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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*

(Received for publication, April 27, 1996, and in revised form, August 30, 1996)

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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 between 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 28080)-sensitive ATP dependent phosphorylation capacity. Mutants E795Q and E820Q formed a phosphorylated intermediate, which, like the wild type enzyme, was hydroxylamine-sensitive, indicating that an acylphosphate was formed. Formation of the phosphorylated intermediate from the E795Q mutant was similarly inhibited by K\(^+\) (I\(_{50}\) = 0.4 mM) and SCH 28080 (I\(_{50}\) = 10 nM) as the wild type enzyme, when the membranes were preincubated with these ligands before phosphorylation. The dephosphorylation reaction was K\(^+\)-sensitive, whereas ADP had hardly any effect. Formation of the phosphorylated intermediate of mutant E820Q was much less sensitive toward K\(^+\) (I\(_{50}\) = 4.5 mM) and SCH 28080 (I\(_{50}\) = 1.7 mM) than the wild type enzyme. The dephosphorylation reaction of this intermediate was not stimulated by either K\(^+\) or ADP. In contrast to the wild type enzyme and mutant E795Q, mutant E820Q did not show any K\(^+\)-stimulated ATPase activity. These findings indicate that residue Glu\(^{820}\) might be involved in K\(^+\) binding and transition to the \(E_2\) form of gastric H\(^+\),K\(^+\)-ATPase.

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\(^{2+}\)-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

* This work was supported by the Netherlands Foundation for Scientific Research, Division of Medical Sciences Grant 902-22-086. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: SERCA, sarcoplasmic and endoplasmic reticulum Ca\(^{2+}\)-ATPase; E-P, phosphorylated intermediate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SCH 28080, 3-(cyanomethyl)-2-methyl-8(phenylmethoxy)imidazo[1,2a]pyridine.

Printed in U.S.A.


29764 This paper is available on line at http://www-jbc.stanford.edu/jbc/
transmembrane segments (14). Lutsenko et al. (15) recently showed that extensive tryptic digestion of Na\textsuperscript{+},K\textsuperscript{+}-ATPase led to membrane release of a water-soluble fragment (Gln\textsuperscript{787} - Arg\textsuperscript{829}), which included the putative M5-M6 hairpin. Occlusion of K\textsuperscript{+}, 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 \(\alpha\)-helix and (ii) this part of the \(\alpha\)-subunit plays a role in cation binding and transport.

The putative M5-M6 region in gastric H\textsuperscript{+},K\textsuperscript{+}-ATPase contains at least three conserved negatively charged residues (Glu\textsuperscript{795}, Glu\textsuperscript{820}, and Asp\textsuperscript{824}). Moreover, quite close to the C terminus of M6 there are three other negatively charged residues (Glu\textsuperscript{834}, Glu\textsuperscript{837}, and Asp\textsuperscript{839}), 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\textsuperscript{+},K\textsuperscript{+}-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 Lys\textsuperscript{791} (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\textsuperscript{+},K\textsuperscript{+}-ATPase as compared with the original models for Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+},K\textsuperscript{+}-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 Cu\textsuperscript{2+}-ATPase of both the SERCA (3, 5, 22–24) and the plasma membrane type (25) as well as in Na\textsuperscript{+},K\textsuperscript{+}-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\textsuperscript{+},K\textsuperscript{+}-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 mutations D824N, E824Q, E825Q, or D829Q prevents the formation of a phosphorylated intermediate. The mutation E820Q results in a phosphorylated intermediate with a markedly reduced sensitivity toward both K\textsuperscript{+} and the specific H\textsuperscript{+},K\textsuperscript{+}-ATPase inhibitor 3-(cyanomethyl)-2-methyl-8(phenylmethoxy)-imidazo[1,2-\(a\)]pyridine (SCH 28080) in the phosphorylation reaction. The hydrolysis of the phosphointermediate is not stimulated by K\textsuperscript{+}, and no K\textsuperscript{+}-stimulated ATPase activity can be determined. This emphasizes the importance of these negatively charged residues in the function of H\textsuperscript{+},K\textsuperscript{+}-ATPase.
previous reported H⁺,K⁺-ATPase obtained with the conventional baculovirus method as described before.

Production of Recombinant H⁺,K⁺-ATPase—Sf9 cells were grown at 27 °C in either 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 DL2βSAs or BGia mutated viruses and incubated for 3 days. Occasionally the multiplicity of infection was varied from 0.1 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 aspecific binding sites for antibodies were blocked by incubation for 30–60 min in PBS, 0.05% polyoxyethylene sorbitan mono- laurate (Tween 20), 1% (w/v) gelatin, and 2% fetal bovine serum. From this point 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 Tween 20, 1% (w/v) gelatin, and 2% fetal bovine serum. Free antibodies were removed by washing the cells as above. Next, the cells were incubated with a fluorescein-labeled secondary antibody and finally, blinks were incubated in a Hoechst dye solution. The ATase activity is presented as the percentage of the activity in the absence of added K⁺ (5 μM), which is 70–150 pmol of P, liberated per mg per h. The latter activity is endogenously present in membranes of Sf9 cells.

ATP Phosphorylation Capacity—ATP phosphorylation was deter- mined as described before (37). Sf9 membranes (40–100 μg) were incubated at 0 °C in 25 mM Tris-acetic acid (pH 6.0), 1 mM MgCl₂ with and without 0.1 mM SCH 28080 in 0.2% (v/v) ethanol in a volume of 50 μl. After 30–60 min of preincubation, 10 μl of 0.6 μM [γ-32P]ATP was added, and the mixture was incubated for another 10 s at 0 °C. The reaction was stopped by adding 5% trichloroacetic acid in 0.1 M phosphoric acid, and the phosphorylated protein was collected by filtration over a 0.8-μm membrane filter (Schleicher and Schuell, Dassel, Germany). After repeated washing, the filters were analyzed by liquid scintillation analysis. The SCH 28080-sensitive part is defined as the H⁺,K⁺-ATPase-dependent phosphorylation; the activity in the presence of SCH 28080 is the background phosphorylation.

Dephosphorylation Studies—After ATP phosphorylation as described above, the reaction mixture was diluted from 60 to 200 μl with nonradioactive ATP (final concentration 1 μM) in order to prevent rephosphorylation with radioactive ATP and the ligands to be tested (5 mM ADP, 10 mM K⁺) (46). The mixture was further incubated for 5 or 10 s at 0 °C. Thereafter, the reaction was stopped as described above, and the residual phosphorylation level was determined.

Hydroxylamine Sensitivity of the Phosphorylated Intermediate—Af- ter ATP phosphorylation, the acid-denaturated membranes present on the membrane filters were washed with 0.5 M imidazole-HCl (pH 7.5). After exposure of the filter to either ice-cold 0.5 mM hydroxylamine-imidazole (pH 7.5) or 0.5 mM imidazole-HCl (pH 7.5) for 10 min, the membranes were washed with 5% trichloroacetic acid in 0.1 M phosphoric acid and analyzed by liquid scintillation analysis.

Analysis of Data—The I50 values for K⁺ and SCH 28080 were iteratively determined by fitting the concentration relationship to the logistic equation $Y = A + \frac{B - A}{1 + (C/A)^D}$ (where A represents the bottom plateau, B is the top plateau, C is I50, 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.

Chemicals—[γ-32P]ATP (3000 Ci mmol⁻¹, Amersham, Buckingham- shire, UK) was diluted with nonradioactive Tris-ATP (pH 6.0) to a specific radioactivity of 20–100 Ci mmol⁻¹. SCH 28080, kindly provided by Dr. A. Barnett (Schering-Plough, Bloomfield, NJ), was dissolved in ethanol and diluted to its final concentration of 0.1 mM in 0.2% ethanol. The antibodies 2G11, HKB, and HKCa2 were gifts of Drs. J. Forte (University of California, Berkeley), M. Caplan (Yale University), and A. Smolka (University of South Carolina), respectively.

RESULTS

Six baculoviruses were produced, each of which contains coding sequences for the β-subunit as well as for a mutated α-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 α-subunit present in the membrane fractions of Sf9 cells infected with these mutated viruses has the same apparent molecular mass as the α-subunit of the enzyme of pig gastric mucosa (47). The antibody used to detect the α-subunit on the
Mutation of amino acid residues in a protein might not only affect the function of the protein but could also lead to disturbances in the routing of the α-subunit. In all the mutants the confocal image was similar to that of the wild type enzyme, indicating that the routing of the α-subunit was not disturbed.

One of the characteristic properties of the P-type ATPases is the formation of an acid-stable phosphorylated intermediate during 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 E₁ ↔ E₂ equilibrium to the right (45). Fig. 4 (top panel) shows that in all the mutants the confocal image was similar to that of the wild type enzyme, indicating that the routing of the α-subunit was not disturbed.

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. 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), Bg-da (wild type virus) (C), DLZβASa-E795Q (D), Bg-da-E820Q (E), DLZβASa-D839N (F), DLZβASa-E834Q (G), Bg-da-E837Q (H), and DLZβASa-D839N (I). Bar, 10 μm.

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-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\textsuperscript{+},K\textsuperscript{-}-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\textsuperscript{+},K\textsuperscript{-}-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\textsuperscript{+},K\textsuperscript{-}-ATPase (42). By comparing the amount of immunoreactive protein with that of the pig enzyme, the amount of baculovirus-produced (mutated) H\textsuperscript{+},K\textsuperscript{-}-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\textsuperscript{+},K\textsuperscript{-}-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\textsuperscript{+},K\textsuperscript{-}-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\textsuperscript{+},K\textsuperscript{-}-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\textsuperscript{+} or SCH 28080. Fig. 7A shows that the K\textsuperscript{+} sensitivity of mutant E795Q (I50 = 0.45 ± 0.10 mM; n = 3), is similar to that of the wild type enzyme (I50 = 0.38 ± 0.04 mM; n = 5). The I50 value of mutant E820Q, however, is 10 times higher (I50 = 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\textsuperscript{+} or choline\textsuperscript{+} (not shown), indicating that the inhibitory effect of high [K\textsuperscript{+}] 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 I50 value of 1.7 ± 0.6 μM (n = 4) was found, whereas the wild type enzyme has an I50 value of 14 ± 3 mM (n = 4) and mutant E795Q has an I50 value of 8 ± 3 mM (n = 4).

The effects of K\textsuperscript{+}, 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.](image-url)
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 $E₁^-P → E₂^-P$ 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 thapsigargin, vanadate, and ouabain have no effect on the level of this phosphorylated product (not shown) but SERCA-type

![Diagram](Image 67x549 to 279x729)

**Fig. 6.** Correlation between the expression level of H⁺,K⁺-ATPase of wild type and mutant H⁺,K⁺-ATPase membranes. Sf9 cells were infected with viruses expressing the wild type H⁺,K⁺-ATPase and were incubated for 2–5 days with a varying multiplicity of infection (0.01–10). The expression level (% H⁺,K⁺-ATPase) of the obtained membrane preparations is plotted as a function of the SCH 28080-dependent phosphorylation level (squares) and calculated by means of linear regression analysis ($y = 0.48x + 0.11, r = 0.78$). The mutant H⁺,K⁺-ATPase membrane data (circles) are those from Table I, in which the multiplicity of infection was 3 and the incubation time was 3 days. The experiments with the wild type virus in which similar infection and incubation conditions were used are for comparison given as closed squares. The open squares represent experiments with wild type virus using other infection and/or incubation conditions.

![Diagram](Image 308x570 to 554x729)

**Fig. 7.** Effects of K⁺ and SCH 28080 on the ATP phosphorylation capacity of H⁺,K⁺-ATPase obtained from Sf9 membranes infected with recombinant baculoviruses. Membranes obtained from Sf9 cells infected with wild type virus (closed circles), mutant E795Q (open circles), or mutant E820Q (open squares) were preincubated for 60 min at 0 °C in the presence of 1 mM MgCl₂ and 20 mM Tris-acetic acid (pH 6.0) in the presence of either K⁺ (A) or SCH 28080 (B) in the concentrations indicated. After phosphorylation for 10 s at 0 °C with 0.1 μM [γ⁻³²P]ATP, the residual phosphorylation level ($E-P$) was determined and expressed as a percentage of the control and plotted as a function of the K⁺ or SCH 28080 concentration. Bars represent S.E. values for three to five experiments.

![Diagram](Image 308x511 to 554x729)

**Fig. 8.** Effects of K⁺ and ADP on the dephosphorylation reaction of the phosphorylated intermediate of H⁺,K⁺-ATPase obtained from Sf9 membranes infected with recombinant baculoviruses. Membranes obtained from Sf9 cells infected with wild type virus (A), mutant E795Q (B), and mutant E820Q (C) 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 10 s ($t = 0$) the incubation medium was diluted from 60 to 200 μl with nonradioactive ATP (final concentration 1 mM) in 25 mM Tris-acetic acid (pH 6.0) in order to prevent dephosphorylation with radioactive ATP and incubated for either 5 or 10 s without further addition (squares) or with either 5 mM ADP (triangles) or 10 mM KCl (circles). The residual phosphorylation level ($E-P$) was expressed as a percentage of the control and plotted as a function of time.
Ca-ATPases and Na\textsuperscript{+},K\textsuperscript{+}-ATPase can be excluded as candidates for this 140-kDa protein.

Phosphorylation and dephosphorylation are key steps in the catalytic cycle of H\textsuperscript{+},K\textsuperscript{+}-ATPase. Fig. 10 shows the K\textsuperscript{+} 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 \muM) had to be used in order to obtain significant stimulation by K\textsuperscript{+}. The wild type enzyme showed a biphasic activation curve with a maximum at 1 mM K\textsuperscript{+}. This activation could be completely blocked by 100 \muM SCH 28080 (not shown). A similar biphasic activation curve was found with the pig enzyme using a comparable low (5 versus 10 \muM) ATP concentration (45). The E820Q mutant showed, like the uninfected cells, no K\textsuperscript{+}-dependent ATPase activity. Low K\textsuperscript{+} 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\textsuperscript{+}, 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\textsuperscript{+} (Fig. 9).

**DISCUSSION**

In the present study we converted six negatively charged amino acid residues present in the M5-M6 region of the \alpha\textsubscript{-}subunit of gastric H\textsuperscript{+},K\textsuperscript{+}-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) \alpha\textsubscript{-}subunit and the \beta\textsubscript{-}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 \alpha\textsubscript{-}subunit using a specific enzyme-linked immunosorbent assay (40). Although the amount of immunoreactive \alpha\textsubscript{-}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\textsuperscript{+} 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\textsuperscript{+},K\textsuperscript{+}-ATPase mutant D824N was also not active in terms of phosphorylation capacity by ATP. This Asp residue is completely conserved in Na\textsuperscript{+},K\textsuperscript{+}-ATPase and Ca\textsuperscript{2+}-ATPases from both sarcoplasmic reticulum and plasma membrane. Mutation of the similar Asp residue in either Na\textsuperscript{+},K\textsuperscript{+}-ATPase (26, 28) or plasma membrane Ca\textsuperscript{2+}-ATPase (25) did not result in active enzyme either. In SERCA1a Ca\textsuperscript{2+}-ATPase, the similar mutant D800N did not show Ca\textsuperscript{2+} occlusion or Ca\textsuperscript{2+}-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 Glu\textsuperscript{795} 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 $\alpha$-subunit. This might be due to the production of more inactive $\alpha$-subunit. Mutational studies of the similar residue in SERCA Ca$^{2+}$-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 Glu$^{770}$ 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 Glu$^{770}$ by a Lys even resulted in an increase in cation affinity in the latter system. Moreover, Glu$^{770}$ 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 Ca$^{2+}$-ATPase, mutation of the corresponding residue, Glu$^{771}$, into Gln results in inhibition of Ca$^{2+}$ transport (3) and Ca$^{2+}$ occlusion (55). Phosphorylation from ATP at 2.5 mM Ca$^{2+}$ and Ca$^{2+}$-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 into a Gln, using the baculovirus system, resulted in an active enzyme with only reduced cation affinity (29). Replacement of Glu$^{771}$ by a Lys even resulted in an increase in cation affinity in the latter system. Moreover, Glu$^{771}$ 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.

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 $E_1' \rightarrow E_2$ 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 $E_2$ form of the enzyme, thus preventing formation of a phosphorylated intermediate (57, 58). ADP stimulates hydrolysis of the $E_1'$ form of the enzyme (46). The K$^+$ insensitivity of the phosphorymate of mutant ES20Q is not due to inhibition of the $E_1'$-P to $E_2$-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 $E_2$ 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 Glu$^{770}$ thus affords 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 Asp$^{386}$. The presented results in this paper suggest that this process is no longer possible in mutant E820Q. This suggests that Glu$^{920}$ 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 Asn. In Na$^+$.K$^-$/ATPase, mutation of this residue (Asn$^{979}$) into alanine abolished Ca$^{2+}$ uptake and phosphorylation from ATP (25). In SERCA1a Ca$^{2+}$-ATPase, the N796A mutation resulted in the absence of ATP-dependent phosphorylation or Ca$^{2+}$ occlusion, but the mutant still showed Ca$^{2+}$-dependent inhibition of the phosphorylation from inorganic phosphate (3). This is explained by the assumption that for ATP-phosphorylation binding of two Ca$^{2+}$ ions is necessary, whereas binding of a single Ca$^{2+}$ 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 Glu$^{770}$ are involved in cation binding.

In summary, the present study emphasizes the importance of Glu$^{770}$ 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.

Acknowledgments—We thank Drs. Michael Caplan, John Forte, and Adam Smolka for the generous gift of antibodies and Dr. Feico Schuurmans Stekhoven for critical reading of the manuscript.

REFERENCES

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