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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 α- and β-subunits of H⁺,K⁺-ATPase. Both subunits of all mutants were expressed 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 D830N showed no 3-(cyanomethyl)-2-methyl-8(phenylmethoxy)imidazo[1,2-alpyridine (SCH 28080)-sensitive ATP-dependent phosphorylation capacity. Mutants E795Q and E820Q formed a phosphorylated intermediate from the E795Q mutant was similarly inhibited by K⁺ (I₅₀ = 0.4 mM) and SCH 28080 (I₅₀ = 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⁺ (I₅₀ = 4.5 mM) and SCH 28080 (I₅₀ = 1.7 μM) 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 E₂ 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 α-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 Ca²⁺-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.

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† The abbreviations used are: SERCA, sarcoplasmic and endoplasmic reticulum Ca²⁺-ATPase; E-P, phosphorylated intermediate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SCH 28080, 3-(cyanomethyl)-2-methyl-8(phenylmethoxy)imidazo[1,2-alpyridine.
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 (Gln⁷⁰⁷–Arg⁸²⁹), which included the putative M5-M6 hairpin. Oclusion 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 (Glu⁷⁸⁰, Glu⁸²⁰, and Asp⁸²⁴). Moreover, quite close to the C terminus of M6 there are three other negatively charged residues (Glu³⁸³, Glu⁷³⁷, and Asp⁸³⁹), 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 Lys⁷⁹¹ (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 Ca⁺⁺-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 Cu²⁺-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, E834Q, E837Q, or D839N 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 pUC19BglII-HKα (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 fragment (base pairs 2238–3400) was inserted in SphI-digested pBluescript (Stratagene, La Jolla, CA). This vector was digested with BamHI and purified of the mutant by HPLC, and the dephosphorylation product obtained from viral DNA isolated from nuclei from infected Sf9 cells. The wild type H⁺,K⁺-ATPase (16, 17). After digestion with BamHI, and dephosphorylation, the 3.3-kilobase pair fragment (base pairs 2238–3400) was inserted in BamHI-digested pBluescript (Stratagene, La Jolla, CA). This vector was digested with BamHI, purified of the mutant and a 1.2-kilobase pair BamHI fragment was isolated and purified from agarose gels. The baculovirus transfer vector pAcAS3, containing the code for the β-subunit by Western blotting. The presence of the β-subunit under control of the polyhedrin promoter and of the β-subunit under control of the P10 locus of DLZ-I subunit was digested with BamHI and dephosphorylated with BamHI fragment. The wild type H⁺,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 Cu²⁺-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.

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previous reported H+/K+-ATPase obtained with the conventional bacular method (29).

Production of Recombinant H+/K+-ATPase—Sf9 cells were grown at 27 °C either in 100-mm 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 DL2ΔAs3 or Bgδ12 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 cover slips in complete growth medium and infected with a multiplicity of infection of 3 at 27 °C. After infection, the 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-buffered saline (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 cover slips were dried in a desiccator containing 10% acrylamide according to Laemmli (43). For immunoblotting, the separated proteins were transferred to Immobilon-P PVDF membranes. The antibodies 5B6 (41, 42) and using purified pig H/K+-ATPase and the monoclonal antibody 2G11 (44) evoked against the β-subunit of H+/K+-ATPase were detected as described earlier (32), with the polyclonal antibody HKB (38) recognizing the 565–585 region of the α-subunit of H+/K+-ATPase and the monoclonal antibody 2G11 (44) evoked against the β-subunit of H+/K+-ATPase, respectively.

K+-ATPase Activity Assay—The K+-activated ATPase activity was determined with a radioactive 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 mCi·mmol⁻¹), 1.0 mM MgCl2, 0.3 mM EDTA, 0.1 mM EGTA, 0.1 mM ouabain, 1 mM NaN₃, 20 mM Tris-HCl (pH 7.0), and varying concentrations of KCl. After incubation in PBS, 0.05% Tween 20, 1% (w/v) gelatin, and 2% fetal bovine serum. Free antibodies were removed by washing the cells HKB (38) for 30–60 min in PBS, 0.05% Tween 20, 1% (w/v) gelatin, 2% fetal bovine serum at 4°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 (32P) 3, 3 ml of OptiFluor (Canberra Packard, Tilburg, The Netherlands) was added, and the mixture was analyzed by liquid scintillation analysis. The values of the specific activity in the activity of the supernatant in the presence of added K⁺ (5 µM), which is 70–150 mmol of P, liberated per mg per h. The latter activity is endogenously present in membranes of Sf9 cells.

ATP Phosphorylation Capacity—ATP phosphorylation was determined as described before (37). Sf9 membranes (40–100 µg) were incubated at 0 °C in 25 mM Tris-acetate buffer (pH 6.0), 1 mM MgCl2 with and with 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 mM) 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. Hydroxyamine Sensitivity of the Phosphorylated Intermediate—After ATP phosphorylation, the acid-denatured 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 assayed by liquid scintillation analysis.

Analysis of Data—The IC₅₀ values for K⁺ and SCH 28080 were iteratively determined by fitting the concentration relationship to the log-logistic equation, which is given by

\[ Y = \frac{A - B}{1 + (X/X_0)^{-10^C}} \]

where A represents the bottom plateau, B is the top plateau, C is X₅₀, 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, Buckinghamshire, 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 HKCo2 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 process. In the baculovirus system, H,K-ATPase is, like in the gastric parietal cell, primarily routed to intracellular membrane vesicles. This can be shown by confocal laser scan microscopy (Fig. 3). In gastric parietal cells these vesicles fuse with the apical plasma membrane upon stimulation with agents that increase cyclic AMP or Ca²⁺, but in Sf9 cells this mechanism apparently is absent. Fig. 3 also 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.

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 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 (Fig. 5). The presence of such a phosphorylated protein in the enzyme produced by the wild type virus could be detected by the monoclonal antibody HKb-AS (48) directed against the C terminus of the α-subunit. 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), BgΔ-as (wild type virus) (c), DLZβASα-E795Q (D), BgΔ-as-E820Q (E), DLZβASα-D839N (F), DLZβASα-E834Q (G), BgΔ-as-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. 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), BgΔ-as (wild type virus) (c), DLZβASα-E795Q (D), BgΔ-as-E820Q (E), DLZβASα-D839N (F), DLZβASα-E834Q (G), BgΔ-as-E837Q (H), and DLZβASα-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.
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 (I\textsubscript{50} = 0.45 ± 0.10 mM; n = 3), is similar to that of the wild type enzyme (I\textsubscript{50} = 0.38 ± 0.04 mM; n = 5). The I\textsubscript{50} value of mutant E820Q, however, is 10 times higher (I\textsubscript{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\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 I\textsubscript{50} value of 1.7 ± 0.6 μM (n = 4) was found, whereas the wild type enzyme has an I\textsubscript{50} value of 14 ± 3 mM (n = 4) and mutant E795Q has an I\textsubscript{50} 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
to 100 mM. The E820Q mutant also showed no sensitivity for phosphorylation level (membrane preparations is plotted as function of the SCH 28080-dependent intermediate in the absence of K+ phosphorylated intermediate. We next prepared a phosphorylation with this ion, thus preventing formation of a phospho- specific phosphorylation capacity was investigated by preincu- bation with this ion, thus preventing formation of a phospho-

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hardly any effect on the formation of the phosphorylated inter- mediate 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 preincu- bation with this ion, thus preventing formation of a phospho- rylated intermediate. We next prepared a phosphorylated inter- mediate in the absence of K+ and SCH 28080 and measured the residual amount of phosphorylated intermediate after incu- bation 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 phos- phorylated 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 E₁P → E₂P 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 dephos- phorylation of this SCH 28080-insensitive phosphoprotein 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 hydroxylamine, which converts the acylphosphate into a hydroxy- mate (51). Both the wild type enzyme and the mutants E795Q and E820Q showed similar hydroxylamine sensitivity (Fig. 9), indicating that also the K+-insensitive mutant E820Q had formed an acylphosphate as a phosphorylated intermediate. The figure also shows that the phosphorylated protein present in the membranes of uninfected Sf9 cells is at least in part an acylphosphate too. However, a further identification of the nature of this phosphorylated protein cannot be given. Since thapsigargin, vanadate, and ouabain have no effect on the level of this phosphorylated product (not shown) both SERCA-type
Phosphorylation and dephosphorylation are key steps in the catalytic cycle of H\(^+\),K\(^-\)-ATPase. The studies were carried out in the baculovirus system and aspartate residues for the function of this transport enzyme. The maximal ATPase activity with the wild type enzyme increased by 51% compared to the wild type enzyme and the mutants E795Q and E820Q. In this assay a relatively low ATP concentration (10 \(M\)) 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 \(\mu M\) SCH 28080 (not shown). A similar biphasic activation curve was found with the pig enzyme using a comparable low (5 \(versus\) 10 \(\mu M\)) ATP concentration (45).

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 plasma ATPases. Mutation of the similar Asp residue in either Na\(^-\),K\(^-\)-ATPase (26, 28) or plasma membrane Ca\(^2+\)-ATPase (25) did not result in active enzyme either. In SERCA1a Ca\(^2+\)-ATPase, the similar mutant D800N did not show Ca\(^2+\)-occlusion or Ca\(^2+\)-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\(^{795}\) into Gln results in formation of a phos-
phosphorylated 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+-induced occlusion (55). Phosphorylation from ATP at 2.5 mʊt 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 Andersen 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 $E_1 \rightleftharpoons E_2$ 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 an $E_1$-P form of the enzyme (46). The K+-insensitivity of the phosphorymone of mutant ES20Q is not due to inhibition of the $E_1$-P $\rightarrow$ $E_1$-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 $E_1$ 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 Asp586. The presented results in this paper suggest that this process is no longer possible in mutant E820Q. This suggests that Glu586 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 (Asn854) into Asn or Gln 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 (Asn879) into alanine abolished Ca2+ uptake and phosphorylation from ATP (25). In SERCAa 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 Glu586 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