The negative charge of glutamic acid-820 in the gastric H⁺,K⁺-ATPase α -subunit is essential for K⁺ activation of the enzyme activity

Harm P. H. HERMSEN, Herman G. P. SWARTS, Jan B. KOENDERINK and Jan Joep H. H. M. DE PONT§

Department of Biochemistry, Institute of Cellular Signalling, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

To investigate the role of Glu⁸²⁰, located in transmembrane domain M6 of the α -subunit of gastric H⁺,K⁺-ATPase, a number of mutants was prepared and expressed in Sf9 cells using a baculovirus encoding for both H⁺,K⁺-ATPase subunits. The wild-type enzyme and the E820D (Glu⁸²⁰ → Asp) mutant showed a similar biphasic activation by K⁺ on the ATPase activity (maximum at 1 mM). The mutant E820A had a markedly decreased K⁺ affinity (maximum at 40–100 mM). The other mutants, E820Q, E820N, E820L and E820K, showed no K⁺-activated ATPase activity at all, whereas all mutants formed a phosphorylated intermediate. After preincubation with K⁺ before phosphorylation mutant E820D showed a similar K⁺-sensitivity as the wild-type enzyme. The mutants E820N and E820Q had a 10–20 times lower sensitivity, whereas the other three mutants

were hardly sensitive towards K⁺. Upon preincubation with 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)imidazo[1,2a]-pyridine (SCH 28080), all mutants showed similar sensitivity for this drug as the wild-type enzyme, except mutant E820Q, which could only partly be inhibited, and mutant E820K, which was completely insensitive towards SCH 28080. These experiments suggest that, with a relatively large residue at position 820, the binding of SCH 28080 is obstructed. The various mutants showed a behaviour in K⁺-stimulated-dephosphorylation experiments similar to that for K⁺-activated-ATPase-activity measurements. These results indicate that K⁺ binding, and indirectly the transition to the E₂ form, is only fully possible when a negatively charged residue is present at position 820 in the α -subunit.

INTRODUCTION

The mechanism of coupling between ATP hydrolysis and active ion transport is still one of the unsolved problems in biology. In the P-type ATPases such as Ca²⁺-ATPase, Na⁺,K⁺-ATPase and gastric H⁺,K⁺-ATPase the γ -phosphate residue of ATP forms an acyl-phosphate with a conserved aspartic acid residue. This aspartic acid residue is present in the large intracellular loop of the catalytic subunit, located between the fourth and fifth transmembrane domain. Ions which are transported across the membrane affect the phosphorylation state of these enzymes either by increasing the rate of phosphorylation or dephosphorylation. The phosphorylation of the gastric H⁺,K⁺-ATPase, as well as the sequential countertransport of H⁺ and K⁺, can be described in defined partial reactions summarized in the Post-Albers scheme (Scheme 1) [1]. The binding of the cations is thought to be located within the transmembrane segments of the catalytic subunit. The effect of ions on the phosphorylation reaction is thus a long-range effect in which conformational changes in the protein have to be involved.

Polar amino acid residues and, in particular the negatively charged amino acid residues aspartate and glutamate, might play a key role in the binding and transport of the cations. Previous reports on different P-type ATPases such as Ca²⁺-ATPase [2–5], Na⁺,K⁺-ATPase [6–8] and gastric H⁺,K⁺-ATPase [9,10] indicated a possible role of cation-co-ordinating residues in the fifth and sixth transmembrane regions. Amino acid sequences in these transmembrane regions are well conserved among P-type ATPases [11]. This also accounts for the negatively charged

residues Glu and Asp, suggesting their importance for ion binding and transport.

In a previous study with baculoviruses encoding the gastric H⁺,K⁺-ATPase expressed in Sf9 cells, we observed that mutation of Glu⁸²⁰ into a Gln residue resulted in an enzyme with a normal aspartyl-phosphate as intermediate, but without K⁺-stimulated ATPase activity and K⁺-stimulated dephosphorylation. More-

Scheme 1 Post-Albers scheme, describing the reaction cycle of the gastric H⁺,K⁺-ATPase

Abbreviations used: SERCA, sarcoplasmic and endoplasmic reticulum; E-P, phosphorylated intermediate; SCH 28080, 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)imidazo[1,2a]pyridine; WT, wild-type; IC₅₀, 50% inhibitory concentration; E820D mutant (etc.), Glu⁸²⁰ → Asp (etc.) (single-letter amino acid).

§ To whom correspondence should be addressed (e-mail J.depont@bioch.kun.nl).

Figure 1 Amino acid sequence of gastric H^+,K^+ -ATPase around the M5–M6 region

The sequence was obtained from Shull et al. [37]. The negatively charged residues present in the fifth and sixth transmembrane domains of the gastric H^+,K^+ -ATPase are depicted in **boldface** type. The exact positioning of M5 and M6 is argued in the text.

over, the steady-state ATP-phosphorylation level could hardly be decreased upon preincubation with 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)imidazo[1,2-a]pyridine (SCH 28080), a specific inhibitor for the gastric H^+,K^+ -ATPase [12,13]. This suggests that Glu⁸²⁰ is involved in the coupling between K^+ binding and the dephosphorylation reaction and possibly also in SCH 28080 binding.

The precise location of Glu⁸²⁰ in the M5/M6 region is not known. Most workers locate this residue or its counterpart in other P-type ATPases: D804 (Asp⁸⁰⁴) in Na^+,K^+ -ATPase [7], N796 in SERCA1a Ca^{2+} -ATPase [14] and N879 [15] in plasma-membrane Ca^{2+} -ATPase [15] within the M6 transmembrane segment (SERCA is sarcoplasmic and endoplasmic reticulum). In our previous studies [9,16] we postulated a model for the M5/M6 region in which E820 was in the extracellular domain between M5 and M6. Hydrophobicity plots in this region are not very decisive, and since Lys⁷⁹¹ has been shown to be a cytosolically located tryptic-digestion site [17], this residue has to be within the large intracellular loop between M4 and M5. Moreover, Cys⁸²² was a candidate for the reaction with acid-activated omeprazole [17] and was also placed by us in the extracellular domain between M5 and M6. Recently, however, Besancon et al. [18] showed convincingly that acid-activated omeprazole reacts from the luminal side with Cys⁸¹³, and Falson et al. [19] showed a cleavage site for proteinase K at Gly⁸⁰⁸ in rabbit SERCA1a Ca^{2+} -ATPase, which is analogous to Ala⁸³² in H^+,K^+ -ATPase. If the latter finding is valid for H^+,K^+ -ATPase, it is compatible with the model shown in Figure 1. Both M5 and M6 are then only 18 amino acids long, and the extracellular domain between M5 and M6 contains only four amino acids. Most importantly for the present study, Glu⁸²⁰ is present in M6, slightly closer to the luminal than to the cytosolic space.

To investigate the role of this negatively charged amino acid in more detail we prepared, in addition to E820Q, the mutants E820D, E820N, E820A, E820L and E820K and measured a series of (partial) reactions. Generally it can be concluded that a negatively charged residue at position 820 is involved in K^+ binding and is essential for a normal functioning of the enzyme. Moreover, in the binding pocket for K^+ , other residues are likely to be involved additionally. These results suggest that the binding pocket has other constraints when K^+ enters from the luminal than from the cytosolic side.

EXPERIMENTAL

Site-directed mutagenesis

All DNA manipulations were done according to standard molecular-biology techniques described by Sambrook et al. [20]. The BaculoGold transfer vector pAcUW51 (Pharmingen, San Diego, CA, U.S.A.), containing the full-length cDNA of the rat H^+,K^+ -ATPase α - and β -subunits was used for site-directed mutagenesis using the Unique Site Elimination (Pharmacia Biotech) procedure developed by Deng and Nickoloff [21]. This mutagenesis method utilizes a two-primer system to generate site-specific mutations. The target mutagenic primer introduces the desired mutation in the α -subunit. The second primer, the selection primer, introduces a silent mutation, thereby eliminating a unique *AccI* restriction-endonuclease site which subsequently serves as the basis for selection of the mutated constructs. Selection of the desired mutants was performed using restriction analysis on the *AccI* site, which should be eliminated, and the restriction sites introduced or eliminated in the α -subunit. Finally all mutations were checked by sequence analysis.

Generation of recombinant viruses

The obtained pAcUW51-HK $\beta\alpha$ -wt and pAcUW51-HK $\beta\alpha$ -mutants, with the DNA code of the α -subunit under control of the polyhedrin promoter and that of the β -subunit under control of the P10 promoter were used to produce recombinant viruses. The mutated transfer vectors and linearized AcNPV DNA (BaculoGoldTM DNA) were co-transfected in Sf9 cells according to the instructions of the supplier. By this method the viruses Bg $\beta\alpha$ -wt, Bg $\beta\alpha$ -E820D, Bg $\beta\alpha$ -E820Q, Bg $\beta\alpha$ -E820N, Bg $\beta\alpha$ -E820A, Bg $\beta\alpha$ -E820L and Bg $\beta\alpha$ -E820K were obtained. The viruses were further purified via a plaque assay and were screened for the presence of the α -subunit by Western blotting. The presence of the desired mutation in the viral genome was checked by sequence and restriction analysis.

Production of recombinant H^+,K^+ -ATPase

Sf9 cells were grown at 27 °C either in 100 ml spinner-flask cultures or as monolayer cultures in 175 cm² culture flasks as

described by Klaassen et al. [22]. For production of H⁺,K⁺-ATPase the cells were infected at a multiplicity of infection of 1–3 in the presence of 1 % ethanol [23] and incubated for 3 days.

Preparation of Sf9 membranes

The Sf9 cells were harvested by centrifugation at 2000 *g* for 5 min. After resuspension at 0 °C in 0.25 M sucrose/2 mM EDTA/25 mM Hepes/Tris, pH 7.0, the membranes were sonicated three times for 15 s at 60 W in a Branson (Branson Power Company, Denbury, CT, U.S.A.) instrument. After centrifugation for 30 min at 10000 *g* the supernatant was re-centrifuged for 60 min at 100000 *g* at 4 °C. The pelleted membranes were resuspended in the above-mentioned buffer and stored at –20 °C.

Protein determination

Protein was determined with the modified Lowry method described by Peterson [24] using BSA as a standard.

Quantification of the expression level

The H⁺,K⁺-ATPase α -subunit content of the membrane fraction was determined by a quantitative ELISA [25] using the monoclonal antibody 5B6 [26,27] and using purified pig H⁺,K⁺-ATPase as a reference.

Western blotting

Protein samples from the membrane fraction were solubilized in SDS/PAGE sample buffer and separated on SDS/10 %-acrylamide as described by Laemmli [28]. For immunoblotting the separated proteins were transferred to Immobilon PVDF membranes. The α - and β -subunits of H⁺,K⁺-ATPase were detected as described previously [22] with the polyclonal antibody HKB [29] and the monoclonal antibody 2G11 [30] evoked against the β -subunit of H⁺,K⁺-ATPase respectively.

K⁺-ATPase activity assay

The K⁺-activated ATPase activity was determined with a radiochemical method [31]. For this purpose 0.6–5 μ g of Sf9 membranes were added to 100 μ l of medium, which contained 10 μ M [γ -³²P]ATP (sp. radioactivity 100–500 mCi·mmol^{–1}), 1.0 mM MgCl₂, 0.2 mM EGTA, 0.1 mM EDTA, 0.1 mM ouabain, 1 mM NaN₃, 25 mM Tris/HCl, pH 7.0, and various concentrations of KCl. After incubation for 30 min at 37 °C the reaction was stopped by adding 500 μ l 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 (10000 *g*). To 0.2 ml of the clear supernatant, containing the liberated [³²P]P_i, 3 ml of OptiFluor (Canberra Packard, Tilburg, The Netherlands) was added and the mixture was analysed by liquid-scintillation analysis. In general, blanks were prepared by incubating in the absence of enzyme. The ATPase activity is presented as a percentage of the activity in the absence of added K⁺ (which is 70–150 nmol of P_i liberated/h per mg). The latter activity is endogenously present in membranes of Sf9 cells.

ATP-phosphorylation capacity

ATP phosphorylation was determined as described previously [23]. Sf9 membranes (40–100 μ g) were incubated at 0 °C in 25 mM Tris/acetic acid (pH 6.0)/1 mM MgCl₂, with or without 100 μ M SCH 28080 or 10 mM KCl in 0.2 % (v/v) ethanol in a volume of 50 μ l. After 30–60 min preincubation, 10 μ l of 0.6 μ M

[γ -³²P]ATP was added and the incubation continued for 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 μ m membrane filter (Schleicher and Schuell, Dassel, Germany). After repeated washing, the filters were analysed by liquid-scintillation analysis.

Dephosphorylation studies

After ATP phosphorylation as described above, the reaction mixture was diluted from 60 μ l to 200 μ l with non-radioactive 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⁺) [32]. The mixture was further incubated for 3 s at 0 °C. Thereafter the reaction was stopped as described above and the residual phosphorylation level was determined.

SDS/PAGE of the phosphorylated proteins

After phosphorylation of 50–100 μ g of membranes with [γ -³²P]ATP, the reaction was terminated upon addition of 0.5 ml of stopping solution. After 30 s centrifugation at 16000 *g*, the pellets were washed with 0.5 ml 30 % (w/v) sucrose and resuspended in 40 μ l of 2 $\times$ sample buffer, containing 100 mM Tris/HCl, pH 6.8, 5 % (w/v) dithioerythritol, 4 % (w/v) SDS, 20 % glycerol (v/v) and 0.012 % Bromophenol Blue. The protein was solubilized during 30 min incubation at room temperature. Samples of 5 μ l (2–10 pCi) of ³²P-labelled protein, were subjected to SDS/PAGE on a Mini-Protein II (Bio-Rad, Richmond, CA, U.S.A.) apparatus. Running gels were prepared with 6 % polyacrylamide/*N,N'*-bismethyleneacrylamide (ratio 4000:107) in 100 mM sodium phosphate buffer (pH 6.5)/0.2 % SDS, whereas the stacking gel contained 4 % polyacrylamide. In the same 100 mM sodium phosphate buffer the electrophoresis was performed at 0 °C, first during 20 min at 30 mV and then for 3–4 h at 60 mV. The upper electrophoresis buffer contained 0.1 % SDS. After fixation in 5 % (v/v) methanol/7.5 % (v/v) acetic acid for 15–30 min at room temperature, the gels were autoradiographed at –20 °C on Kodak XOMAT AR film (Eastman Kodak Company, Rochester, NY, U.S.A.), for 3–16 h.

Analysis of data

The IC₅₀ values for K⁺ and SCH 28080 were iteratively determined by fitting the concentration relationship to the logistic equation:

$$Y = A + (B - A) / (1 + (10^C / 10^X)^D)$$

where *A* is the bottom plateau (0.48 pmol/mg of protein), *B* is the top plateau, *C* is the IC₅₀ and *D* the Hill coefficient; the values of *X* and *C* were entered as the logarithm of concentration using the non-linear regression computer program InPlot (GraphPAD Software for Science, San Diego, CA, U.S.A.). All data are presented as means $\pm$ S.E.M. Differences from the mean were tested for significance by means of Student's *t* test.

Chemicals

[γ -³²P]ATP (3000 Ci·mmol^{–1}; Amersham International, Amersham, Bucks., U.K.) was diluted with non-radioactive Tris-ATP (pH 6.0) to a sp. radioactivity of 20–100 Ci·mmol^{–1}. SCH 28080, kindly provided by Dr. A. Barnett (Schering-Plough, Bloomfield, NJ, U.S.A.) was dissolved in ethanol and diluted to its final concentration of 0.1 mM in 0.2 % ethanol. The antibodies 2G11 and HK α C2 were gifts from Dr. J. Forte (Department of Molecular and Cell Biology, University of California, Berkeley, CA, U.S.A.) and A. Smolka (Department of Medicine, Uni-

versity of South Carolina, Charleston, SC, U.S.A.) respectively. Dr. M. Caplan (Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT, U.S.A.) provided the antibodies HKB and HK9.

RESULTS

As a follow-up of a recent study in which a number of negatively charged residues, including Glu⁸²⁰ present in the α -subunit of rat gastric H⁺,K⁺-ATPase, were mutated into their acid amides [9], a number of additional mutants were constructed: E820D, E820N, E820A, E820L and E820K. Baculoviruses were produced containing coding sequences for the β -subunit as well as for the (mutated) α -subunit and were used to infect Sf9 cells.

Expression of the H⁺,K⁺-ATPase

All mutants show a normal expression pattern as compared with the wild-type according to Western-blotting analysis (Figure 2) and confocal-image analysis (results not shown). The α -subunit present in the membrane fractions of Sf9 cells after infection with these mutated viruses has the same apparent molecular mass as the α -subunit of the enzyme of pig gastric mucosa [33] and was absent in the membrane fractions obtained from mock-infected Sf9 cells. Figure 2 shows a Western blot performed with the HKB antibody, which recognizes a part of the intracellular loop between the fourth and fifth transmembrane segment [34]. Similar results were obtained using the antibodies HK α C2 [35] and HK9 [36] directed against the C- and N-terminal domain of the α -subunit respectively (results not shown). This indicates that all mutants were fully expressed and no proteolytic breakdown occurred. The monoclonal antibody 2G11 directed against the β -subunit revealed both a carbohydrate-free and a core-glycosylated form of the β -subunit similar to the results found previously using the baculovirus expression system ([22,30]; results not shown).

K⁺-activated ATPase activity

The K⁺-dependence of the overall ATPase activity of the various mutants was compared with that of the wild-type enzyme (Figure 3). A relatively low ATP concentration (10 μ M) had to be used in these experiments in order to obtain a significant stimulation by K⁺. At higher ATP concentrations the background ATPase activity became too high, so that measurement of the K⁺-stimulated activity was rather inaccurate. The wild-type enzyme as well as the E820D mutant showed a biphasic activation curve, with a maximum at 1 mM K⁺. The rising part of these curves suggests that the K⁺ affinity of the E820D mutant might be slightly higher than that of the wild-type enzyme. A similar K⁺

Figure 2 Western blot of H⁺,K⁺-ATPase mutants

Membranes (10–20 μ g) isolated from Sf9 cells infected either with wild-type virus or mutated viruses expressing the α - and β -subunit of H⁺,K⁺-ATPase were blotted and the presence of the α -subunit was detected using the polyclonal antibody HKB [29]. For comparison the enzyme isolated from gastric mucosa (pig) and membranes isolated from mock-infected cells are shown.

Figure 3 Effect of K⁺ on the ATPase activity in Sf9 membranes infected with recombinant baculoviruses

Membranes obtained from Sf9 cells infected with either wild-type virus (○) or the mutants E820D (●), E820Q (□), E820N (■), E820A (△), E820L (▲) and E820K (◇) were isolated and the ATPase activity was measured as described in the Experimental section with the K⁺ concentrations as indicated. In all experiments the activity in the absence of added K⁺ was set at 100%. Mean values from three experiments with S.E.M. values are presented.

activation curve was also observed with pig gastric H⁺,K⁺-ATPase when a low ATP concentration was used [31]. The activation of the ATPase activity by K⁺ could be blocked completely by 100 μ M SCH 28080. The E820D mutant and the wild-type enzyme showed similar IC₅₀ values for SCH 28080 (0.2–0.4 μ M; results not shown). The mutants E820Q, E820N, E820L and E820K showed no K⁺ stimulation. The E820A mutant showed a maximal K⁺ activation at much higher K⁺ concentration (40–100 mM) than the wild-type enzyme and the E820D mutant (1 mM). The maximal activity of mutant E820A, taken as the difference between total activity and activity of the endogenous ATPase, was similar to that of the wild-type enzyme.

Phosphorylation by ATP

The formation of an acid-stable phosphorylated intermediate during the catalytic cycle is a characteristic property of the P-type ATPases. In gastric H⁺,K⁺-ATPase phosphorylation occurs upon addition of Mg²⁺-ATP [31]. In wild-type as well as in all the mutants the phosphorylated α -subunit was revealed as a 100 kDa band upon autoradiography after SDS/PAGE of the H⁺,K⁺-ATPase containing Sf9 membranes (Figure 4, left column). The 140 kDa band also present in these gels appears to be an inherent property of an endogenous ATPase present in Sf9 cell membranes, since it was also present in mock-infected or uninfected cells [9].

After preincubation with either 10 mM K⁺ or 0.1 mM SCH 28080 for 60 min at pH 6.0 before addition of ATP, some differences between the wild-type and the mutants could be observed. The 100 kDa phosphorylated band of the wild-type enzyme could hardly or not be detected after preincubation with either K⁺ or SCH 28080. Similar results were obtained with the E820D mutant. Preincubation with either K⁺ or SCH 28080 did not reduce the amount of phosphoenzyme of mutants E820Q

Figure 4 Autoradiogram of SDS/PAGE of the ATP-phosphorylated Sf9 membranes infected with recombinant baculoviruses expressing wild-type and mutated gastric H⁺,K⁺-ATPase

Membranes isolated from Sf9 cells infected with wild-type virus or the mutant viruses E820D, E820Q, E820N, E820A, E820L and E820K were phosphorylated at 0 °C with 0.1 μ M [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 either 100 μ M SCH 28080 or 10 mM KCl. The acid-quenched samples were solubilized and subjected to SDS/PAGE at pH 6.5 as described in the Experimental section. The position of the α -subunit of pig gastric H⁺,K⁺-ATPase on the gels is indicated by arrows.

Table 1 Characteristics of the isolated membranes obtained from Sf9 cells infected with recombinant baculovirus

Sf9 cells were infected with recombinant baculovirus expressing the (mutant) gastric H⁺,K⁺-ATPase {wild-type, E820D, E820Q, E820N, E820A, E820L, E820K or with the control virus DZ1 (mock) [22]}. After 72 h, membranes were isolated according to the protocol described in the Experimental section. The phosphorylation level (E-P) of the membranes was determined after incubation for 10 s at 0 °C at pH 6.0 by a filtration method after preincubation with or without 10 mM K⁺ or 100 μ M SCH 28080. The amount of gastric H⁺,K⁺-ATPase was determined by an ELISA method and expressed as a percentage of the total protein content taking highly purified pig H⁺,K⁺-ATPase as standard; after infection with virus DZ1, no signal was obtained in this assay. All values are given as means $\pm$ S.E.M. for the number of membrane preparations (*n*) shown. Differences of the results compared with the mock-infected cells were tested for significance by means of Student's *t* test (**P* < 0.1; ***P* < 0.05; ****P* < 0.01)

Mutant	Phosphorylation level (pmol/mg of protein)			H ⁺ ,K ⁺ -ATPase (% of total)	<i>n</i>
	E-P	E-P + K ⁺	E-P + SCH 28080		
Mock	0.45 $\pm$ 0.05	0.48 $\pm$ 0.06	0.50 $\pm$ 0.07	0.00 $\pm$ 0.00	5
Wild-type	2.03 $\pm$ 0.29***	0.56 $\pm$ 0.05	0.54 $\pm$ 0.06	1.18 $\pm$ 0.16***	10
E820D	1.04 $\pm$ 0.11***	0.60 $\pm$ 0.05	0.61 $\pm$ 0.05	1.16 $\pm$ 0.11***	10
E820Q	1.07 $\pm$ 0.14***	0.67 $\pm$ 0.03**	0.78 $\pm$ 0.08**	0.87 $\pm$ 0.14***	7
E820N	1.76 $\pm$ 0.19***	1.03 $\pm$ 0.20***	0.50 $\pm$ 0.06	1.09 $\pm$ 0.18***	7
E820A	2.67 $\pm$ 0.49***	2.77 $\pm$ 0.42***	0.58 $\pm$ 0.09	1.40 $\pm$ 0.24***	7
E820L	0.89 $\pm$ 0.12**	0.85 $\pm$ 0.12*	0.68 $\pm$ 0.08	0.95 $\pm$ 0.14***	8
E820K	0.90 $\pm$ 0.11***	0.69 $\pm$ 0.07*	0.92 $\pm$ 0.10***	0.84 $\pm$ 0.08***	6

and E820K. On the other hand the mutants E820N, E820L and E820A showed no effect of preincubation with K⁺ on the amount of phosphorylated intermediate, whereas preincubation with SCH 28080 led to a (nearly complete) disappearance of the phosphorylated intermediate band.

The amount of phosphorylated intermediate of Sf9 cell membranes obtained after infection with baculoviruses coding for the various mutants was quantified after filtration on membrane filters. The amount of phosphorylated intermediate varied from 0.89 to 2.67 pmol/mg of protein. These values were all significantly higher than that of mock-infected cells (0.45 pmol/mg of protein; see Table 1, first column). The phosphorylation level of the E820A mutant was even higher than that of the wild-type enzyme, whereas the phosphorylation level of the E820L and E820K mutants was rather low. Table 1 also shows the residual phosphorylation level after preincubation with either 10 mM K⁺ (column 2) or 100 μ M SCH 28080 (column 3). The qualitative results from the gels shown in Figure 4 are confirmed quantitatively. A complete inhibition by K⁺ was only obtained with the wild-type enzyme and with mutant E820D. With SCH 28080, all mutants were inhibited, except mutant E820K, which was completely insensitive, and mutant E820Q, which was only partly inhibited. The amount of H⁺,K⁺-ATPase protein expressed was measured in the same membrane fractions using an ELISA [25] based on specific binding of the produced protein to the monoclonal antibody 5B6 [26].

Quantification of the amount of baculovirus-produced H⁺,K⁺-ATPase (mutants) was established by comparison of the amount of immunoreactive protein with that of the pig enzyme. Table 1 (fourth column) shows that the amount of immunoreactive protein in the isolated membrane fractions varied from 0.84 to 1.40 % of total protein. It is clear that the variation in the level of phosphorylated intermediate is not due to differences in expression level.

K⁺ and SCH 28080 sensitivity

The above-described experiments were carried out in more detail by using varying K⁺ and SCH 28080 concentrations during the preincubation period in order to determine the half-maximal inhibitory K⁺ and SCH 28080 concentrations. As shown in Figure 5, mutant E820D shows a similar K⁺-sensitivity (IC₅₀ = 0.4 $\pm$ 0.1 mM; *n* = 3) to the wild-type enzyme (IC₅₀ = 0.6 $\pm$ 0.2 mM; *n* = 3). The other mutants, all lacking a negative charge, have much lower K⁺-sensitivities than the wild-type enzyme. Figure 5 shows that the K⁺-sensitivity for the two mutants with an acid amide group E820Q (IC₅₀ = 8.5 $\pm$ 0.5 mM; *n* = 3) and E820N (IC₅₀ = 12.8 $\pm$ 0.8 mM; *n* = 3), is 10–20 times less than that of the wild-type enzyme. The mutants E820A (IC₅₀ = 40 $\pm$ 10 mM; *n* = 3), E820L (IC₅₀ = 23 $\pm$ 7 mM; *n* = 3) and E820K (IC₅₀ = 10 $\pm$ 2.5 mM; *n* = 3) also have a reduced K⁺ sensitivity.

The effect of SCH 28080 on the ATP-phosphorylation capacity (Figure 6) was also quantified. The mutants E820D (IC₅₀ = 7.8 $\pm$ 1.1 nM; *n* = 3), E820N (IC₅₀ = 23 $\pm$ 0.1 nM; *n* = 3) and E820A (IC₅₀ = 126 $\pm$ 5 nM; *n* = 3) showed an SCH 28080-sensitivity comparable with that of the wild-type enzyme (IC₅₀ = 20 $\pm$ 0.4 nM; *n* = 3). The IC₅₀ value for mutant E820Q, however, was 0.6 $\pm$ 0.1 μ M, which value is slightly lower than found previously (1.7 $\pm$ 0.6 μ M; [9]). Mutants E820K and E820L were not, or hardly, inhibited by 100 μ M SCH 28080.

K⁺-stimulated dephosphorylation

In order to study whether the effects of K⁺ on the ATPase activity and on the steady-state phosphorylation level are due to a stimulation of the hydrolysis of the phosphorylated intermediate, a dephosphorylation experiment was carried out. The membranes were phosphorylated with radioactive ATP and, after dilution with unlabelled ATP, incubated for 3 s in the presence of 0, 1, 10 or 100 mM KCl. Next the residual amount of radio-

Figure 5 Effect of K^+ on ATP-phosphorylation capacity of H^+,K^+ -ATPase obtained from Sf9 membranes infected with recombinant baculoviruses

Membranes obtained from Sf9 cells infected with either wild type virus (○) or the mutants E820D (●), E820Q (□), E820N (■), E820A (△), E820L (▲) and E820K (◇) were preincubated for 60 min at 0 °C in the presence of 1 mM $MgCl_2$ and 20 mM Tris/acetic acid, pH 6.0, in the presence of various K^+ concentrations. After phosphorylation for 10 s at 0 °C with 0.1 μ M [γ - ^{32}P]ATP the phosphorylation level (E-P) was determined, expressed as pmol/mg of protein (pmol E-P) and plotted as a function of the K^+ concentrations. The phosphorylation level of mock-infected cells is indicated by a broken line. Bars represent S.E.M. values for three separate experiments.

Figure 6 Effect of SCH 28080 on ATP-phosphorylation capacity of H^+,K^+ -ATPase obtained from Sf9 membranes infected with recombinant baculoviruses

Membranes obtained from Sf9 cells infected with either wild-type virus (○) or the mutants E820D (●), E820Q (□), E820N (■), E820A (△), E820L (▲) and E820K (◇) were preincubated for 60 min at 0 °C in the presence of 1 mM $MgCl_2$ and 20 mM Tris/acetic acid, pH 6.0, in the presence of various SCH 28080 concentrations. After phosphorylation for 10 s at 0 °C with 0.1 μ M [γ - ^{32}P]ATP the phosphorylation level (E-P) was determined, expressed as pmol/mg of protein (pmol E-P) and plotted as a function of the SCH 28080 concentration. The phosphorylation level of mock-infected cells is indicated by a broken line. Bars represent S.E.M. values for three separate experiments.

labelled phosphorylated intermediate was determined. Figure 7 shows that the wild-type enzyme has a K^+ -sensitive phosphorylated intermediate. The higher the K^+ concentration the less phosphorylated intermediate was left after the 3 s chase period. The E820D mutant showed a K^+ -sensitivity very similar to that of the wild-type enzyme. The E820N mutant showed, like the E820Q [9], E820K and E820L mutants (not shown), no K^+ -sensitivity at all. The amount of phosphorylated intermediate of the E820A mutant was not reduced by 10 mM KCl, but was

significantly reduced by 100 mM KCl. The low K^+ -sensitivity of this mutant is reminiscent of the similar low sensitivity in the ATPase assay. These findings indicate that the K^+ -sensitivity in the ATPase reaction is due to its effect on the dephosphorylation reaction.

If mutagenesis led to an inhibition of the conformational change between E_1 -P and E_2 -P one would expect that an ADP-dependent dephosphorylation would have been found. However, no effect of 5 mM ADP on the resulting amount of phosphoryl-

Figure 7 Effect of K⁺ on the dephosphorylation reaction of the phosphorylated intermediate of (mutant) H⁺,K⁺-ATPase obtained from Sf9 membranes infected with recombinant baculoviruses

Membranes obtained from Sf9 cells infected with either wild-type virus or the mutants E820D, E820N or E820A were preincubated for 60 min at 0 °C in the presence of 1 mM MgCl₂ and 20 mM Tris/acetic acid, pH 6.0, with or without 100 μ M SCH 28080 and were phosphorylated at 0 °C with 0.1 μ M [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 with non-radioactive ATP (final concentration 1 mM) in 25 mM Tris/acetic acid, pH 6.0, in order to prevent rephosphorylation with radioactive ATP and incubated for 3 s with no further addition (○), 1 mM KCl (●), 10 mM KCl (□) or 100 mM KCl (■). The difference between the phosphorylation level without and with SCH 28080 (E-P) was determined and expressed as a percentage of the control.

ated intermediate could be observed (results not shown), indicating that the mutants were not blocked in the E₁-P to E₂-P transition.

DISCUSSION

Negatively charged residues present in the M5/M6 region of P-type ATPases are thought to play an important role in cation binding and translocation. In a previous study [9] we investigated the role of a number of these negatively charged residues in rat gastric H⁺,K⁺-ATPase by mutating carboxylic acid side chains into acid amides. Of these mutants, E820Q was a very interesting one, since it was able to form a phosphorylated intermediate, but did not show K⁺-induced dephosphorylation and thus no K⁺-stimulated ATPase activity. This indicates an important role of residue Glu⁸²⁰ in the coupling between ATP phosphorylation and K⁺ transport. For a better understanding of the function of Glu⁸²⁰, additional mutants with various amino acid residues at this position were constructed. The different α -subunit mutants were expressed in Sf9 insect cells using a baculovirus encoding both H⁺,K⁺-ATPase subunits. The expression of α - and β -subunits was controlled by the polyhedrin and p10 promoters respectively, which become activated during the late stage of the viral infection cycle [9,22,23].

All mutants, E820Q, E820D, E820N, E820L, E820A and E820K, showed a 100 kDa band which reacted with antibodies against the N-terminus, the C-terminus and the large intracellular loop of the α -subunit of gastric H⁺,K⁺-ATPase, indicating that no proteolytic breakdown occurred in any mutant. Confocal-imaging analysis showed that, as in the previous study [9], nearly all α -subunit was present in intracellular membranes.

All mutants could be phosphorylated by ATP, suggesting that Glu⁸²⁰ is not essential for the enzyme to become phosphorylated, although the level of phosphorylation varies among the mutants.

The results with K⁺-stimulated ATPase activity perfectly matched with those of the experiments in which K⁺-stimulated dephosphorylation was measured. The E820D mutant showed a similar behaviour to the wild-type enzyme, the E820A mutant showed a very low K⁺ affinity in both assays, whereas all other mutants showed no K⁺-stimulated ATPase activity nor any effect of K⁺ on the dephosphorylation reaction.

The homologous residue for Glu⁸²⁰ in the related enzyme Na⁺,K⁺-ATPase is an Asp. Mutation of Glu⁸²⁰ into Asp yielded an enzyme that in all assays gave similar results as the wild-type enzyme. This suggests that a negatively charged residue at position 820 is necessary and sufficient to obtain a wild-type-like activity. The shorter length of the Asp side chain as compared with the Glu side chain has apparently no measurable effect on the K⁺ activation of ATPase and dephosphorylation reaction. Asano et al. [10] reported recently, using rabbit H⁺,K⁺-ATPase expressed in HEK 293 cells, that the K⁺-affinity of the E820D mutant was unchanged, whereas the K⁺-stimulated ATPase activity was 60 % lower than that of the wild-type enzyme. Those authors used rabbit H⁺,K⁺-ATPase in which the similar amino acid is Glu⁸²².

The mutant E820A, lacking a negative charge, still showed a K⁺ stimulated ATPase activity, although with reduced K⁺ affinity. The dephosphorylation reaction could only be stimulated by high (100 mM) K⁺. A 20-fold-reduced affinity of this mutant was recently also reported by Asano and co-workers [10]. We found that all other mutants, all lacking a negative charge and all containing larger residues than Ala do not show any K⁺-stimulated ATPase activity or any K⁺-stimulated dephosphorylation, despite the fact that phosphorylation occurs.

These findings indicate that Glu⁸²⁰ is involved in K⁺ binding from the extracellular side resulting in dephosphorylation and probably K⁺ transport. This function can be fully taken over by another negatively charged residue at this position and only

partially when a methyl group is present as side chain. With other (larger) residues no K^+ stimulation was found. We do not find it likely that the methyl group in the E820A mutant is directly involved in K^+ binding, but assume that the K^+ -binding pocket contains other co-ordinating groups in addition to Glu⁸²⁰, such as Glu⁸²⁴. When a residue larger than a methyl group is present on this site, the other liganding groups cannot any longer be reached by the K^+ ion, so that K^+ -stimulated dephosphorylation can no longer occur.

The results where the membranes were preincubated with K^+ before ATP phosphorylation were in part different from those where the K^+ -sensitivity of the dephosphorylation reaction was measured. Most strikingly the E820N and the E820Q mutants were absolutely insensitive towards K^+ in the dephosphorylation studies (Figure 7), but showed some sensitivity towards K^+ when present during the preincubation experiments (Figure 5). In previous studies with intact gastric vesicles [31] we showed that the effect of K^+ during preincubation occurs on the cytosolic side, whereas it is well known that the effect of K^+ during dephosphorylation takes place on the extracellular side. The preincubation effect is due to a shift of the $E_2 \rightleftharpoons E_1$ equilibrium to the left, thus preventing phosphorylation (Scheme 1). These different effects could mean that the binding pocket for K^+ , when approached from the extracellular side, has other specificities than when approached from the cytosolic side. The mutants with an acid amide residue are apparently not able to bind K^+ from the extracellular side, but still do so in part when the ion approaches the binding pocket from the intracellular side. If the model for the M5/M6 region shown in Figure 1 is correct, residue 820 is located rather close to the extracellular side of the membrane. If we suppose that Glu⁸²⁴ is also involved in K^+ binding, it is likely that the K^+ binding pocket is more in the centre of the membrane. One can imagine that K^+ can reach this binding pocket from the intracellular side, even if Glu⁸²⁰ is mutated, whereas attainability from the extracellular side is obstructed. In addition it is reasonable to assume that the carbonyl group of the acid amide residues in Gln and Asn contributes more to K^+ binding than the neutral or positively charged residues of Ala, Leu and Lys. Further (mutational) studies are needed to test this hypothesis.

As we have already demonstrated previously [9] the SCH 28080 affinity is diminished in the E820Q mutant, suggesting a role for this residue in the SCH 28080 binding to the enzyme. The decrease in affinity could be measured by preincubation of the enzyme with SCH 28080 before measurement of the steady-state phosphorylation level. In the wild-type enzyme this leads to a reduction in the phosphorylation level probably caused by a shift of the $E_1 \rightleftharpoons E_2$ equilibrium to the E_2 conformation. The phosphorylation of the mutants E820D, E820N and E820A was inhibited by SCH 28080 similarly to the wild-type enzyme. On the other hand the mutants E820K and E820L showed, like E820Q, no or only a minor inhibition by SCH 28080. The effects observed on SCH 28080 inhibition are not likely to be due to a change in charge, but might be caused by a change in the size of the amino acid located at position 820.

The present study shows the important role of Glu⁸²⁰ for coupling between K^+ binding and ATP phosphorylation in gastric H^+ , K^+ -ATPase. The direct involvement of this residue in SCH 28080 inhibition is less likely, although the binding of this drug is influenced by some, but not all, mutations of Glu⁸²⁰.

We thank Dr. A. Barnett for providing SCH 28080 and Dr. A. Smolka, Dr. M. Caplan and Dr. J. Forte for generously providing the various antibodies. We thank Dr.

P. H. G. M. Willems and Dr. F. M. A. H. Schuurmans Stekhoven for their advice during the study and for their critical reading of the manuscript before its submission. Part of this work was sponsored by the Netherlands Foundation for Scientific Research (NWO-SLW) through grant 805-05.041.

REFERENCES

- Rabon, E. C. and Reuben, M. A. (1990) *Annu. Rev. Physiol.* **52**, 321–344
- Clarke, D. M., Loo, T. W. and MacLennan, D. H. (1990) *J. Biol. Chem.* **265**, 22223–22227
- Rice, W. J. and MacLennan, D. H. (1996) *J. Biol. Chem.* **271**, 31412–31419
- Møller, J. V., Juul, B. and Le Maire, M. (1996) *Biochim. Biophys. Acta* **1286**, 1–51
- Andersen, J. P. and Vilsen, B. (1994) *J. Biol. Chem.* **269**, 15931–15936
- Van Huysse, J. W., Kuntzweiler, T. A. and Lingrel, J. B. (1996) *FEBS Lett.* **389**, 179–185
- Kuntzweiler, T. A., Arguello, J. M. and Lingrel, J. B. (1996) *J. Biol. Chem.* **271**, 29682–29687
- Pedersen, P. A., Rasmussen, J. H., Nielsen, J. M. and Jørgensen, P. L. (1997) *FEBS Lett.* **400**, 206–210
- Swarts, H. G. P., Klaassen, C. H. W., De Boer, M., Fransen, J. A. M. and De Pont, J. J. H. H. M. (1996) *J. Biol. Chem.* **271**, 29764–29772
- Asano, S., Matsuda, S., Tega, Y., Shimizu, K., Sakamoto, S. and Takeguchi, N. (1997) *J. Biol. Chem.* **272**, 17668–17674
- Lutsenko, S. and Kaplan, J. H. (1995) *Biochemistry* **34**, 15607–15613
- Wallmark, B., Briving, C., Fryklund, J., Munson, K., Jackson, R., Mendlein, J., Rabon, E. and Sachs, G. (1987) *J. Biol. Chem.* **262**, 2077–2084
- Keeling, D. J., Taylor, A. G. and Schudt, C. (1989) *J. Biol. Chem.* **264**, 5545–5551
- MacLennan, D. H., Brandl, C. J., Korczak, B. and Green, N. M. (1985) *Nature (London)* **316**, 696–700
- Verma, A. K., Filoteo, A. G., Stanford, D. R., Wieben, E. M., Penniston, J. T., Strehler, E. E., Fisher, R., Heim, R., Vogel, G., Mathews, S. et al. (1988) *J. Biol. Chem.* **263**, 14152–14159
- Klaassen, C. H. W. and De Pont, J. J. H. H. M. (1994) *Cell. Physiol. Biochem.* **4**, 115–134
- Besancon, M., Shin, J. M., Mercier, F., Munson, K., Miller, M., Hersey, S. and Sachs, G. (1993) *Biochemistry* **32**, 2345–2355
- Besancon, M., Simon, A., Sachs, G. and Shin, J. M. (1997) *J. Biol. Chem.* **272**, 22438–22446
- Falson, P., Menguy, T., Corre, F., Bouneau, L., GomezdeGracia, A., Soulie, S., Centeno, F., Møller, J. V., Champeil, P. and Lemaire, M. (1997) *J. Biol. Chem.* **28**, 17258–17262
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbour, NY
- Deng, W. P. and Nickoloff, J. A. (1992) *Anal. Biochem.* **200**, 81–88
- Klaassen, C. H. W., Van Uem, T. J. F., De Moel, M. P., De Caluwe, G. L. J., Swarts, H. G. P. and De Pont, J. J. H. H. M. (1993) *FEBS Lett.* **329**, 277–282
- Klaassen, C. H. W., Swarts, H. G. P. and De Pont, J. J. H. H. M. (1995) *Biochem. Biophys. Res. Commun.* **210**, 907–913
- Peterson, G. L. (1983) *Methods Enzymol.* **91**, 95–106
- Brenna, E., Swarts, H. G. P., Klaassen, C. H. W., De Pont, J. J. H. H. M. and Waldum, H. L. (1994) *Gut* **35**, 1547–1550
- Van Uem, T. J. F., Peters, W. H. M. and De Pont, J. J. H. H. M. (1990) *Biochim. Biophys. Acta* **1023**, 56–62
- Van Uem, T. J. F., Swarts, H. G. P. and De Pont, J. J. H. H. M. (1991) *Biochem. J.* **280**, 243–248
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Blostein, R., Zhang, R. P., Gottardi, C. J. and Caplan, M. J. (1993) *J. Biol. Chem.* **268**, 10654–10658
- Chow, D. C. and Forte, J. G. (1993) *Am. J. Physiol.* **265**, C1562–C1570
- Swarts, H. G. P., Klaassen, C. H. W., Schuurmans Stekhoven, F. M. A. H. and De Pont, J. J. H. H. M. (1995) *J. Biol. Chem.* **270**, 7890–7895
- Helmich-de Jong, M. L., Van Ernst-De Vries, S. E., De Pont, J. J. H. H. M., Schuurmans Stekhoven, F. M. A. H. and Bonting, S. L. (1985) *Biochim. Biophys. Acta* **821**, 377–383
- Swarts, H. G. P., Van Uem, T. J. F., Hoving, S., Fransen, J. A. M. and De Pont, J. J. H. H. M. (1991) *Biochim. Biophys. Acta* **1070**, 283–292
- Gottardi, C. J. and Caplan, M. J. (1993) *J. Biol. Chem.* **268**, 14342–14347
- Smolka, A., Alverson, L., Fritz, R., Swiger, K. and Swiger, R. (1991) *Biochem. Biophys. Res. Commun.* **180**, 1356–1364
- Gottardi, C. J. and Caplan, M. J. (1993) *J. Cell Biol.* **121**, 283–293
- Shull, G. E. and Lingrel, J. B. (1986) *J. Biol. Chem.* **261**, 16788–16791