity for K\(^{+}\) was 10 times and 100 times lower than that of the wild-type enzyme, respectively, whereas mutant L819G was dephosphorylated by only 30\% in the presence of 50 mM KCl (Fig. 3C). These results suggest that the bulkiness or length of the side chain of the amino acid at this position (position 819) is critical for determining the affinity for K\(^{+}\) in the dephosphorylation step of the H\(^{+}\)-K\(^{+}\)-ATPase.

Expression level of mutants L833G was 31\% of that of the wild-type (Table II), which was comparable with that of mutant L833A. However, the expression levels of mutants L833V and L833M were significantly higher than that of mutant L833A. Mutants L833A and L833G lost K\(^{+}\)-ATPase activity and phosphorylation capacity, whereas mutants L833V and L833M retained them. These results suggest that the bulkiness or length of the side chain of the amino acid at this position (position 833) is important for the expression of the \(\alpha\) subunit as well as the dephosphorylation step of the H\(^{+}\)-K\(^{+}\)-ATPase.

We also prepared a double mutant, E822D/D826E to study the interaction between Glu822 and Asp826. E822D/D826E did not retain the K\(^{+}\)-ATPase activity and phosphorylation capacity, indicating that the roles of these negative side chains are not interchangeable.

Phosphorylation Capacity at Higher Temperature or after Longer Incubation—It is apparently contradictory that mutants I821A, L823A, T825A, P829A, and V831A considerably retained the H\(^{+}\)-K\(^{+}\)-ATPase and K\(^{+}\)-ATPase activities, whereas they almost completely lost the ATP-dependent phosphorylation capacity as shown in Fig. 2. In the experiments shown in Table I and Fig. 2, the ATPase activities and phosphorylation capacity were assayed at 37 \(^\circ\)C and 0 \(^\circ\)C, respectively. It is expected that phosphorylation capacity may be observed in these mutants at higher temperatures. Hence, here we studied the phosphorylation capacity of these mutants at 10 \(^\circ\)C. Mutants I821A, L823A, and T825A were phosphorylated from ATP at 10 \(^\circ\)C, and their phosphorylation levels were 42\%, 57\%, and 65\% of that of the wild-type, respectively (Fig. 6). Mutant P829A was partly phosphorylated from ATP. It is hard to observe whether mutants D826A, L827A, and V831A were phosphorylated from ATP at 10 \(^\circ\)C, because their phosphorylation levels were very low and not significantly different from that of the wild-type in the presence of 50 \(\mu\)M SCH28080. However, it is noteworthy that the phosphorylation levels of these mutants were significantly higher than that of mutant D827N (as a negative control for phosphorylation), indicating that very low levels of phosphorylation were found in these mutants. These results altogether indicate that mutants I821A, L823A, T825A, and P829A retained phosphorylation capacity at 10 \(^\circ\)C. Unfortunately, we cannot estimate the phosphorylation levels at higher temperatures because of the high background phosphorylation.

It is expected that the mutations decrease the rate of phosphorylation resulting in the apparent loss of phosphorylation capacity at 0 \(^\circ\)C. Here, we studied the time course of phosphorylation between 15 and 60 s. Wild-type enzyme attained the steady state of phosphorylation within 15 s, whereas other mutants (I821A, L823A, T825A, D826A, L827A, and P829A) did not (Fig. 7). Mutants I821A, L823A, and T825A reached the steady state of phosphorylation within 30 s, and their phosphorylation levels were 40–60\% of that of the wild-type, indicating that the rate of phosphorylation of these mutants was decreased compared with the wild-type. However, a clear steady state of phosphorylation was not observed in mutants D826A and L827A because of their low phosphorylation levels.

**DISCUSSION**

In this study, we performed alanine-scanning mutagenesis of all the amino acid residues in the M6 segment of gastric H\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\) subunit, which is regarded as one of the major transmembrane segments involved in ion recognition and transport.

Single replacements of Leu819, Asp826, Ile827, or Leu833 with alanine abolished the K\(^{+}\)-ATPase activity. In addition, Glu822 is involved in determining the affinity for K\(^{+}\) in the K\(^{+}\)-ATPase activity (7, 8) and its dephosphorylation step as shown in the present study. The negative charge of this glutamic acid was

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**Fig. 4.** ADP and K\(^{+}\) sensitivity of the phosphorylated intermediates of the wild-type H\(^{+}\)-K\(^{+}\)-ATPase and the M6 mutants. Fifty micrograms of each membrane protein were used. The wild-type H\(^{+}\)-K\(^{+}\)-ATPase and mutants L819G, L819A, L819M, and E822A were phosphorylated from 1 \(\mu\)M [\(\gamma\)-\(\text{P}\)]ATP at 0 \(^\circ\)C for 15 s (lane a). The phosphorylation was followed by the incubation with 1 mM non-radioactive ATP alone (lane b) or with 1 mM ADP and 1 mM ATP (lane c) or with 10 mM KCl and 1 mM ATP (lane d) at 0 \(^\circ\)C for 15 s. The reaction was quenched by the addition of the ice-cold stop solution. Precipitated proteins were separated on SDS-polyacrylamide gel, and the radioactivity associated with the H\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\) subunit was visualized by digital autoradiography. Dephosphorylation patterns in the presence of K\(^{+}\) were presented in Fig. 3A and presented here again for comparison.
The expression levels of mutant H\textsuperscript{+},K\textsuperscript{+}-ATPase \(a\)-subunits were estimated from the standard curve obtained using gastric vesicle preparation and expressed as percentages of the expression of the wild-type. All values of K\textsuperscript{+}-ATPase and H\textsuperscript{+},K\textsuperscript{+}-ATPase activities are given as means \(\pm\) S.E. for more than three membrane fractions. K\textsuperscript{+}-ATPase and H\textsuperscript{+},K\textsuperscript{+}-ATPase activities of the mutants were expressed as percentages of those of wild-type and normalized on the same expression levels.

| Mutation | Expression | K\textsuperscript{+}-ATPase activity % | H\textsuperscript{+},K\textsuperscript{+}-ATPase activity % |
|----------|------------|----------------------------------------|----------------------------------|
| Wild-type | 100        | 1.43 \(\pm\) 0.04                      | 0.80 \(\pm\) 0.04                 |
| L819G    | 78         | –0.07 \(\pm\) 0.01                     | 0                                |
| L819V    | 89         | 0.18 \(\pm\) 0.03                      | 0.22 \(\pm\) 0.03                 |
| L819M    | 104        | 0.39 \(\pm\) 0.00                      | 0.47 \(\pm\) 0.01                 |
| IS27V    | 80         | 0.49 \(\pm\) 0.07                      | 0.46 \(\pm\) 0.07                 |
| IS27M    | 71         | 0.50 \(\pm\) 0.06                      | 0.38 \(\pm\) 0.04                 |
| L833G    | 31         | –0.09 \(\pm\) 0.02                     | 0                                |
| L833V    | 58         | 0.62 \(\pm\) 0.07                      | 0.65 \(\pm\) 0.05                 |
| L833M    | 78         | 0.83 \(\pm\) 0.06                      | 0.64 \(\pm\) 0.01                 |
| E822D/D826E | 84 | 0.08 \(\pm\) 0.07    | 0.05 \(\pm\) 0.01 |

\[\text{Fig. 5. Phosphorylation of the membrane fractions of the cells transfected with the wild-type, mutants I827A, IS27V, IS27M, or E822D/D826E a-subunit cDNAs in combination with the wild-type b-subunit cDNA.}\]

\[\text{Fig. 6. Phosphorylation of the membrane fractions of the cells transfected with either the wild-type or the M6 mutant a-subunit cDNA in combination with the wild-type b-subunit cDNA at 10 °C.}\]

proposed to inhibit the dephosphorylation step of H\textsuperscript{+},K\textsuperscript{+}-ATPase in the absence of K\textsuperscript{+}, and it was proposed that K\textsuperscript{+} stimulates the dephosphorylation by neutralizing the negative charge (30). It is very interesting that amino acid residues such as Leu\textsuperscript{819}, Glu\textsuperscript{822}, Asp\textsuperscript{826}, and Leu\textsuperscript{833}, which are important for charge (30). It is very interesting that amino acid residues such as Leu\textsuperscript{819}, Glu\textsuperscript{822}, Asp\textsuperscript{826}, and Leu\textsuperscript{833} are involved in different reaction steps of H\textsuperscript{+},K\textsuperscript{+}-ATPase and the M6 mutants. Fifty microgram of membrane fractions of the cells co-transfected with the cDNA of the H\textsuperscript{+},K\textsuperscript{+}-ATPase \(\beta\)-subunit, plus the cDNA of the wild-type H\textsuperscript{+},K\textsuperscript{+}-ATPase \(a\)-subunit, and mutants I827A, IS27V, IS27M, or E822D/D826E were phosphorylated in the presence and absence of 50 \(\mu\text{g}\) SCH28080. Bands representing the H\textsuperscript{+},K\textsuperscript{+}-ATPase \(a\)-subunit are shown by the arrow.

\[\text{Fig. 7. Time course of phosphorylation of the wild-type H\textsuperscript{+},K\textsuperscript{+}-ATPase and the M6 mutants. Fifty microgram of membrane fractions obtained from the cells co-transfected with the cDNA of the H\textsuperscript{+},K\textsuperscript{+}-ATPase \(\beta\)-subunit, plus the cDNA of the wild-type H\textsuperscript{+},K\textsuperscript{+}-ATPase \(a\)-subunit, and mutants I821A (\(\square\)), L823A (\(\triangledown\)), T825A (\(\triangle\)), D826A (\(\Delta\)), I827A (\(\times\)), or P829A (\(\blacksquare\)) were phosphorylated at 0 °C for 15 to 60 s. The phosphorylation level of the membrane fraction of the cells expressing \(\beta\)-subunit alone was subtracted from that of each sample. The phosphorylation level was expressed as the percentage of the phosphorylation level of the wild-type enzyme at each time.}\]

ent a hypothesis that Leu\textsuperscript{819}, Glu\textsuperscript{822}, Asp\textsuperscript{826}, and Leu\textsuperscript{833} face to Asp\textsuperscript{826}, may support this structure. Similar helical wheel model of the carboxyl-terminal half (cytoplasmic side) of the M6 segment (Fig. 8A), Ile\textsuperscript{821}, Leu\textsuperscript{823}, and Thr\textsuperscript{825} face the putative ion-channel-like structure (or putative ion-translocating pore), which consists of several transmembrane segments, M4, M5, and M6. The side chain of Ile\textsuperscript{827}, which is close to Asp\textsuperscript{826}, may support this structure. Similar helical wheel models were previously presented for the M4 segments of Na\textsuperscript{+},K\textsuperscript{+}-ATPase and H\textsuperscript{+},K\textsuperscript{+}-ATPase (31). We also found that two groups of amino acid residues that are involved in different reaction steps of H\textsuperscript{+},K\textsuperscript{+}-ATPase show a unique distribution; that is, amino acid residues involved in the phosphorylation step such as Asp\textsuperscript{826}, Ile\textsuperscript{827}, and Leu\textsuperscript{833} are located in the carboxyl-terminal half (cytoplasmic side) of the M6 segment, whereas those involved in the K\textsuperscript{+}-dependent dephosphorylation step such as Leu\textsuperscript{819} and Glu\textsuperscript{822} are located in the amino-terminal half (luminal side) of the M6 segment (Fig. 8B).
Amino acid residues involved in K$^+$ membrane topology of the M6 segment. These mutants showed phosphorylation (Table II and Fig. 3B). Leu$^{821}$, Leu$^{823}$, Thr$^{825}$, and Pro$^{829}$ are involved in phosphorylation (shown by blue circles). Ile$^{821}$, Leu$^{823}$, Thr$^{825}$, and Pro$^{829}$ are involved in phosphorylation step. However, a mutation to Met and Val partly retained the K$^+$-ATPase activity and phosphorylation were observed in these mutants. On the other hand, the Val and Met mutants were expressed at the level of 60–80% of the wild-type and partly retained the K$^+$-ATPase activity and phosphorylation. Therefore, the bulkiness of the side chain at this position is primarily important for the stable expression of the α-subunit and is also likely to be involved in the phosphorylation.

Next, we discuss the role of bulkiness of the side chains at the position of amino acids 819, 826, 827, and 833 on the function of H$^+$,K$^+$-ATPase. At the position of amino acid 819, mutations of Leu to Ala and Gly abolished the K$^+$-ATPase activity and K$^+$-dependent dephosphorylation. These mutants showed much lower or almost no sensitivity to K$^+$ in the dephosphorylation step. However, a mutation to Met and Val partly retained the K$^+$-ATPase activity and K$^+$-dependent dephosphorylation (Table II and Fig. 3C). These mutants showed intermediate affinity for K$^+$ between the wild-type and the alanine mutant. Therefore, the bulkiness of the side chain at this position is very important for the K$^+$-dependent dephosphorylation and determining the affinity for K$^+$, and small side chains cannot support the function.

At the position of amino acid 826, not only the Ala mutation but also the isosteric mutation to Asn and the isocharge mutation to Glu abolished the K$^+$-ATPase activity and phosphorylation (7). Therefore, both the present residue size of Asp and negative charge at this position are very important to retain the phosphorylation capacity. It is also noteworthy that the K$^+$-ATPase activity was not recovered when Glu$^{822}$ was replaced by Asp in combination with the mutation of Asp$^{826}$ to Glu (Table II). Therefore, there is no compensation of the size of the side chain between Glu$^{822}$ and Asp$^{826}$. These two amino acids may play different roles in the binding (or coordination) of ions in the ion pocket as reported for the roles of Asn$^{796}$ and Asp$^{800}$ of sarcoplasmic Ca$^{2+}$-ATPase (32, 33). There are two Ca$^{2+}$-binding pockets, the sites I and II. Asp$^{800}$ is involved in coordination of one Ca$^{2+}$ in the site II, whereas Asp$^{800}$ is involved in coordination of Ca$^{2+}$ both in the sites I and II.

At the position of amino acid 827, a mutation of Ile to Ala abolished the K$^+$-ATPase activity and phosphorylation, whereas mutations to Val and Met partly retained the K$^+$-ATPase activity and phosphorylation. Therefore, the bulkiness of the side chain at this position is important for the phosphorylation, and small side chains cannot support the function.

Leu$^{833}$ is localized at the end of the M6 segment (at the boundary between the M6 segment and cytoplasmic loop). Mutations of Leu to Gly and Ala significantly decreased the expression level, suggesting that this residue is involved in correct membrane insertion of the α-subunit. No K$^+$-ATPase activity and phosphorylation were observed in these mutants. On the other hand, the Val and Met mutants were expressed at the level of 60–80% of the wild-type and partly retained the K$^+$-ATPase activity and phosphorylation. Therefore, the bulkiness of the side chain at this position is primarily important for the stable expression of the α-subunit and is also likely to be involved in the phosphorylation.

Rice and MacLennan (17) performed scanning mutagenesis of amino acid residues in the transmembrane segments, including the M6 of sarcolemmal Ca$^{2+}$-ATPase. They found a motif, (E/D)/GLP(A/T/V), in the M4 and M6 segments, which is partly conserved in other P-type ATPases. In rabbit gastric H$^+$,K$^+$-ATPase, the sequence in the M6 segment corresponding to this motif is D/P/DPSV (34), which is not well conserved. The same group also highlighted Asn$^{796}$, Asp$^{800}$, and Gly$^{801}$ of sarcoplasmic Ca$^{2+}$-ATPase as residues important for Ca$^{2+}$ transport (3, 4, 34), and Pro$^{803}$ as a residue important for determining the affinity for Ca$^{2+}$ (35). The corresponding amino acid residues in rabbit gastric H$^+$,K$^+$-ATPase are Glu$^{822}$, Asp$^{826}$, Ile$^{827}$, and Pro$^{829}$, respectively. Although Glu$^{822}$ is not conserved between H$^+$,K$^+$-ATPase and Ca$^{2+}$-ATPase, this residue is also important for determining the affinity for Ca$^{2+}$ (35). The binding of one Ca$^{2+}$ requires bulky amino acids (Gly or Ala) at the position of 801 to retain its activity; the K$^+$-ATPase requires small amino acids (32, 33). There are two binding pockets, the sites I and II. Asp$^{800}$ is involved in coordination of Ca$^{2+}$ both in the sites I and II.

Therefore, both the present residue size of Asp and negative charge at this position are very important to retain the phosphorylation capacity. It is also noteworthy that the K$^+$-ATPase activity was not recovered when Glu$^{822}$ was replaced by Asp in combination with the mutation of Asp$^{826}$ to Glu (Table II). Therefore, there is no compensation of the size of the side chain between Glu$^{822}$ and Asp$^{826}$. These two amino acids may play different roles in the binding (or coordination) of ions in the ion pocket as reported for the roles of Asn$^{796}$ and Asp$^{800}$ of sarcoplasmic Ca$^{2+}$-ATPase (32, 33). There are two Ca$^{2+}$-binding pockets, the sites I and II. Asp$^{800}$ is involved in coordination of one Ca$^{2+}$ in the site II, whereas Asp$^{800}$ is involved in coordination of Ca$^{2+}$ both in the sites I and II.

At the position of amino acid 827, a mutation of Ile to Ala abolished the K$^+$-ATPase activity and phosphorylation, whereas mutations to Val and Met partly retained the K$^+$-ATPase activity and phosphorylation. Therefore, the bulkiness of the side chain at this position is important for the phosphorylation, and small side chains cannot support the function.

\[ \text{SITE-directed Mutagenesis of Gastric H}^+\text{,K}^+\text{-ATPase} \]
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Alanine-scanning Mutagenesis of the Sixth Transmembrane Segment of Gastric H⁺ ,K⁺-ATPase α-Subunit
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J. Biol. Chem. 2001, 276:31265-31273.
doi: 10.1074/jbc.M103698200 originally published online June 7, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103698200

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