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Role of Negatively Charged Residues in the Fifth and Sixth Transmembrane Domains of the Catalytic Subunit of Gastric H\(^+\),K\(^+\)-ATPase*

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The role of six negatively charged residues located in or around the fifth and sixth transmembrane domain of the catalytic subunit of gastric H\(^+\),K\(^+\)-ATPase, which are conserved in P-type ATPases, was investigated by site-directed mutagenesis of each of these residues. The acid residues were converted into their corresponding acid amides. Sf9 cells were used as the expression system using a baculovirus with coding sequences for the \(\alpha\) and \(\beta\)-subunits of H\(^+\),K\(^+\)-ATPase behind two different promoters. Both subunits of all mutants were expressed like the wild type enzyme in intracellular membranes of Sf9 cells as indicated by Western blotting experiments, an enzyme-linked immunosorbent assay, and confocal laser scan microscopy studies. The mutants D824N, E834Q, E837Q, and D839N showed no 3-(cyanomethyl)-2-methyl-8(phenylmethoxy)-imidazo[1,2a]pyridine (SCH 3614260; Fax: +31-24-3540525; E-mail: J.dePont@bioch.kun.nl.

TRANSPORT ATPASES ARE ABLE TO CONVERT THE ENERGY FROM ATP INTO ACTIVE ION TRANSPORT. ATPases of the P-type class (1) form an acid-stable phosphorylated intermediate during the catalytic cycle. This phosphorylated intermediate contains an aspartyl phosphate residue present in a conserved domain in the large intracellular loop, which in mammalian P-type ATPases is located between the fourth and fifth transmembrane segments of the catalytic subunit (2).

Phosphorylation of this residue and ion transport are coupled in such a way that specific binding of the cation that has to be transported to the extracellular or intravesicular medium stimulates phosphorylation, whereas binding of the cation to be transported into the cytosol stimulates dephosphorylation. The latter process has only been demonstrated unequivocally in Na\(^+\),K\(^+\)-ATPase and the gastric H\(^+\),K\(^+\)-ATPase. The molecular mechanism of the coupling between the phosphorylation process on the one side and ion binding and transport on the other side is still far from being elucidated.

It is generally assumed that polar amino acid residues present in the transmembrane domains might play a key role in transmembrane ion transport. In particular, negatively charged residues like those originating from aspartate and glutamate are likely candidates for such a role (3, 4). The presence of four transmembrane segments in the N-terminal part of the catalytic subunits of these proteins is generally accepted. In these four transmembrane regions there is only one conserved negatively charged amino acid residue, which might be involved in transmembrane ion transport (3, 5–8). In the C-terminal part of the catalytic subunit, however, the secondary structure is still disputed. Most authors assume the presence of six transmembrane segments in this area, but several models with only four transmembrane segments have been proposed too (9, 10). In the last transmembrane segment there is a pair of negatively charged conserved amino acid residues, but mutational studies up to now give no indication for an important role in Na\(^+\),K\(^+\)-ATPase (11, 12).

Most negatively charged residues are present in the fifth and sixth transmembrane segments (see Fig. 1) that are assumed to be immediately C-terminal of the large intracellular loop. This region of P-type ATPases, however, is rather peculiar. Because of the relatively large number of negatively charged and other polar residues, the hydropathy index is rather low. Moreover, this region contains a number of proline residues, which generally give a break in an a-helix structure. In vitro translation studies with H\(^+\),K\(^+\)-ATPase (13) did not show membrane insertion properties for the fifth and sixth transmembrane segments. For sarcoplasmic and endoplasmic reticulum (SERCA)\(^3\)-type Ca\(^+\)-ATPase, only a stop-transfer signal was found for the fifth but not for the sixth transmembrane segment. No signal anchor sequence was found for either of these segments.

This paper is available online at http://www-jbc.stanford.edu/jbc/
transmembrane segments (14). Lutsenko et al. (15) recently showed that extensive tryptic digestion of Na⁺,K⁺-ATPase led to membrane release of a water-soluble fragment (Gln⁷⁹⁷–Arg⁸²⁹), which included the putative M5–M6 hairpin. Occlusion of K⁺, however, prevented the release of this fragment from the membrane. These findings suggest that (i) the putative transmembrane segments M5 and M6 might be present in the membrane in a form different from a classical α-helix and (ii) this part of the α-subunit plays a role in cation binding and transport.

The putative M5-M6 region in gastric H⁺,K⁺-ATPase contains at least three conserved negatively charged residues (Glu⁷⁹⁷, Glu⁸²⁰, and Asp⁸²⁴), and quite close to the C terminus of M6 there are three other negatively charged residues (Glu³⁸⁴, Glu³⁸⁷, and Asp³⁸⁹), which are also conserved in other ATPases. Although most models for P-type ATPases (16, 17) do not consider these residues as being present in transmembrane segments, some models (see Fig. 1) do (18–21). One of the reasons for the discrepancy between the models for H⁺,K⁺-ATPase and other mammalian P-type ATPases is the presence of two cysteine residues in the M5-M6 region, at least one of which is the target for extracellularly applied acid-activated omeprazole, an inhibitor of gastric acid secretion (19). Moreover, there is a cytosolically located tryptic digestion site at Lys⁷⁹⁴ (19, 20), of which the corresponding amino acid residue in most models is placed within M5. This results in a more C-terminally located position of the transmembrane segments M5 and M6 in H⁺,K⁺-ATPase as compared with the original models for Ca²⁺-ATPase and Na⁺,K⁺-ATPase (16, 17).

Despite this uncertainty, a number of site-directed mutagenesis studies aimed at elucidating the function of these negatively charged residues in the M5-M6 region in Ca²⁺-ATPase of both the SERCA (3, 5, 22–24) and the plasma membrane type (25) as well as in Na⁺,K⁺-ATPase (26–29) have been performed recently. From these studies, several candidate amino acids for a role in transmembrane cation transport have been proposed, but a consistent model has not yet been obtained.

With the gastric H⁺,K⁺-ATPase (18, 21, 30), only one study with mutants has been published until now (31), since functional expression of this enzyme system has only recently been successfully carried out (31–33). We report here mutational studies in which six negatively charged amino acid residues within or close to the fifth and sixth transmembrane segments of the catalytic subunit have been converted into the corresponding acid amides. The study shows that the mutation E₇₉₅Q has no effect, whereas the mutation D₈₂₄N, E₈₂₇Q, E₈₃₇Q, or D₈₃₉Q prevents the formation of a phosphorylated intermediate. The mutation E₈₂₀Q results in a phosphorylated intermediate with a markedly reduced sensitivity toward both K⁺ and the specific H⁺,K⁺-ATPase inhibitor 3-(cyanomethyl)-2-methyl-8-phenylmethoxy)-imidazo[1,2α]pyridine (SCH 28080) in the phosphorylation reaction. The hydrolysis of the phosphointermediate is not stimulated by K⁺, and no K⁺-stimulated ATPase activity can be determined. This emphasizes the importance of these negatively charged residues in the function of H⁺,K⁺-ATPase.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis—**All DNA manipulations were done according to standard molecular biology techniques described by Sambrook et al. (34). The construct pUC19BglIII-HKo (32), containing the full-length cDNA of the rat H⁺,K⁺-ATPase α-subunit was digested with Sphi. After purification from agarose gels, a 1.2-kilobase pair Sphi fragment (base pairs 2228–3400) was inserted in Sphi-digested M13mp18. Nucleotide substitutions were introduced in the rat H⁺,K⁺-ATPase cDNA according to the method of Vandeyar et al. (35), using the T7-GEN In Vitro Mutagenesis Kit (U.S. Biochemical Corp.), resulting in a substitution of Glu⁷⁹⁵→Gln, Glu⁸₃⁴→Glu, Glu⁸₃⁷→Gln, Glu⁸₃⁸→Gln, and Asp⁸₃⁹→Glu. Not only a change in the sequence of rat cDNA as desired was created, but also a recognizable sequence of a restriction site was either introduced or deleted. After selection, digestion with Sphi, and purification of the mutant Sphi fragment from agarose gels, this fragment was ligated into the dephosphorylated pUC19BglII-HK fragment. The mutated clones were digested with BglII and DraI, and a 3.3-kilobase pair BglII fragment was isolated and purified from agarose gels. The baculovirus transfer vector pAcAs3, containing the code for the β-galactosidase (36), was digested with BamHI and dephosphorylated. The 3.3-kilobase pair BglII fragment was ligated into this vector, and the pAcAs mutants were obtained.

**Generation of Recombinant Viruses—**The mutated transfer vectors were used for recombination into the P10 locus of DLZJi virus (32) generating the mutant viruses DLZJiAsbE₇₉₅Q, DLZJiAsbE₈₂₇Q, DLZJiAsbE₈₃₇Q, and DLZJiAsbE₈₃₉Q. The viruses were purified by conventional screening for blue plaques after the addition of 5-bromo-4-chloro-3-indoyl-D-galactosidase (36), using the T7-GEN In Vitro Mutagenesis Kit (U.S. Biochemical Corp.), resulting in a substitution of Glu⁷⁹⁵→Gln, Glu⁸₃⁴→Glu, Glu⁸₃⁷→Gln, and Asp⁸₃⁹→Glu. Not only a change in the sequence of rat cDNA as desired was created, but also a recognizable sequence of a restriction site was either introduced or deleted. After selection, digestion with Sphi, and purification of the mutant Sphi fragment from agarose gels, this fragment was ligated into the dephosphorylated pUC19BglII-HK fragment. The mutated clones were digested with BglII and DraI, and a 3.3-kilobase pair BglII fragment was isolated and purified from agarose gels. The baculovirus transfer vector pAcAs3, containing the code for the β-galactosidase (36), was digested with BamHI and dephosphorylated. The 3.3-kilobase pair BglII fragment was ligated into this vector, and the pAcAs mutants were obtained.

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previous reported H⁺,K⁺-ATPase obtained with the conventional bacular ASSay method is described in Site-directed Mutagenesis of Gastric H⁺,K⁺-ATPase

Production of Recombinant H⁺,K⁺-ATPase—Sf9 cells were grown at 27 °C either in 100-ml spinner flask cultures or as monolayer cultures in 175-cm² culture flasks as described by Klaassen et al. (32). For production of H⁺,K⁺-ATPase, the cells were infected at a multiplicity of infection of 3 in the presence of 1% ethanol (37) with the DLβ2As or βGts mutated viruses and incubated for 3 days. Occasionally the multiplicity of infection was varied from 0.01 to 10, and the Sf9 cells were incubated up to 5 days.

Confocal Laser Scan Microscopy—Sf9 cells were grown on sterile microscope coverslips in complete growth medium and infected with a multiplicity of infection of 3 at 27 °C. After infection, cells were incubated at 27 °C for 48 h in complete growth medium with additions as indicated. Cells were washed three times for 5 min with phosphate-buffered saline (PBS; pH 7.4) followed by fixation in 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. Further processing was done by permeabilization at ~20 °C in 100% methanol for 5 min. Next, the coverslides were dried at room temperature, and aspiric binding sites for antibodies were blocked by incubation with 30–60 min in PBS, 0.05% polyoxyethylene sorbitan monolaurate (Tween 20), 1% fetal bovine serum, and 2% gelatin for 5 min. Next, the coverslides were dried for 5 min each, the cells were incubated with the polyclonal antibody HKB (38) for 30–60 min in PBS, 0.05% Tween 20, 1% (w/v) gelatin, 2% fetal bovine serum. Free antibodies were removed by washing the cells as above. After washing, the cells were incubated for 5 min with 1% (w/v) gelatin, 2% fetal bovine serum, and 1% Tween 20, with gentle rocking. After washing with PBS, 0.05% Tween 20 three times for 5 min each, the cells were incubated with the polyclonal antibody HKB (38) for 30–60 min in PBS, 0.05% Tween 20, 1% (w/v) gelatin, 2% fetal bovine serum. Free antibodies were removed by washing the cells as above. Next, the cells were incubated with a fluorescently labeled secondary antibody, Gilostrup, HistoGene (Palo Alto, CA). Coverslides were washed with 1% PBS, 0.05% Tween 20, 1% gelatin, and 2% fetal bovine serum. From this moment on, the coverslides were kept in the dark as much as possible. After washing as above, the cells were desalted by a short wash with water, dehydrated with 100% methanol, dried, and mounted in 10% acetic acid for 15–30 min at room temperature. After centrifugation for 30 min at 100,000 × g at 4 °C. These viruses were used to infect Sf9 cells. Fig. 2 shows that these viruses were used to infect Sf9 cells.}

WESTERN BLOTTING—Protein samples from the membrane fraction were solubilized in SDS-PAGE sample buffer and separated on SDS-gels containing 10% acrylamide according to Laemmli (43). For immunoblotting, the separated proteins were transferred to Immobilon PVDF membranes. The a- and β-subunit of H⁺,K⁺-ATPase were detected as described earlier (32), with the polyclonal antibody HKB (38) recognizing the 556–585 region of the a-subunit of H⁺,K⁺-ATPase and the monoclonal antibody 2G11 (44) evoked against the b-subunit of H⁺,K⁺-ATPase specifically.

K⁺-ATPase Activity Assay—The K⁺-activated ATPase activity was determined with a radiochemical method (45). For this purpose, 0.6–5 μg of Sf9 membranes were added to 100 μl of medium, which contained 10 μM [γ-32P]ATP (specific activity 100–500 μCi/mmol⁻¹), 1.0 mM MgCl₂, 0.2 mM EDTA, 0.1 mM EDTA, 0.1 mM ouabain, 1 mM NaN₃, 25 mM Tris-HCl (pH 7.0), and varying concentrations of KCl. After incubation for 30 min at 37 °C, the reaction was stopped by adding 500 μl of ice-cold 10% (w/v) charcoal in 6% (w/v) trichloroacetic acid, and after 10 min at 0 °C, the mixture was centrifuged for 10 s (10,000 × g). To 0.2 ml of the clear supernatant, containing the liberated inorganic phosphate (35P), 3 ml of OptiFluor (Canberra Packard, Tilburg, The Netherlands) was added, and the mixture was analyzed by liquid scintillation analysis.

Analysis of Data—The L₃ values for K⁺ and SCH 28080 were iteratively determined by fitting the concentration relationship to the logistic equation

\[ Y = A + \frac{B - A}{1 + (X/Xₜₜ)} \]

where A represents the bottom plateau, B is the top plateau, C is the IC₅₀ and D is the Hill coefficient; the values of X and C were entered as the logarithm of concentration) using the nonlinear regression computer program InPlot (GraphPad Software for Science, San Diego, CA). All data are presented as mean values with standard error of the mean, and differences of average were tested for significance by means of Student’s t test.

RESULTS

Six baculoviruses were produced, each of which contains coding sequences for the β-subunit as well as for a mutated a-subunit of gastric H⁺,K⁺-ATPase. In each of the mutants, one of the negatively charged carboxyl residues located in or around the fifth and sixth transmembrane segments of the α-subunit had been converted into an acid amide residue. These viruses were used to infect Sf9 cells. Fig. 2 shows that the a-subunit present in the membrane fractions of Sf9 cells infected with these mutated viruses has the same apparent molecular mass as the a-subunit of the enzyme of pig gastric mucosa (47). The antibody used to detect the a-subunit on the
Mutation of amino acid residues in a protein might not only decrease the amount of phosphorylated intermediate but could also lead to disturbances in the catalytic cycle. In gastric H⁺,K⁺-ATPase phosphorylation occurs upon the addition of Mg²⁺-ATP (45). K⁺ ions not only decrease the amount of phosphorylated intermediate by stimulating the dephosphorylation reaction, but they also prevent formation of it by shifting the Eᵡ = Eᵣ equilibrium to the right (45). Fig. 4 (top panel) shows that the presence of such a phosphorylated protein in the enzyme produced by the wild type virus can be visualized upon autoradiography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a band with an apparent molecular mass of 100 kDa. This band is absent when 10 mM K⁺, 0.1 mM SCH 28080, or 1 mM vanadate was present in the incubation medium before ATP was added. The specific Na⁺,K⁺-ATPase inhibitor ouabain (1 mM) did not affect the appearance of the 100 kDa band. Fig. 4 shows an additional band of about 140 kDa in the membrane fraction obtained from the Sf9 cells infected with the recombinant virus. This product is absent in the purified pig enzyme but is also present in the membranes of uninfected Sf9 cells (32). It is absent when 10 mM K⁺ is added. The specific Na⁺,K⁺-ATPase (49,50) has been reported in studies in which Na⁺,K⁺-ATPase (49,50) has been expressed with the baculovirus system. In the mutants, the H⁺,K⁺-ATPase-specific phosphorylated intermediate was only found in the mutants E795Q and E820Q (Fig. 5). No intermediate was found in the mutants D824N, E834Q, E837Q, and

**Fig. 3. Confocal image analysis of sections of Sf9 cells expressing H⁺,K⁺-ATPase.** Sf9 cells were infected with baculoviruses expressing the (mutated) α-subunit and the β-subunit of H⁺,K⁺-ATPase, and the presence of the α-subunit was visualized as described under “Experimental Procedures.” Label was found in the cytoplasm around the nucleus in all mutants and the wild type. The following viruses were used: uninfected (A); DZ1 (32) (control) (B), Bgla-wt (wild type virus) (C), DLZβASα-E795Q (D), Bgla-E820Q (E), DLZβASα-D824N (F), DLZβASα-E834Q (G), Bgla-E837Q (H), and DLZβASα-D839N (I). Bar, 10 μm.

**Fig. 2. Western blot of H⁺,K⁺-ATPase mutants.** Membranes (10–20 μg) isolated from Sf9 cells infected either with wild type virus or mutated viruses expressing the α- and β-subunits of H⁺,K⁺-ATPase were blotted, and the presence of the α-subunit was detected using the polyclonal antibody HKB (38). For comparison, the enzyme isolated from gastric mucosa (pig) and membranes isolated from uninfected cells are shown.

**Fig. 4. Autoradiogram of SDS-polyacrylamide gel of the ATP-phosphorylated Sf9 membranes infected with wild type and mutated baculoviruses.** Membranes isolated from Sf9 cells infected with wild type virus or the mutant viruses E795Q and E820Q were phosphorylated at 0 °C with 0.1 μM [γ-³²P]ATP in the presence of 1 mM MgCl₂, and 20 mM Tris-acetic acid (pH 6.0) after preincubation for 60 min at 0 °C with 100 μM SCH 28080, 10 mM KCl, 1 mM ouabain, or 1 mM vanadate. The acid-quenched samples were solubilized and subjected to SDS-PAGE at pH 6.5 as described under “Experimental Procedures.” Purified pig gastric H⁺,K⁺-ATPase was used as control.
The amount of phosphorylated intermediate was measured both after preincubation with 0.1 mM SCH 28080 and in its absence and was expressed as pmol of E-\(\text{P}\)-ing of protein. In the presence of SCH 28080, the amount of phosphorylated intermediate was in most cases not different between the mutants and the wild type enzyme (Table I); only with mutant E820Q this background phosphorylation was significantly higher than with the wild type enzyme (see below). The difference between the ATP phosphorylation level in the absence of SCH 28080 and in its presence was taken as the H\(^+\),K\(^-\)-ATPase-specific phosphorylated intermediate. Table I shows that in the four mutants in which no 100-kDa phosphoprotein could be seen in the SDS gels (Fig. 5), the amount of specific phosphorylated intermediate was not significantly different from zero. The amount of phosphorylated intermediate of the two mutants of which a 100-kDa phosphoprotein could be visualized on the SDS gel (E795Q and E820Q) indeed showed a significant phosphorylation capacity.

The amount of H\(^+\),K\(^-\)-ATPase protein expressed was measured using an enzyme-linked immunosorbent assay (40), based on specific binding of the produced protein to the monoclonal antibody 5B6 (41), which recognizes an epitope in the intracellular loop between the fourth and fifth transmembrane domain of the catalytic subunit of pig gastric H\(^+\),K\(^-\)-ATPase (42). By comparing the amount of immunoreactive protein with that of the pig enzyme, the amount of baculovirus-produced (mutated) H\(^+\),K\(^-\)-ATPase could be quantitated. Table I shows that the amount of immunoreactive protein in the particulate membrane fractions varied from 0.84 to 4.13% of the total amount of protein. Although no explanation can be given for this variation, the table clearly shows that all viruses produced a considerable amount of mutated H\(^+\),K\(^-\)-ATPase protein, indicating that the lack of activity of the mutants D824N, E834Q, E837Q, and D839N is not due to lack of biosynthesis. The amount of phosphorylated intermediate produced by the mutants E795Q and E820Q either expressed per mg of protein or per mg of H\(^+\),K\(^-\)-ATPase was significantly reduced compared with the wild type enzyme.

In a series of experiments, the incubation conditions for the wild type virus have been optimized. The period of infection and the multiplicity of infection were varied, and in these experiments both the amount of SCH 28080-sensitive phosphorylated intermediate and the amount of immunoreactive H\(^+\),K\(^-\)-ATPase produced by the insect cells were determined. Fig. 6 shows that for the wild type enzyme there is a positive correlation between these two parameters, although individual experiments do deviate considerably from the calculated regression line. From this kind of experiment the conditions used in the present study (3 days of infection and a multiplicity of infection of 3) were chosen. The figure clearly shows that in addition to the mutants D824N, E834Q, E837Q, and D839N, which have no measurable phosphorylation capacity, mutant E795Q has a relatively low phosphorylation capacity, compared with its expression level. The phosphorylation capacity of mutant E820Q was slightly lower than that of the wild type enzyme.

The two mutants E795Q and E820Q, which show the presence of a SCH 28080-sensitive phosphorylated intermediate, have been studied in more detail. The membranes containing the (mutated) enzyme were preincubated for 60 min at 0°C (pH 6.0) with varying concentrations of either K\(^+\) or SCH 28080. Fig. 7A shows that the K\(^+\) sensitivity of mutant E795Q (I\(_{50}\) = 0.45 ± 0.10 mM; n = 3), is similar to that of the wild type enzyme (I\(_{50}\) = 0.38 ± 0.04 mM; n = 5). The I\(_{50}\) value of mutant E820Q, however, is 10 times higher (I\(_{50}\) = 4.5 ± 1.2 mM; n = 4), and complete inhibition is not reached at 30 mM. Some reduction was also found with either 100 mM Na\(^+\) or choline\(^+\) (not shown), indicating that the inhibitory effect of high [K\(^+\)] on the level of the phosphorylated intermediate of E820Q might even be in part nonspecific. This mutant has additionally a 100 times lower sensitivity toward SCH 28080 (Fig. 7B). An I\(_{50}\) value of 1.7 ± 0.6 mM (n = 4) was found, whereas the wild type enzyme has an I\(_{50}\) value of 14 ± 3 mM (n = 4) and mutant E795Q has an I\(_{50}\) value of 8 ± 3 mM (n = 4).

The effects of K\(^+\), SCH 28080, ouabain, and vanadate, if present during the preincubation step, on the ATP phosphorylation level are also reflected in the autoradiograms of the SDS-PAGE gels (Fig. 4, middle and lower panels) of these two mutants. The figure clearly shows that these reagents have

**Fig. 5. Autoradiogram of SDS-polyacrylamide gel of the ATP-phosphorylated Sf9 membranes infected with mutated recombinant baculoviruses.** Wild type enzyme and the H\(^+\),K\(^-\)-ATPase mutants E795Q, E820Q, D824N, E834Q, E837Q, and D839N were phosphorylated at 0°C with 0.1 mM \(\gamma\)-\(\text{P}\)-ATP in the presence of 1 mM MgCl\(_2\) and 20 mM Tris-acetic acid (pH 6.0). The acid-quenched samples were solubilized and subjected to SDS-PAGE at pH 6.5 as described under “Experimental Procedures.” Uninfected Sf9 membranes as well as gastric H\(^+\),K\(^-\)-ATPase (pig) were used as control.

**Table I**

Properties of Sf9 membranes after infection with recombinant baculoviruses

Sf9 cells were infected with recombinant baculoviruses containing the information for either the wild type H\(^+\),K\(^-\)-ATPase or the H\(^+\),K\(^-\)-ATPase mutants E795Q, E820Q, D824N, E834Q, E837Q, and D839N with a multiplicity of infection of 3. After 72 h, the membranes were isolated, and the H\(^+\),K\(^-\)-ATPase expression level (percentage of H\(^+\),K\(^-\)-ATPase) and the SCH 28080-sensitive (E-P) and insensitive background (Bkg) ATP-phosphorylation capacity were determined as described under “Experimental Procedures.” Averages with standard error of the mean for the indicated number of preparations (n) are presented. Differences of the data compared with the wild type enzyme are tested for significance by means of Student’s t test (*, p < 0.1; **, p < 0.05; ***, p < 0.01).

<table>
<thead>
<tr>
<th>mutant</th>
<th>n</th>
<th>Bkg</th>
<th>E-P</th>
<th>H(^+),K(^-)-ATPase</th>
<th>E-P</th>
</tr>
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<tbody>
<tr>
<td>Uninfected</td>
<td>5</td>
<td>0.87 ± 0.18</td>
<td>−0.03 ± 0.04</td>
<td>0.00 ± 0.00</td>
<td></td>
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<tr>
<td>Wild type enzyme</td>
<td>9</td>
<td>0.69 ± 0.09</td>
<td>1.15 ± 0.11</td>
<td>1.50 ± 0.17</td>
<td>78.1 ± 10.2</td>
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<tr>
<td>E795Q</td>
<td>5</td>
<td>0.78 ± 0.16</td>
<td>0.66 ± 0.12(^{**})</td>
<td>4.13 ± 0.86(^{***})</td>
<td>19.7 ± 6.0</td>
</tr>
<tr>
<td>E820Q</td>
<td>4</td>
<td>1.05 ± 0.15(^{**})</td>
<td>0.52 ± 0.04(^{****})</td>
<td>1.86 ± 0.45</td>
<td>33.2 ± 7.1</td>
</tr>
<tr>
<td>D824N</td>
<td>5</td>
<td>0.77 ± 0.16</td>
<td>0.42 ± 0.02(^{****})</td>
<td>3.13 ± 0.82(^{**})</td>
<td>2.4 ± 1.3</td>
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<tr>
<td>E834Q</td>
<td>6</td>
<td>0.76 ± 0.17</td>
<td>0.02 ± 0.03(^{****})</td>
<td>1.36 ± 0.23</td>
<td>−4.5 ± 4.0</td>
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<tr>
<td>E837Q</td>
<td>3</td>
<td>0.53 ± 0.13</td>
<td>−0.03 ± 0.05(^{****})</td>
<td>0.84 ± 0.28(^{#})</td>
<td>−9.3 ± 5.6</td>
</tr>
<tr>
<td>D839N</td>
<td>5</td>
<td>0.67 ± 0.08</td>
<td>0.07 ± 0.04(^{****})</td>
<td>1.97 ± 0.40</td>
<td>4.3 ± 1.8</td>
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hardly any effect on the formation of the phosphorylated intermediate of mutant E820Q in contrast to both the wild type enzyme and mutant E795Q.

The reduced sensitivity of mutant E820Q for SCH 28080 has another consequence. The background phosphorylation (Table I, column 2) has been defined as the SCH 28080 (100 μM)-insensitive phosphorylation capacity. Since SCH 28080 even at 1 mM is not able to inhibit the formation of the phosphorylated intermediate of E820Q completely (see Fig. 7B), the background phosphorylation, attributed to the 140-kDa product, has been overestimated. This explains the significantly higher background phosphorylation level of mutant E820Q as compared with the wild type enzyme (Table I) and implicitly suggests that the specific phosphorylation level of this mutant has been underestimated.

In the above mentioned studies, the effect of K⁺ on the specific phosphorylation capacity was investigated by preincubation with this ion, thus preventing formation of a phosphorylated intermediate. We next prepared a phosphorylated intermediate in the absence of K⁺ and SCH 28080 and measured the residual amount of phosphorylated intermediate after incubation for 5 and 10 s in the presence of either K⁺ or ADP. Fig. 8 shows that the phosphorylated intermediate obtained with the wild type virus is K⁺-sensitive as expected. There is hardly any effect of ADP on the dephosphorylation rate of this phosphorylated intermediate, in contrast to the situation with the pig enzyme, where a small effect has been observed (46). A similar behavior was found with the E795Q mutant. The E820Q mutant, however, was insensitive toward added K⁺ up to 100 mM. The E820Q mutant also showed no sensitivity for ADP, suggesting that this mutation did not lead to a blockade of the $E_1-P \rightarrow E_2^-P$ conversion, which would have resulted in an ADP-sensitive phosphorylated intermediate. Surprisingly, the SCH 28080-insensitive phosphorylation, which in the SDS gel is responsible for the band of 140 kDa and is also present in uninfected cells, also decreased with time. The rate of dephosphorylation of this SCH 28080-insensitive phosphorylated protein was increased by ADP but not by the presence of K⁺ (not shown).

In order to test whether the phosphorylated intermediate of the wild type enzyme and of the mutants E795Q and E820Q was an acylphosphate, the intermediate was treated with hydrazinotriacetic and the resulting acid-soluble products were separated by high-speed centrifugation. The results are shown in Fig. 8. The wild type intermediate contained a band at 140 kDa, which was not present in the intermediate of E820Q. This band was identified as the acylphosphate by treatment with hydrazinotriacetic and subsequent nondenaturing polyacrylamide gel electrophoresis. The acylphosphate was not sensitive to SH-3,3′-dithiobispropionimidate, which is a specific inhibitor of acylphosphatases (51). The E820Q mutant also contained an acylphosphate, which was not present in the intermediate of E795Q. These results suggest that the difference in sensitivity of the mutants to SCH 28080 is due to a difference in the structure of the phosphorylated intermediate.
Ca-ATPases and Na⁺,K⁺-ATPase can be excluded as candidates for this 140-kDa protein.

Phosphorylation and dephosphorylation are key steps in the catalytic cycle of H⁺,K⁺-ATPase. Fig. 10 shows the K⁺ dependence of the overall ATPase activity of membranes of uninfected Sf9 cells and cells infected with viruses expressing the wild type enzyme and the mutants E795Q and E820Q. In this assay a relatively low ATP concentration (10 μM) had to be used in order to obtain significant stimulation by K⁺. The wild type enzyme showed a biphasic activation curve with a maximum at 1 mM K⁺. This activation could be completely blocked by 100 μM SCH 28080 (not shown). A similar biphasic activation curve was found with the pig enzyme using a comparable low (5 versus 10 μM) ATP concentration (45). The E820Q mutant showed, like the uninfected cells, no K⁺-dependent ATPase activity. Low K⁺ activated the ATPase activity of mutant E795Q, although the maximal level reached was less than that of the wild type enzyme, as is also the case for the phosphorylation capacity of this mutant (Fig. 6). At high K⁺, however, less inhibition was found with this mutant than with the wild type enzyme. The maximal ATPase activity with the wild type enzyme was only 60% above the background activity, whereas the steady-state phosphorylation level was 170% above the control level (Table I). The relatively minor increase in the ATPase assay is due to the high basal ATPase activity of the membranes of Sf9 cells. It is tempting to speculate that the high basal ATPase activity is related to the relatively high dephosphorylation rate of uninfected cells in the absence of K⁺ (Fig. 9).

**DISCUSSION**

In the present study we converted six negatively charged amino acid residues present in the M5-M6 region of the α-subunit of gastric H⁺,K⁺-ATPase in their acid amide counterpart as a first approach to establish the importance of these glutamate and aspartate residues for the function of this transport enzyme. The studies were carried out in the baculovirus system, in which we were able to express the enzyme functionally, by constructing viruses with the (mutated) α-subunit and the β-subunit behind two different promoters (32, 37). As a main functional parameter, we measured the presence of a phosphorylated intermediate both qualitatively by autoradiography of the 100-kDa phosphorylated intermediate and quantitatively by measuring the SCH 28080-sensitive ATP-phosphorylation level. We also determined the biosynthesis of immunoreactive α-subunit using a specific enzyme-linked immunosorbent assay (40). Although the amount of immunoreactive α-subunit varied considerably from experiment to experiment, we have no indication that proteolytic breakdown of one of the mutants was enhanced. Moreover, we have no indication of routing problems with any of the mutants.

None of the mutants E834Q, E837Q, and D839N showed any ATP phosphorylation capacity, suggesting that each of these residues is essential for the enzyme to become phosphorylated. It might be that these residues are involved in H⁺ binding, which is essential for ATP phosphorylation. Similar residues in other P-type ATPases have met less attention until now, due to the fact that in nearly all models these residues are located in the intracellular loop between M6 and M7 and not in transmembrane segments (see Fig. 1). Further studies on the precise location of these residues and mutational studies in other P-type ATPases are needed to reach more definite conclusions.

The H⁺,K⁺-ATPase mutant D824N was also not active in terms of phosphorylation capacity by ATP. This Asp residue is completely conserved in Na⁺,K⁺-ATPase and Ca²⁺-ATPases from both sarcoplasmic reticulum and plasma membrane. Mutation of the similar Asp residue in either Na⁺,K⁺-ATPase (26, 28) or plasma membrane Ca²⁺-ATPase (25) did not result in active enzyme either. In SERCA1a Ca²⁺-ATPase, the similar mutant D800N did not show Ca²⁺ occlusion or Ca²⁺-induced phosphorylation by ATP (52). Thus, it is possible that this residue is involved in the binding of cations by all P-type ATPases, and thus amino acid substitutions affect phosphorylation from ATP.

Mutation of Glu⁷⁹⁵ into Gln results in formation of a phos-
phorylated intermediate with apparently normal behavior toward K⁺ and SCH 28080. The amount of this intermediate formed is smaller than that for the wild type enzyme, as might be expected from the measurement of immunoreactive α-subunit. This might be due to the production of more inactive α-subunit. Mutational studies of the similar residue in SERCA Ca²⁺-ATPase and Na⁺,K⁺-ATPase suggest a more important role of this residue in these two P-type ATPases. The obtained results, however, markedly depend on the type of the amino acid residue chosen to replace the glutamate present in these two enzymes and possibly on the expression system used. Replacement of Glu⁷⁷⁹ into Asp or Leu in Na⁺,K⁺-ATPase expressed in HeLa cells did not result in active enzyme, whereas mutation of this Glu into Gln or Ala gave an active enzyme (27). Mutation of the same Glu residue into an Asp, using the baculovirus system, resulted in an active enzyme with only reduced cation affinity (29). Replacement of Glu⁷⁷⁹ by a Lys even resulted in an increase in cation affinity in the latter system. Moreover, Glu⁷⁷⁹ in Na⁺,K⁺-ATPase is the target for the carboxyl-specific reagent 4-(diazomethyl)-7-(diethylamine)-coumarin (53, 54), which inactivates the enzyme in a cation-protective way. In SERCA Ca²⁺-ATPase, mutation of the corresponding residue, Glu⁷⁷¹, into Gln results in inhibition of Ca²⁺ transport (3) and Ca²⁺ occlusion (55). Phosphorylation from ATP at 2.5 mM Ca²⁺ and Ca²⁺-induced inhibition of phosphorylation by inorganic phosphate still occurs (22). The dephosphorylation reaction of the ADP-insensitive intermediate was blocked in this mutant. Mutation of this Glu residue by either Gly or Ala resulted in similar effects. Replacement of Glu by Lys resulted in a mutant in which Ca²⁺ had no effect on phosphorylation from either ATP or inorganic phosphate (56). Moreover the dephosphorylation step was not inhibited by Ca²⁺ in this mutant. These experiments led Andersson to the suggestion that Glu⁷⁷¹ might participate in countertransport of two protons/ Ca²⁺-ATPase cycle (56).

The most interesting mutant made in the present study is ES20Q. This mutant yields a phosphorylated intermediate from ATP, but preincubation with either K⁺ or SCH 28080 had, in contrast to the wild type enzyme, hardly any effect on the steady-state ATP phosphorylation level. The hydrolysis of this phosphorylated intermediate was insensitive to both ADP and K⁺, and no K⁺-activated ATPase activity could be detected in this mutant. In the pig enzyme, K⁺ lowers the steady-state phosphorylation level both by shifting the E₁⁻P = E₂ equilibrium to the right, which is assumed to occur through a cytosolic K⁺-binding site (45), and by increasing the rate of dephosphorylation, which occurs through an extracellular accessible K⁺-binding site. SCH 28080, a K⁺ antagonist, is assumed to stabilize the E₂ form of the enzyme, thus preventing formation of a phosphorylated intermediate (57, 58). ADP stimulates hydrolysis of an E₁⁻P form of the enzyme (46). The K⁺ insensitivity of the phosphoryzation of mutant ES20Q is not due to inhibition of the E₁⁻P → E₂⁻P conversion, since ADP does not increase the hydrolysis rate of the phosphorylated intermediate. It is also not due to formation of an abnormal intermediate, since the hydroxylamine sensitivity of the phosphorylated intermediate indicates that also in this mutant an acylphosphate had been formed. The finding that SCH 28080 does not prevent formation of a phosphorylated intermediate can be explained by assuming that the drug is no longer able to convert the mutated enzyme to the E₁ form. A similar explanation can be given for the fact that vanadate does not completely preclude ATP phosphorylation. However, the mutation could also affect the binding of these drugs. The mutation of residue Glu⁸²⁰ thus affects an extracellularly accessible K⁺-binding site and possibly also a cytosolically accessible K⁺-site.

Binding of extracellular K⁺ to the pig enzyme results in a long range conformational change, which enhances the hydrolysis rate of the E-P at Asp⁸⁰⁴. The presented results in this paper suggest that this process is no longer possible in mutant ES20Q. This suggests that Glu⁸²⁰ is directly involved in K⁺ binding. There are indications that the similar residue in other ATPases is also involved in cation binding. In addition, it is striking that in the K⁺-sensitive P-type ATPases this residue is an Asp or a Glu, whereas in the K⁺-insensitive Ca²⁺-ATPases this residue is an Asn. In Na⁺,K⁺-ATPase, mutation of this residue (Asp⁸⁰⁴) into Asn or Glu resulted in an inactive enzyme as measured by the inability to confer ouabain resistance to ouabain-sensitive cells (26, 28). In plasma membrane Ca²⁺-ATPase, mutation of this residue (Asn⁷⁷⁹) into alanine abolished Ca²⁺ uptake and phosphorylation from ATP (25). In SERCA α Ca²⁺-ATPase, the N796A mutation resulted in the absence of ATP-dependent phosphorylation or Ca²⁺-occlusion, but the mutant still showed Ca²⁺-dependent inhibition of the phosphorylation from inorganic phosphate (3). This is explained by the assumption that for ATP-phosphorylation binding of two Ca²⁺ ions is necessary, whereas binding of a single Ca²⁺ ion can already inhibit phosphorylation from inorganic phosphate (52). All of these studies indicate that in all P-type ATPases the amino acids present on the site similar to Glu⁸²⁰ are involved in cation binding.

In summary, the present study emphasizes the importance of Glu⁸²⁰ for coupling between ATP phosphorylation and K⁺ transport in gastric H⁺,K⁺-ATPase. Further studies with this enzyme are necessary to understand the structural basis for the specificity of and the kinetic differences between the various P-type ATPases.

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REFERENCES

Site-directed Mutagenesis of Gastric H\(^{+}\),K\(^{-}\)-ATPase