Mimicking of K^+ Activation by Double Mutation of Glutamate 795 and Glutamate 820 of Gastric H^+,K^+-ATPase†

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ABSTRACT: Six double mutants of Glu^{795} and Glu^{820} present in transmembrane domains 5 and 6 of the α-subunit of rat gastric H^+,K^+-ATPase were generated and expressed with the baculovirus expression system. Five of the six mutants exhibited an SCH 28080-sensitive ATPase activity in the absence of K^+. The activity levels decreased in the following order: E795Q/E820A > E795Q/E820Q > E795Q/E820D ≅ E795A/E820A > E795L/E820Q. The E795L/E820D mutant possessed no constitutive activity. The relative low ATPase activity of the E795L/E820Q mutant is due to its low phosphorylation rate so that the dephosphorylation step was no longer rate-limiting. The constitutively active mutants showed a much lower vanadate sensitivity than the wild-type enzyme and K^+-sensitive mutants, indicating that these mutants have a preference for the E_1 conformation. In contrast to the constitutively active single mutants generated previously, the double mutants exhibited a high spontaneous dephosphorylation rate at 0 °C compared to that of the wild-type enzyme. In addition, the H^+,K^+-ATPase inhibitor SCH 28080 increased the steady-state phosphorylation level of the constitutively active mutants, due to the formation of a stable complex with the E_2−P form. These studies further substantiate the idea that the empty ion binding pockets of some mutants apparently mimic the K^+-filled binding pocket of the native enzyme.

Accumulating evidence suggests that negatively charged residues present in the transmembrane parts of P_2-type ATPases are involved in cation binding and transport (*1*−*9*). Recently, Glu^{343} (*10*) in transmembrane domain 4, Glu^{795} (*7*) in transmembrane domain 5, and Glu^{820} (*6*, *11*) and Asp^{824} (*4*) in transmembrane domain 6 have been identified as important amino acids in gastric H^+,K^+-ATPase[1] functioning. In these studies, it was established that a negative charge on residue 820 was required for K^+-stimulated ATP hydrolysis and dephosphorylation of the phosphorylated intermediate (*11*). Importantly, it was observed that replacing Glu^{820} with a neutral amino acid residue (E820Q, E820N, or E820A) increased the ATPase activity in the absence of K^+ (*6*). This activity was coined "constitutive ATPase activity". Moreover, these mutants exhibited a high K^+-independent (spontaneous) dephosphorylation rate, when measured at 20 °C (*6*). It was postulated that neutralization of the negative charge on position 820 modified the K^+-binding pocket in such a way that the K^+-filled pocket was mimicked. However, the K^+-independent dephosphorylation of these mutants required a higher temperature than the K^+-dependent dephosphorylation of the wild-type enzyme, indicating that the imitation of the K^+-filled pocket was not complete.

Intriguingly, recent work showed that preincubation of the constitutively active mutant E820Q with SCH 28080[2] increased rather than decreased the steady-state level of ATP phosphorylation (*12*). It was postulated that this behavior reflects an increased preference of the mutant for the E_1 conformation (Figure 1). As a result, the enzyme is not trapped in the E_2−SCH 28080 conformation but after addition of ATP in the E_2−P.SCH 28080 conformation. This latter complex is relatively stable as compared to the E_2−P complex, leading to its accumulation in time. However, in the same study it was observed that the levels of ATP phosphorylation obtained with the constitutively active E820A and E820N mutants were decreased by SCH 28080, but only by 25 and 62%, respectively, whereas the wild-type enzyme was not phosphorylated.

We recently demonstrated that the presence of a carbonyl residue on position 795 is sufficient for K^+ to exert its stimulatory effect (*7*). A constitutive activity was found with the E795L and E795A mutants. However, the magnitude of this activity was considerably lower than that obtained with mutants E820Q, E820N, and E820A.

In summary, the results obtained in the above-mentioned studies (*6*, *7*) show that certain mutants of Glu^{820} and Glu^{795} display a constitutive ATPase activity that in some, but not all, cases is accompanied by an increased level of ATP phosphorylation after preincubation with SCH 28080. At present, the structural basis for this phenomenon is unclear.

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[1] The mutated residues in the α-subunit of gastric H^+,K^+-ATPase are present within the following sequences: $IPE^{795}LT$ and $FIE^{820}LC$.

[2] Abbreviations: SR, sarcoplasmic reticulum; E−P, phosphorylated intermediate; SCH 28080, 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)-imidazo[1,2*a*]pyridine; WT, wild type; QQ, E795Q/E820Q; QA, E795Q/E820A; QD, E795Q/E820D; AA, E795A/E820A; LQ, E795L/E820Q; LD, E795L/E820D.

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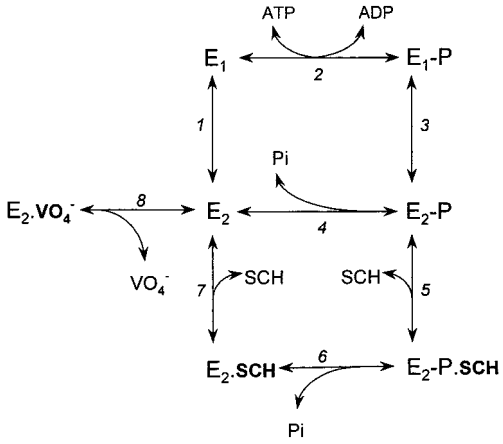

FIGURE 1: Post-Albers reaction scheme for H^+,K^+-ATPase, including the SCH 28080 and vanadate inhibition mechanism. The clockwise reactions are indicated in the text with a positive (+) sign and the counterclockwise reactions with a minus (−) sign. Reaction +4 is the conversion of E_2−P to E_2. The E_1 forms are assumed to have a high affinity for H^+ ions and a low affinity for K^+ ions. The E_2 forms have a high affinity for K^+ and a low affinity for H^+ ions. H^+ transport takes place in step 3 and K^+ transport in step 1.

One of the questions that remains to be answered is how constitutive activity and SCH 28080-induced ATP phosphorylation are related. Another intriguing question is whether the imitation of the K^+-filled pocket can be further improved. To gain more insight into these issues, we generated a series of double mutants of Glu^{795} and Glu^{820} and analyzed their properties. In this study, we show that the constitutive ATPase activity is increased further by certain double mutations. The highest constitutive ATPase activity was found when the two glutamate residues were replaced with a glutamine at position 795 and either a glutamine or an alanine at position 820.

EXPERIMENTAL PROCEDURES

Preparation of Mutants. All DNA manipulations were carried out according to standard molecular biology techniques described by Sambrook et al. (*13*). The cDNAs of the previously constructed Glu^{820} mutants (E820Q, E820A, and E820D) of the α-subunit of rat gastric H^+,K^+-ATPase (*11*) were cloned in the pAlter-I vector. To introduce the Glu^{795} mutations in this vector, site-directed mutagenesis was performed as described previously (*7*). After selection and subcloning of the mutated α-subunits in the pFastbacdual vector (which already contained the *β*-subunit), the mutations were checked by sequence analysis. Recombinant baculoviruses and enzyme preparations were produced as described previously (*7*). Protein was quantified with the modified Lowry method according to the method of Peterson (*14*) using bovine serum albumin as a standard.

Western Blotting. Samples from the membrane fraction were solubilized in SDS−PAGE sample buffer and separated on SDS gels containing 10% acrylamide according to the method of Laemmli (*15*). For immunoblotting, the separated proteins were transferred to Immobilon polyvinylidenefluoride membranes (Millipore Corp., Bedford, MA). The α-subunit of the gastric H^+,K^+-ATPase was detected with the polyclonal antibody HKB (*16*).

ATPase Assay. ATPase activity was determined with a radiochemical method, as described in detail previously (*17*). Sf9 membranes were added to 100 μL of assay medium containing 10 μM [γ-^{32}P]ATP (Amersham, Buckinghamshire, U.K.; specific activity of 100−500 mCi mmol^{-1}), 1.3 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM EDTA, 0.1 mM ouabain, 1 mM NaN_3, 25 mM Tris-HCl (pH 7.0), with or without 100 μM SCH 28080 (kindly provided by C. D. Strader, Schering-Plough, Kenilworth, NJ), and varying concentrations of KCl and incubated for 30 min at 37 °C. The amount of membrane preparation varied between 0.5 and 2.0 μg and was chosen such that the extent of substrate conversion was less than 25%. After the reaction had been quenched, further analysis was performed as described previously (*4*).

Phosphorylation and Dephosphorylation Assays. ATP phosphorylation was performed as described previously (*6*). Briefly, 2−20 μg of Sf9 cell membranes was added to 50 μL of assay medium containing 50 mM Tris-acetic acid (pH 6.0), 1 mM $MgCl_2$, and 0.2 mM EDTA, with or without 0.1 mM SCH 28080, and preincubated for 30−60 min at either 0 or 21 °C. The reaction was started by the addition of 10 μL of 0.6 μM [γ-^{32}P]ATP, and the reaction mixture was incubated for the indicated period of time. At the end of this incubation period, the reaction was quenched by the addition of 5% (w/v) trichloroacetic acid in 0.1 M phosphoric acid. For dephosphorylation studies, phosphorylation was carried out at 0 °C as described above. At the end of the phosphorylation period, the reaction mixture was diluted 8.3-fold with ice-cold assay medium containing nonradioactive ATP (final concentration of 10 μM) to prevent rephosphorylation with radioactive ATP. The reaction was quenched at the indicated time points. Phosphorylated protein was collected by filtration over a 0.8 μm membrane filter (Schleicher and Schüll, Dassel, Germany) (*4*). The filters were washed twice and analyzed by liquid scintillation analysis. The dephosphorylation rate was calculated from the amounts of phosphorylated intermediate 0, 3, and 5 s after dilution, assuming first-order rate kinetics.

Hydroxylamine 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 the filters had been exposed to either 0.5 M hydroxylamine imidazole (pH 7.5) or 0.5 M imidazole-HCl (pH 7.5) for 5 min, the membranes were washed with 5% trichloroacetic acid in 0.1 M phosphoric acid and analyzed by liquid scintillation analysis.

RESULTS

Expression. A series of Glu^{795}/Glu^{820} double mutants of rat gastric H^+,K^+-ATPase was produced and expressed in Sf9 insect cells using the baculovirus expression system. To determine the relative expression level of the mutant enzymes, membranes were isolated and subjected (20 μg of protein) to Western blot analysis. The blots were probed with the polyclonal antibody HKB directed against the α-subunit of gastric H^+,K^+-ATPase. Figure 2 shows that all mutants were expressed at a level similar to that of the wild-type enzyme.

ATPase Activity. The ATPase activity of the various mutant enzymes was measured in the presence of 10 μM ATP as described previously (*6*, *7*). To correct for the

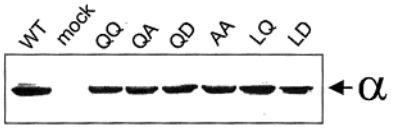

FIGURE 2: Western blot of the double mutants of gastric H^+,K^+-ATPase. Membranes (20 μg) isolated from Sf9 cells infected with a baculovirus containing either wild-type H^+,K^+-ATPase or the double mutants were blotted. The presence of the α-subunit was detected using the polyclonal antibody HKB (*16*). Membranes isolated from mock-infected cells are shown as a negative control.

endogenous activity present in insect membranes, we routinely subtracted the activity value obtained in the presence of 100 μM SCH 28080 from that obtained in the absence of this inhibitor. The activity measured in the presence of SCH 28080 was in these mutants not significantly different from that measured in membranes isolated from mock-infected cells [0.13 ± 0.03 μmol of P_i (mg of protein)$^{-1}$ h^{-1} (n = 3)].

Figure 3 (●) shows that all double mutants, except the E795L/E820D mutant (Figure 3E), displayed a marked SCH 28080 inhibitable ATPase activity in the absence of added K^+ ions, which is termed constitutive activity (*7*). The magnitude of this activity decreased in the following order: E795Q/E820A ≅ E795Q/E820Q > E795Q/E820D ≅ E795A/E820A > E795L/E820Q. The ATPase activity of these mutants decreased at K^+ concentrations of >10 mM, which is probably due to a shift in the $E_1 \leftrightarrow E_2$ equilibrium to the right. The ATPase activity of the E795L/E820D mutant was maximally stimulated by K^+ to 0.08 ± 0.02 μmol of P_i (mg of protein)$^{-1}$ h^{-1} (n = 2) at a cation concentration of 30 mM. For comparison, Figure 3 includes the previously reported activities of the corresponding single mutants (*6*, *7*) and of the wild-type enzyme. K^+, at a concentration of 1 mM, maximally increased the activity of the wild-type enzyme by 0.28 ± 0.04 μmol of P_i (mg of protein)$^{-1}$ h^{-1} (n = 4). This increase in the ATPase activity was completely inhibited by 100 μM SCH 28080 and was of a size equal to that reported previously (*6*, *7*).

One of the striking findings presented in this figure is the appearance of a K^+-insensitive double mutant with a marked constitutive activity, the E795Q/E820D mutant (Figure 3B), based on two parent mutants, E795Q and E820D, each of which possesses a K^+-stimulated ATPase activity. The figure also shows that mutation of Glu^{795} and Glu^{820} yielded constitutively active double mutants with an activity that was much higher (E795Q/E820A), slightly higher (E795Q/E820Q and E795A/E820A), or even considerably lower (E795L/E820Q) than the maximal activity of the most active parent mutant.

To obtain information about the SCH 28080 sensitivity of the various double mutants, we prepared dose−inhibition curves at a K^+ concentration of 1 mM. At this concentration, the wild-type enzyme and most of the K^+-sensitive mutants were maximally active and K^+ had no effect on the activity

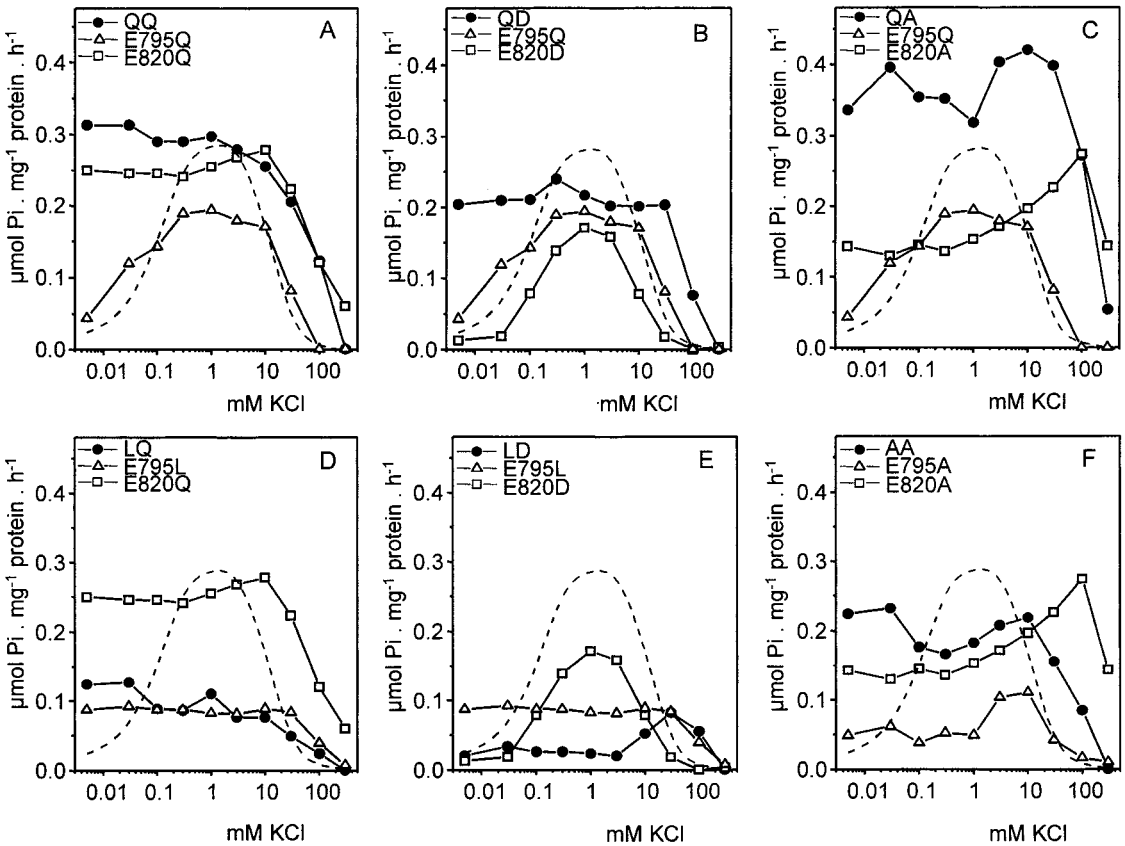

FIGURE 3: Effect of K^+ on the ATPase activity of the double and single mutants. The SCH 28080 sensitive ATPase activities of the double mutants (●) are plotted as a function of the K^+ concentration used. In the absence of additional K^+ ions, the following activities, expressed in μmol of P_i (mg of protein)$^{-1}$ h^{-1}, were measured for double mutants E795Q/E820A (0.34 ± 0.04), E795Q/E820Q (0.31 ± 0.07), E795Q/E820D (0.20 ± 0.03), E795A/E820A (0.22 ± 0.05), E795L/E820Q (0.12 ± 0.03), and E795L/E820D (0.02 ± 0.01). For comparison, the activities measured for the corresponding Glu^{795} mutants (△; from Figure 2 and ref *7*) and the Glu^{820} mutants (□; from Figure 1 and ref *6*) are given. The behavior of the wild-type enzyme is given as a dotted line. Mean values are given for two to four independent membrane preparations.

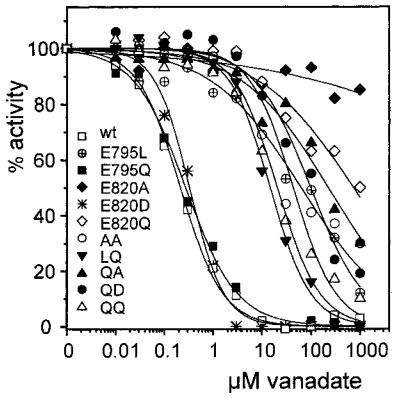

FIGURE 4: Effect of vanadate on the ATPase activity of the single and double mutants. The ATPase activities of the wild-type enzyme and the various mutants were measured in the presence of 1 mM K^+ and the indicated concentrations of vanadate. The data were corrected for the values of mock-infected cells obtained with the same vanadate concentrations. For each preparation, the activity in the absence of vanadate was set at 100%. The results are the means of two independent preparations. The meaning of the symbols is indicated in the figure.

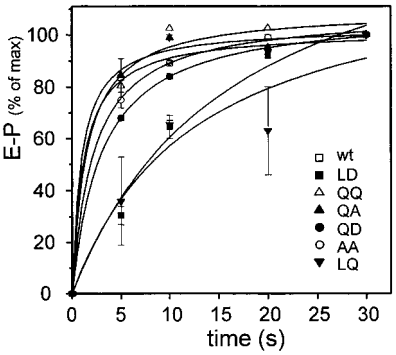

FIGURE 5: Time dependence of ATP phosphorylation of H^+,K^+-ATPase and its mutants. Phosphorylation levels were measured at 0 °C at indicated times. The results presented are the means ± standard error for two independent preparations. The lines were calculated assuming first-order rate kinetics. The symbols are similar to those in Figure 4. The following rate constants (s^{-1}) were calculated: 0.58 ± 0.10 for the wild type, 0.46 ± 0.18 for E795Q/E820Q, 0.23 ± 0.08 for E795Q/E820D, 0.69 ± 0.28 for E795Q/E820A, 0.05 ± 0.03 for E795L/E820Q, 0.04 ± 0.01 for E795L/E820D, and 0.32 ± 0.01 for E795A/E820A. The meaning of the symbols is indicated in the figure.

of the constitutively active mutants. The curves obtained were of the same shape as described previously (ref *6* and Figure 3). The calculated IC_{50} values of the E795Q/E820D [0.4 ± 0.2 μM ($n = 2$)], E795Q/E820A [0.3 ± 0.2 μM ($n = 2$)], and E795A/E820A [0.3 ± 0.1 μM ($n = 2$)] mutants were not different from that of the wild-type enzyme [0.3 ± 0.1 μM ($n = 2$)]. The mutants E795Q/E820Q [$IC_{50} = 1.1 \pm 0.2$ μM ($n = 2$)] and E795L/E820Q [$IC_{50} = 1.0 \pm 0.7$ μM ($n = 2$)] exhibited a slightly lower SCH 28080 sensitivity relative to that of the wild-type enzyme. The activity of the E795L/E820D mutant was too low to measure the inhibitor sensitivity reliably.

Figure 4 shows vanadate inhibition curves of the wild-type enzyme, the five active double mutants, and the parent single mutants of these double mutants. The preparations can be divided in two groups: the vanadate-sensitive preparations (wild type, E820D, and E795Q; $IC_{50} = 0.2-0.4$ μM) and the mutants with a low vanadate sensitivity (E820Q, E820A, E795L, E795Q/E820Q, E795Q/E820A, E795Q/E820D, E795L/E820Q, and E795A/E820A; $IC_{50} > 15$ μM). All three preparations with a high vanadate sensitivity possessed K^+-stimulated ATPase activity. The mutants with a low vanadate sensitivity were all constitutively active. Since vanadate is assumed to react with the E_2 form of the enzyme, these findings indicate that the constitutively active mutants are for only a relatively short period of the reaction cycle in the latter conformation and thus have a preference for the E_1 conformation.

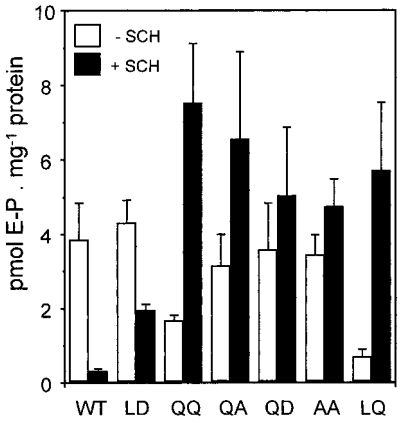

FIGURE 6: Effect of SCH 28080 on the steady-state ATP phosphorylation capacity in the absence and presence of 100 μM SCH 28080. The level of ATP phosphorylation was determined as described in Experimental Procedures. The membrane preparations were preincubated with or without 100 μM SCH 28080 and phosphorylated with 0.1 μM ATP at 21 °C. The phosphorylation levels (picomoles of EP per milligram of protein) were determined and corrected for those of mock-infected cells [1.0 ± 0.1 pmol of EP (mg of protein)$^{-1}$]. The bars represent the means ± standard error of three enzyme preparations. The meaning of the symbols is indicated in the figure.

Time Dependence of the Phosphorylation Reaction. Like the wild-type enzyme and the single mutants, all double mutants can be phosphorylated by ATP. Figure 5 shows the time dependence of the phosphorylation process with 0.1 μM ATP. All presented values were corrected for the phosphorylation levels of mock-infected cells. The figure shows that the phosphorylation rates of the E795L/E820Q and E795L/E820D mutants at 0 °C were markedly lower than those of the wild-type enzyme and the other mutants. This suggests that in these mutants H^+ binding needed for the phosphorylation process may be hampered.

Effect of SCH 28080 on the Steady-State Phosphorylation Level. Figure 6 shows the steady-state phosphorylation levels measured after 10 s at 21 °C using 0.1 μM ATP in the absence and presence of 100 μM SCH 28080. At this temperature, steady-state phosphorylation levels were reached for both the wild-type enzyme and all the mutants within this time period. The presented values were corrected for the phosphorylation level measured in membranes isolated from mock-infected cells [1.0 ± 0.1 pmol of EP (mg of protein)$^{-1}$]. In the absence of SCH 28080, the phosphorylation levels obtained with the E795Q/E820Q and E795L/E820Q mutants were considerably lower than that obtained with the wild-type enzyme.

ATP phosphorylation of the wild-type enzyme was nearly completely inhibited by preincubation with SCH 28080 (Figure 6). The phosphorylation level of all mutants, except E795L/E820D, was higher when 100 μM SCH 28080 was present, as was found before for the E820Q mutant (*12*).

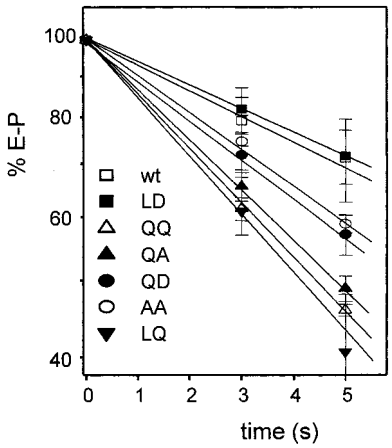

FIGURE 7: Spontaneous dephosphorylation of the phosphorylated intermediate of the wild-type enzyme and the Glu^{795}/Glu^{820} mutants. Membrane preparations were phosphorylated for 10 s at 0 °C as described in Experimental Procedures. Dephosphorylation was started by addition of excess nonradioactive ATP. After 3 and 5 s, the reaction was stopped and the phosphorylation levels were determined. The residual phosphorylation level after 3 and 5 s was expressed as the percentage of the phosphorylation level before the start of the dephosphorylation step. The results presented are the means ± standard error for two independent preparations. The rate constant of the dephosphorylation process was calculated for each experiment assuming first-order rate kinetics. The values (s^{-1}) were averaged and are as follows (mean ± standard error; s^{-1}): 0.07 ± 0.01 for E795L/E820D, 0.07 ± 0.02 for the wild type, 0.11 ± 0.01 for E795Q/E820D, 0.11 ± 0.01 for E795A/E820A, 0.14 ± 0.01 for E795Q/E820A, 0.16 ± 0.01 for E795Q/E820Q, and 0.18 ± 0.02 for E795L/E820Q.

Thus, the steady-state phosphorylation level measured with these mutants in the absence of SCH 28080 underestimates the theoretically maximal phosphorylation capacity.

Dephosphorylation Rate of the Phosphorylated Intermediate. One would expect that mutants with a high constitutive ATPase activity exhibit a high dephosphorylation rate in the absence of added K^+. The wild-type enzyme and the six double mutants were therefore phosphorylated at 0 °C and further incubated with nonradioactive ATP. The residual phosphorylation level was measured after 3 and 5 s and expressed as a percentage of the phosphorylated level at time zero. Figure 7 shows that this spontaneous dephosphorylation process obeys first-order rate kinetics. The dephosphorylation rates of the phosphorylated intermediates increased in the following order: E795L/E820D ≅ wild type < E795A/E820A ≅ E795Q/E820D < E795Q/E820A ≅ E795Q/E820Q ≅ E795L/E820Q. When under the same conditions the dephosphorylation of the wild-type enzyme was studied in the presence of 1 mM K^+, only 30% of the original amount of phosphorylated intermediate was left (*11*). Thus, the K^+-stimulated phosphorylation rate of the wild-type enzyme is considerably faster than that of the mutant with the highest dephosphorylation rate.

Hydroxylamine Sensitivity of the Phosphorylated Intermediate. ATP phosphorylation takes place at Asp^{386} present in the large intracellular loop between transmembrane segments 4 and 5. A property of acyl phosphates is that they are sensitive to hydroxylamine. To test whether the phosphorylated intermediate formed in the mutants is indeed an acyl phosphate, the E820Q mutant was phosphorylated in the presence and absence of 100 μM SCH 28080. The formed phosphorylated intermediate was treated with ice-cold 0.5 M hydroxylamine imidazole (pH 7.5) for 5 min. The residual level of phosphorylated intermediate of the mutant was reduced to 20% of the initial value, both with and without SCH 28080. This value is similar to that of the wild-type enzyme (*4*), indicating that in the presence as well as in the absence of SCH 28080 an acyl phosphate was formed.

DISCUSSION

Recent mutagenesis studies on the role of two negatively charged residues present in transmembrane domains 5 and 6 of gastric H^+,K^+-ATPase resulted in two new and exciting observations. First, some mutants of Glu^{820} (*6*) and Glu^{795} (*7*) showed an SCH 28080-sensitive ATPase activity that was independent of K^+. These mutants are therefore termed constitutively active. The magnitude of this constitutive activity, however, was highly variable between the various mutants. Second, with one of these mutants (E820Q), the steady-state phosphorylation level was enhanced rather then decreased following preincubation with SCH 28080 (*12*). The steady-state phosphorylation level of the other constitutively active mutants was decreased by SCH 28080, although less than the wild-type enzyme.

We proposed previously (*6*) that the constitutive activity reflects a mutation-induced simulation of the K^+-bound state. One of the questions that emerged from these studies was whether this simulation can be improved. A second intriguing question was whether the constitutive activity and the SCH 28080-induced increase or decrease in ATP-phosphorylation level are related. To answer these questions, a series of double mutants at positions 820 and 795 of gastric H^+,K^+-ATPase was prepared and analyzed. At each of these positions, amino acids were introduced that gave either constitutive activity (E795L, E795A, E820Q, and E820A) or K^+-stimulated activity (E795Q and E820D) as in the wild-type enzyme.

The data presented here show that five of the six double mutants prepared in this study showed constitutive ATPase activity. Only the E795L/E820D mutant showed hardly any activity in the absence of K^+ and could slightly be activated at high K^+ concentrations. In four of the constitutively active mutants, either one or both of the parent single mutants showed also constitutive activity. Intriguingly, constitutive ATPase activity was also found in a double mutant (E795Q/E820D), of which the parent single mutants had a normal K^+-stimulated activity.

The ATPase activity of double mutants E795Q/E820A and E795Q/E820Q was significantly higher than the highest constitutive activity measured thus far (E820Q) (*6*, *7*). The constitutive activities of the other double mutants, however, were only slightly higher than, similar to, or even lower than that of E820Q or the most active parent mutant. Thus, there is no simple rule to predict when constitutive activity is found and what the magnitude of this activity is.

The constitutively active double mutants showed an enhanced spontaneous dephosphorylation rate already, whereas with single mutants this could only be measured at 21 °C (*6*, *7*). This indicates that in the double mutants the conformational change that mimics the K^+-activated state occurs already at lower temperatures than with the single mutants. However, the dephosphorylation rate obtained with the most active double mutant at 0 °C (0.17 s^{-1}) was still

considerably lower than that obtained with the K^+-stimulated wild-type enzyme (>0.40 s^{-1}) (*11*). In this context, one would assume that the conformation of these mutants still does not completely resemble that induced by K^+ in the wild-type enzyme.

One of the intriguing properties of the E820Q mutant is that it displays increased levels of steady-state phosphorylation following pretreatment with SCH 28080 (*12*). Here we demonstrate that this effect of SCH 28080 was also observed with a number of double mutants. This is in sharp contrast to the wild-type enzyme where this treatment effectively lowers the steady-state phosphorylation level. For the wild-type enzyme, the inhibitory effect of SCH 28080 has been explained (*18*−*20*) by assuming that during the preincubation in the absence of ATP the enzyme is mainly present in the E_2 form, which then forms a complex with the inhibitor (Figure 1, step 7). A similar mechanism explains the inhibition of phosphorylation by vanadate (Figure 1, step 8). As a result, no phosphorylated intermediate is produced upon addition of ATP. As indicated by the low sensitivity for vanadate, the constitutively active mutants have a high preference for the E_1 form and therefore do not form a complex with SCH 28080 (see Figure 1). Upon addition of ATP, phosphorylation occurs resulting in the formation of the E_2−P form (Figure 1, reactions +2 and +3). In the presence of SCH 28080, this phosphorylated enzyme will, at least in part, be converted into the E_2−P.SCH form (reaction +5), which is rather stable (*12*). The remaining E_2−P will be rapidly hydrolyzed, and the mutant will be phosphorylated again (reactions +2 and +3). It is likely that after a number of cycles the majority of the enzyme molecules will be present in the E_2−P.SCH form, which explains the SCH 28080-induced increase in the steady-state phosphorylation level observed with many of the mutants.

Four of the five double mutants with constitutive activity (E795Q/E820Q, E795Q/E820A, E795Q/E820D, and E795A/E820A) have both an increased spontaneous dephosphorylation rate and a steady-state phosphorylation level that is increased by SCH 28080. Although the E795L/E820Q mutant has the highest spontaneous dephosphorylation rate and shows a maximal stimulation of the steady-state phosphorylation level by SCH 28080, the constitutive ATPase activity of this mutant is relatively low. This can be attributed to the fact that the phosphorylation rate of this mutant, like that of the E795L/E820D mutant, is lower than the dephosphorylation rate so that the latter step is no longer rate-limiting. This also explains the low steady-state phosphorylation level of this mutant. The rate constants of the phosphorylation process of the E795L/E820Q (0.05 ± 0.03 s^{-1}) and E795L/E820D (0.04 ± 0.01 s^{-1}) mutants are rather similar to that of the E795L single mutant (0.11 ± 0.01 s^{-1}; calculated from ref *7*). It might be that the presence of a hydrophobic Leu residue at position 795 has a negative effect on the binding of H^+ during phosphorylation and thus indirectly leads to a decrease in the phosphorylation rate.

This study in combination with our previous studies (*6*, *7*) shows that a mutation-induced loss of K^+-stimulated ATPase activity does not automatically lead to a gain of constitutive activity. The double mutant E795L/E820D and some of the previously described single mutants E820L, E820K (*11*), E795D, and E795N (*6*) have lost their K^+ sensitivity of the dephosphorylation process but do not possess constitutive activity. In other words, well-defined changes in the structural elements involved in K^+-stimulated enzyme activity are required to obtain a constitutively active enzyme.

The constitutive activity of H^+,K^+-ATPase mutants might be due to H^+ ions that take over the role of K^+ in the dephosphorylation process. In that sense, it may resemble Na^+,K^+-ATPase mutants with a high Na^+-ATPase activity. High Na^+-ATPase activities have been found with both the E781A mutant (*21*) and the D804A mutant (*22*), which correspond to residues Glu^{795} and Glu^{820} in gastric H^+,K^+-ATPase, respectively.

Up to now, ion transport studies with these mutants have not yet been possible. Therefore, we do not know whether mutants with a constitutive activity transport H^+ ions. However, it has been found that the D684N mutant (corresponding to D824N in gastric H^+,K^+-ATPase) in the *Arabidopsis thaliana* plasma membrane H^+-ATPase hydrolyzes ATP without transporting protons (*23*).

This study shows that modification of the amino acid side chains at positions 795 and 820 can transform gastric H^+,K^+-ATPase into a K^+-insensitive enzyme with a high constitutive activity. Very recently, the first atomic structure of a P-type ATPase, the Ca^{2+}-ATPase from rabbit sarcoplasmic reticulum, has been reported (*8*). In this structure, there are two Ca^{2+}-binding sites. The residue homologous to Glu^{795} (Glu^{771}) is involved in Ca^{2+}-binding site I, whereas the residue homologous to Glu^{820} (Asn^{796}) provides one of the oxygen-donating groups in Ca^{2+}-binding site II. If it is assumed that the structure of the ion binding sites in the catalytic subunit of gastric H^+,K^+-ATPase resembles that of Ca^{2+}-ATPase, it is difficult to visualize the conformational changes underlying the K^+-mimetic effects of these mutants, and in particular why the effect is larger with one of the double mutants. It might be important that in gastric H^+,K^+-ATPase a positively charged amino acid (Lys^{791}) is uniquely present just below Glu^{795}. For instance, in pyruvate kinase it is known the protonated ϵ-amino group of a lysine can serve as a fixed internal monovalent cation analogue for K^+ ions (*24*). It might be that upon mutation of neighboring residues the Lys^{791} side chain in gastric H^+,K^+-ATPase becomes better exposed.

We previously postulated (*6*) that with those modifications resulting in constitutive ATPase activity the K^+-filled pocket is imitated, which then signals to the catalytic domain of the enzyme. Alternatively, these modifications may mimic the effect of K^+ binding without changing the K^+-binding site. For instance, the modifications may change the mobility of transmembrane domains 5 and 6 (*25*), thus simulating the presence of K^+ in the binding pocket. Further studies are needed to resolve this fascinating mechanism.

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