Mutation of Aspartate 804 of Na^+,K^+-ATPase Modifies the Cation Binding Pocket and Thereby Generates a High Na^+-ATPase Activity†

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ABSTRACT: A series of six different mutants (D804A, D804E, D804G, D804N, D804Q, and D804S) of aspartate 804 present in transmembrane segment 6 of the rat Na^+,K^+-ATPase α_1-subunit were prepared and expressed in Sf9 cells by use of the baculovirus expression system. In contrast to the wild-type enzyme all mutants except D804Q showed a very high Na^+-ATPase activity, which was hardly further stimulated by the addition of K^+. The ATPase activity of the mutants was already nearly maximal at 10 μM ATP and most of them could be phosphorylated in the absence of Na^+ at pH 6.0 and 21 °C, suggesting that they strongly prefer the E_1 over the E_2 conformation. However, Na^+ dose-dependently lowered the steady-state phosphorylation level, as a consequence of the increased affinity for Na^+ in the dephosphorylation reaction of the mutants compared to the wild-type enzyme. Conversely, the affinity for K^+ in the dephosphorylation reaction was decreased for the mutants as compared to that for the wild-type enzyme. When the pH was increased or the temperature was decreased, the phosphorylation level of the mutants decreased and the Na^+ activation in the phosphorylation reaction became apparent. It is concluded that upon mutation of aspartate 804 the affinity of the cation-binding pocket is changed relatively in favor of Na^+ instead of K^+, as a consequence of which the enzyme has obtained a preference for the E_1 conformation.

Polar, in particular negatively charged, residues present in transmembrane segments of P-type ATPases[1] are assumed to play a role in cation binding and transport. Previous studies of our group with gastric H^+,K^+-ATPase (*1*−*3*) have indicated a pivotal role of Glu820, present in transmembrane domain 6 of the catalytic subunit. A negative charge on this position is essential for K^+-stimulated dephosphorylation (*2*). Most interestingly, three neutral mutants (E820Q, E820N, and E820A) that had nearly or completely lost their K^+ sensitivity showed an ATPase activity in the absence of K^+ and an increased spontaneous dephosphorylation rate (*3*). This constitutive ATPase activity of these mutants could still be inhibited by the specific H^+,K^+-ATPase inhibitor SCH 28080.

These experiments led us to postulate that the empty K^+-binding pocket, in which Glu820 plays a crucial role, inhibits the dephosphorylation process ("inhibited dephosphorylation hypothesis"). When K^+ binds to its pocket the dephosphorylation rate is enhanced, resulting in a K^+-stimulated ATPase reaction. The K^+ activation reaction can be mimicked by mutations in the binding pocket, which remove the negative charge of Glu820.

The catalytic subunits of gastric H^+,K^+-ATPase and Na^+,K^+-ATPase are 63% identical. The location of negatively charged residues within transmembrane domains is conserved between these two ion pumps. Both ATPases form a phosphorylated intermediate, the hydrolysis of which is stimulated by K^+. It is therefore of importance to know whether the above-mentioned hypothesis is also valid for Na^+,K^+-ATPase.

The residue in Na^+,K^+-ATPase that is analogous to Glu820 in gastric H^+,K^+-ATPase is Asp804. Although several mutants of this residue have been prepared before (*4*−*8*), they were not analyzed in such a way that they could validate the "inhibited dephosphorylation hypothesis" for Na^+,K^+-ATPase. We therefore generated six different mutants of Asp804, expressed these in Sf9 cells, and analyzed them similarly as was done for H^+,K^+-ATPase. As guideline for this study, the modified Post−Albers scheme is given in Figure 1 (*9*, *10*). This scheme also includes the mechanism of action of the Na^+,K^+-ATPase inhibitor ouabain.

EXPERIMENTAL PROCEDURES

Expression Constructs. The rat Na^+,K^+-ATPase α_1 subunit cDNA (*11*) *Hin*dIII fragment and the sheep Na^+,K^+-ATPase β_1-subunit cDNA (*12*) *Sma*I and *Spe*I fragment were ligated into the pFastbacdual vector (Life Technologies, Breda, The Netherlands) as described before (*13*). We used the Altered Sites II in vitro mutagenesis systems (Promega, Madison, WI) to introduce the D804A, D804E, D804G, D804N, D804Q, and D804S mutations in the α-subunit of Na^+,K^+-ATPase. The total sequence was checked by sequencing with

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[1] Abbreviations: ATPase, adenosine triphosphatase; Sf, *Spodoptera frugiperda*; $C_{12}E_8$, octaethylene glycol monododecyl ether; SCH 28080, 3-(cyanomethyl)-2-methyl-8(phenylmethoxy)imidazo[1,2-*a*]pyridine; IC_{50}, 50% inhibitory concentration.

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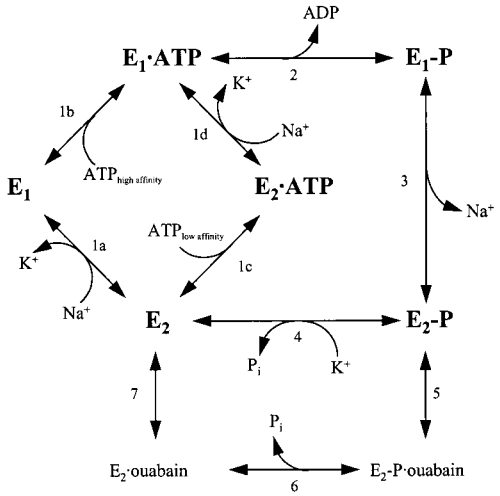

FIGURE 1: Post−Albers reaction scheme for Na^+,K^+-ATPase. ATP binds to the enzyme with both a high and a low affinity. In the presence of high concentrations of ATP the enzyme will first bind ATP, after which the conformation will shift from E_1 to E_2 (steps 1c and 1d). When the ATP concentration is low, the enzyme first has to shift to the E_1 conformation before ATP will bind (steps 1a and 1b). When the enzyme is in the E_1 conformation, Na^+ is bound and K^+ is released intracellularly. In the E_2 conformation, K^+ is bound and Na^+ is released extracellularly. The postulated way of inhibition by ouabain is also included in the scheme.

the Big Dye kit (Perkin-Elmer, Warrington, Great Britain). For mock transformations the baculovirus DZ1, only expressing β-galactosidase, was used (*14*).

Production of Recombinant Viruses. Competent DH10bac *Escherichia coli* cells (Life Technologies, Breda, The Netherlands) harboring the baculovirus genome (bacmid) and a transposition helper vector were transformed with the pFastbacdual transfer vector containing different cDNAs. Upon transition between the Tn7 sites in the transfer vector and the bacmid, recombinant bacmids were selected and isolated (*15*). Subsequently, insect Sf9 cells were transfected with recombinant bacmids by use of Cellfectin reagent (Life Technologies, Breda, The Netherlands). After 3 days, the recombinant baculoviruses were isolated and used to infect Sf9 cells at a multiplicity of infection of 0.1. Four days after infection, the amplified viruses were harvested.

Preparation of Membranes. Sf9 cells were grown at 27 °C in 100 mL spinner flask cultures (*14*). For production of the ATPases subunits $1.0-1.5 \times 10^6$ cells mL^{-1} were infected at a multiplicity of infection of 1−3 in Xpress medium (BioWittaker, Walkersville, MD) containing 1% ethanol (*16*) and incubated for 4 days. The Sf9 cells were harvested by centrifugation at 2000*g* for 5 min and resuspended at 0 °C in 0.25 M sucrose, 2 mM EDTA, and 25 mM Hepes/Tris (pH 7.0). The membranes were sonicated twice for 30 s at 60 W (Branson Power Co., Danbury, CT), after which the disrupted cells were centrifuged at 10000*g* for 30 min. The supernatant was recentrifuged at 100000*g* for 60 min and the pelleted membranes were resuspended in the above-mentioned buffer and stored at −20 °C.

Protein Determination. Protein was determined by the modified Lowry method described by Peterson (*17*) with bovine serum albumin as a standard.

ATPase Activity Assay. The Na^+,K^+-ATPase activity was determined by a radiochemical method (*18*). For this purpose Sf9 membranes were added to 100 μL of medium, which contained 1.3 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM EDTA, 2 mM Tris-N_3, 50 mM Tris−acetic acid (pH 7.0), 100 μM Mg-[γ-^{32}P]ATP (specific activity 100−500 mCi $mmol^{-1}$), 10 mM KCl, and 100 mM NaCl. After incubation at 37 °C, the reaction was stopped by adding 500 μL of 10% (w/v) charcoal in 6% (w/v) trichloroacetic acid, and after incubation at 0 °C, the mixture was centrifuged for 30 s (10000*g*). To 200 μL of the clear supernatant, containing the liberated inorganic phosphate ($^{32}P_i$), was added 4 mL of OptiFluor (Canberra Packard, Tilburg, The Netherlands), and the mixture was analyzed by liquid scintillation analysis. Blanks were prepared by incubation of the reaction medium in the absence of enzyme. The specific activity is presented as the difference between the mock [0.32 ± 0.02 μmol of P_i (mg of protein)$^{-1}$ h^{-1}, $n = 5$] and the expressed enzyme.

Phosphorylation and Dephosphorylation Assays. Sf9 membranes were incubated for 10 s at 21 °C in 50 mM Tris−acetic acid (pH 6.0), 1.2 mM $MgCl_2$, 0.2 mM EDTA, 100 mM NaCl, 0.5 mg/mL $C_{12}E_8$, and 0.1 μM [γ-^{32}P]ATP in a volume of 60 μL (*2*). For dephosphorylation studies, part of the reaction mixture was diluted 10 times with and without KCl or NaCl and was incubated for an additional 3 s. This dephosphorylation solution contained nonradioactive ATP (final concentration 1 mM), to prevent rephosphorylation with radioactive ATP. Thereafter, adding 5% trichloroacetic acid in 0.1 M phosphoric acid stopped the reaction 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 phosphorylation levels of the mutants and the wild-type Na^+,K^+-ATPase were corrected for that of mock-infected cells [∼1.5 pmol of EP (mg of protein)$^{-1}$]. The calculated turnover for the wild-type Na^+,K^+-ATPase was about 9000 min^{-1}, which is close to that of the isolated enzyme (*19*).

Calculations. The IC_{50} value is defined as the value giving 50% inhibition of the maximal activation. Data are presented as mean values with standard error of the mean.

Materials. The cDNA of the rat Na^+,K^+-ATPase α_1-subunit and cDNA of the sheep Na^+,K^+-ATPase β_1-subunit were provided by Dr J. B. Lingrel (Cincinnati). Cellfectin, competent DH10bac *E. coli* cells, and all enzymes used for DNA cloning were purchased from Life Technologies Inc. (Breda, The Netherlands). [γ-^{32}P]ATP (3000 Ci $mmol^{-1}$) was purchased from Amersham (Buckinghamshire, U.K.).

RESULTS

The aspartate (D) 804 residue present in the α-subunit of Na^+,K^+-ATPase was replaced by either an alanine (A), glutamate (E), glycine (G), asparagine (N), glutamine (Q), or serine (S) residue. These mutants and the wild-type Na^+,K^+-ATPase were expressed in Sf9 insect cells by use of the baculovirus expression system. The membrane fractions of these Sf9 cells were isolated and Western blot analysis revealed that the level of expression of the mutant ATPases was similar to that of the wild-type Na^+,K^+-ATPase (data not shown).

The ATPase activity present in membranes of Sf9 cells expressing recombinant Na^+,K^+-ATPase originates from both recombinant and endogenous Na^+,K^+-ATPase. To measure specifically recombinant Na^+,K^+-ATPase activity, the ob-

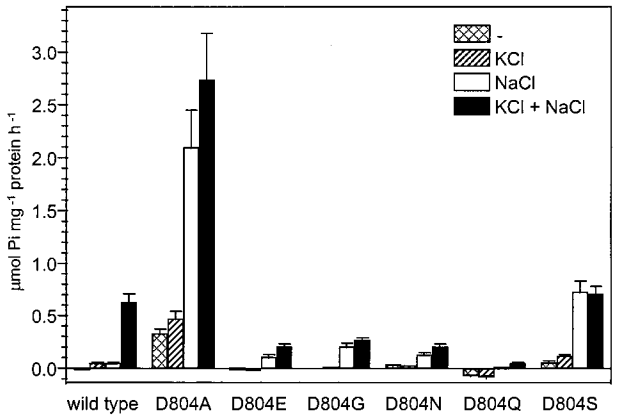

FIGURE 2: Cation dependency of the ATPase activity of the mutant and wild-type Na^+,K^+-ATPases. The assay was performed at 37 °C in the presence of 50 mM Tris−acetic acid (pH 7.0), 1.3 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM EDTA, 2 mM Tris-N_3, and 100 μM ATP. Depending on the condition described in the figure, 10 mM KCl and/or 100 mM NaCl was included in the incubation medium. In parallel experiments, the ATPase activity of membranes of mock-infected Sf9 cells was measured, and the results obtained with the mutants and the wild-type enzyme were corrected for those of mock-infected cells. The values presented are the mean ± SE of three enzyme preparations.

tained values were corrected for those of mock-infected cells (*20*). ATPase activity was measured in the presence of 100 μM ATP and the absence and presence of 10 mM K^+ and/or 100 mM Na^+ at 37 °C and pH 7.0 (Figure 2). Under these conditions the wild-type Na^+,K^+-ATPase possessed an ATPase activity of 0.63 ± 0.08 μmol of P_i (mg of protein)$^{-1}$ h^{-1} (n = 4) in the presence of both K^+ and Na^+. When one of these two cations was omitted the ATPase activity was virtually absent. The D804A mutant showed already a significant ATPase activity in the absence of added Na^+ and K^+ [0.33 ± 0.04 μmol of P_i (mg of protein)$^{-1}$ h^{-1}, n = 4]. The activity only slightly increased upon addition of K^+. However, in contrast to the wild type, a marked stimulation was observed after addition of Na^+ alone [2.10 ± 0.35 μmol of P_i (mg of protein)$^{-1}$ h^{-1}, n = 4]. K^+ further increased this activity up to 2.74 ± 0.44 μmol of P_i (mg of protein)$^{-1}$ h^{-1} (n = 4). The mutants D804E, D804G, and D804N showed no measurable ATPase activity in the absence of cations or in the presence of K^+ alone. However, similarly to the D804A mutant, these mutants possessed already a significant ATPase activity of 0.1−0.2 μmol of P_i (mg of protein)$^{-1}$ h^{-1} in the presence of Na^+ alone. The ATPase activities of these mutants slightly increased when K^+ was also present. The D804S mutant possessed a small ATPase activity in the absence of Na^+. This activity was markedly increased by the addition of Na^+ to 0.73 ± 0.10 μmol of P_i (mg of protein)$^{-1}$ h^{-1} (n = 3) and could not be further enhanced by K^+. Finally, the D804Q mutant did not show any ATPase activity in the absence or presence of either Na^+ or K^+ or the combination of these cations.

The ATPase activity of the D804A mutant in the presence of Na^+ and K^+ was about 4 times that of the wild-type enzyme. Since 100 μM ATP was suboptimal for the wild-type Na^+,K^+-ATPase, we measured the ATP dependence for both the wild type and the D804A mutant with and without K^+ and/or Na^+ (Figure 3). The ATPase activity of the wild-type enzyme in the presence of both Na^+ and K^+ strongly increased upon increasing of the ATP concentration (Figure 3A). In the absence of one or both of these ions the ATPase activity was undetectable up to 300 μM ATP. The ATPase activity of the D804A mutant in the presence of Na^+ or the combination of Na^+ and K^+ was already nearly maximal at 10 μM ATP (Figure 3B). Apparently, this mutant has a very high ATP affinity in the ATPase reaction in the presence of Na^+. In the absence of Na^+, an increase in the ATP concentration up to 1 mM stimulated the relatively low ATPase activity by a factor of 3−4. The ATPase activity of the other mutants was also measured at 10 mM K^+, 100 mM Na^+, and 1 mM ATP, but there was hardly any stimulation of the activity compared to that at 100 μM ATP as presented in Figure 2 (data not shown).

The cation dependence of both the wild-type enzyme and the D804A mutant was further investigated with 1 mM Mg-ATP. In the absence of cations, the ATPase activity of the wild type enzyme was only ~ 0.2 μmol of P_i (mg of protein)$^{-1}$ h^{-1} and addition of either Na^+ (Figure 4A) or K^+ (Figure 4B) did not significantly affect this activity. The

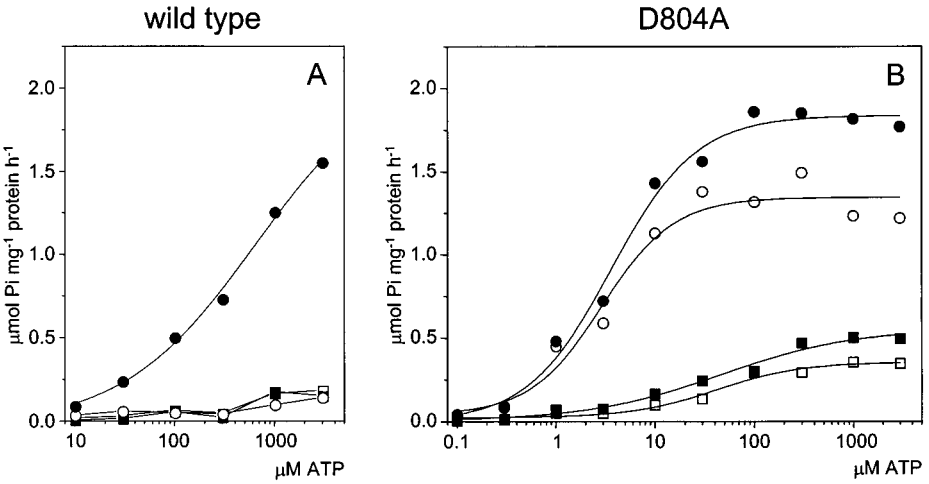

FIGURE 3: ATP dependence of the ATPase activity of the wild-type Na^+,K^+-ATPase (A) and the D804A mutant (B). The assay was performed at 37 °C in the presence of 50 mM Tris−acetic acid (pH 7.0), 1.3 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM EDTA, 2 mM Tris-N_3, and Mg-ATP concentrations between 0.1 and 3000 μM. The following symbols were used: (□) no monovalent cations added; (■) 10 mM KCl added; (○) 100 mM NaCl added; (●) 10 mM KCl and 100 mM NaCl added. The ATPase activity is presented as the measured activity minus that of mock-infected cells.

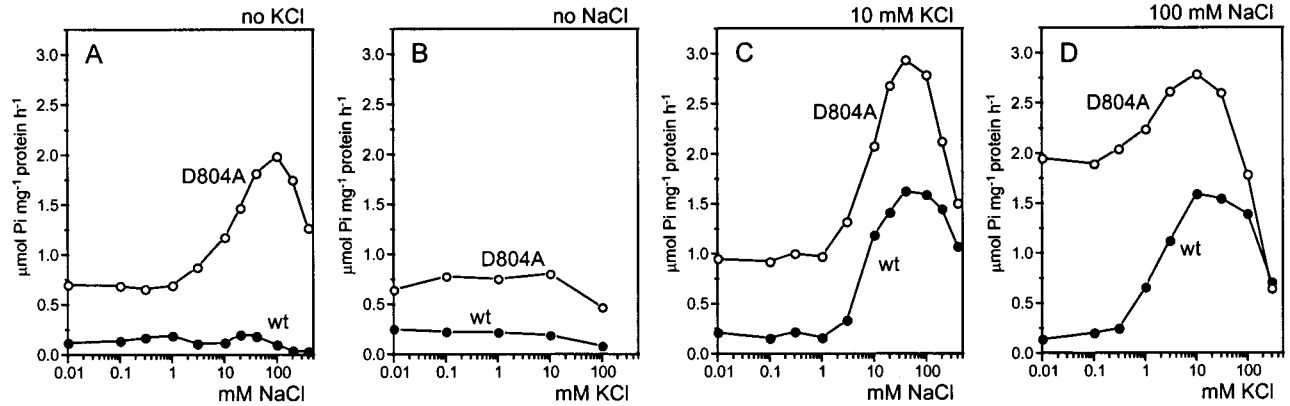

FIGURE 4: Na^+ and K^+ dependence of the ATPase activity of the wild-type Na^+,K^+-ATPase and the D804A mutant. The assay was performed at 37 °C in the presence of 50 mM Tris−acetic acid (pH 7.0), 1.3 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mM EDTA, 2 mM Tris-N_3, and 1 mM Mg-ATP. In panel A, no KCl was present and different concentrations of NaCl (0.01−300 mM) were added. In panel B, no NaCl was present and different concentrations of KCl (0.01−300 mM) were added. In panel C, 10 mM KCl was present and different concentrations of NaCl (0.01−300 mM) were added. In panel D, 100 mM NaCl was present and different concentrations of KCl (0.01−300 mM) were added. The results with the wild-type enzyme are indicated with solid circles (●) and those with the D804A mutant with open circles (○). The ATPase activity is presented as the measured activity minus that of mock-infected cells.

D804A mutant had an activity of ~ 0.7 μmol of P_i (mg of protein)$^{-1}$ h^{-1} that was not affected by K^+ but was markedly increased by Na^+ at concentrations of 3 mM and higher, reaching a maximal value of 2.0 μmol of P_i (mg of protein)$^{-1}$ h^{-1} at 100 mM Na^+. In the presence of 10 mM K^+, the ATPase activity of the wild-type enzyme was stimulated by Na^+, reaching a maximal activity of 1.6 μmol of P_i (mg of protein)$^{-1}$ h^{-1} at 40−100 mM Na^+ (Figure 4C). In both cases, high Na^+ concentrations inhibited the turnover of the enzyme. K^+ drastically stimulated the very low ATPase activity of the wild-type enzyme in the presence of 100 mM Na^+ (Figure 4D). At K^+ concentrations above 30 mM, the ATPase activity decreased again. The high ATPase activity of the D804A mutant in the presence of 100 mM Na^+ was increased by K^+ from 2.0 to 2.8 μmol of P_i (mg of protein)$^{-1}$ h^{-1}. At K^+ concentrations above 10 mM the ATPase activity of the D804A mutant progressively decreased, like that of the wild-type enzyme.

To investigate if the measured ATPase activity of the D804A mutant is inhibitable by ouabain, we determined the IC_{50} for this inhibitor. The IC_{50} value for the wild-type enzyme determined in the presence of 100 μM ATP, 100 mM Na^+, and 10 mM K^+ was 147 μM (data not shown). This relatively high value is in agreement with the low sensitivity of the rat α_1-subunit for ouabain (*21*). The ATPase activity of the D804A mutant in the presence of 100 mM Na^+ alone or the combination of 100 mM Na^+ and 10 mM K^+ could also be inhibited by ouabain (IC_{50} values of 25 and 31 μM, respectively). In the wild-type enzyme K^+ competes with the ouabain binding. This K^+−ouabain antagonism is probably less prominent in the D804A mutant.

In the presence of Na^+ and ATP Na^+,K^+-ATPase forms a phosphorylated intermediate, which accumulates in the absence of K^+ (Figure 1). We measured the phosphorylation capacity of the wild-type enzyme and the Asp804 mutants in the absence of K^+ and in the presence of 0.1 μM ATP and varying concentrations of Na^+ at 21 °C and pH 6.0 (Figure 5). The ATP concentration used (0.1 μM ATP) was about 8 times the $K_{0.5}$ of ATP (*13*). Phosphorylation of the wild-type Na^+,K^+-ATPase was nearly absent in the absence of added Na^+ and was stimulated by this cation up to a

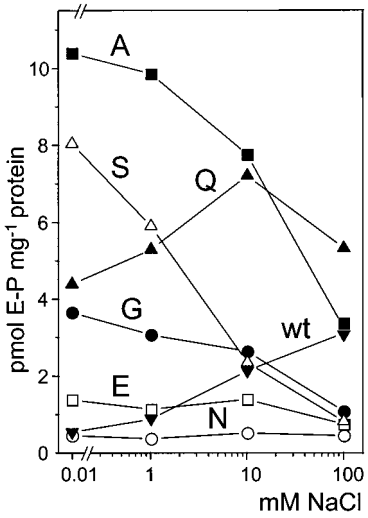

FIGURE 5: Steady-state phosphorylation level of mutants and wild-type Na^+,K^+-ATPases at different concentrations of Na^+. Membrane preparations of the mutant and wild-type Na^+,K^+-ATPase were preincubated for at least 10 min at 21 °C in the presence of 50 mM Tris−acetic acid (pH 6.0), 1.2 mM $MgCl_2$, 0.2 mM EDTA, and the indicated NaCl concentrations. After phosphorylation for 10 s at 21 °C with 0.1 μM [γ-^{32}P]ATP in the presence of 0.5 mg/mL $C_{12}E_8$, the phosphorylation level was determined and corrected for that of the mock-infected cells. Wild type, wt (▼); D804A, A (■); D804E, E (□); D804G, G (●); D804N, N (○); D804Q, Q (▲); D804S, S (△). Mean values of two enzyme preparations are given.

maximal level of 3.0 pmol of EP (mg of protein)$^{-1}$ at 100 mM Na^+. Surprisingly, the D804A mutant was already phosphorylated in the absence of added Na^+ to a level of 10.4 pmol of EP (mg of protein)$^{-1}$. With increasing Na^+ concentration the phosphorylation level of this mutant decreased to 3.2 pmol of EP (mg of protein)$^{-1}$ at 100 mM Na^+. The same pattern of Na^+ dependence was observed with the D804S and D804G mutants, although the obtained phosphorylation levels were much lower. The phosphorylation level of the D804E mutant in the absence of Na^+ was rather low [1.4 pmol of EP (mg of protein)$^{-1}$] and again it was decreased at 100 mM Na^+ [0.7 pmol of EP (mg of protein)$^{-1}$]. The D804N mutant did not form significant amounts of phosphorylated intermediate. The phosphoryla-

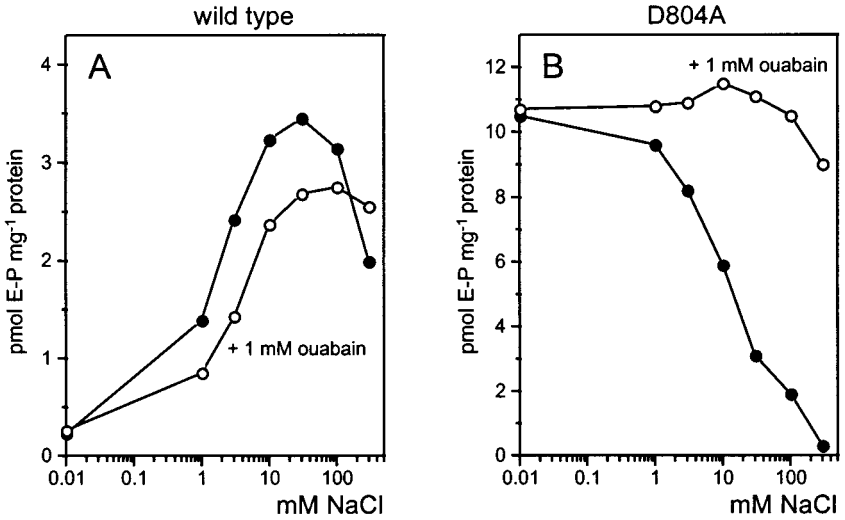

FIGURE 6: Effect of ouabain on the Na^+-dependent steady-state phosphorylation level of the wild-type enzyme and the D804A mutant. Membrane preparations of the mutant D804A and wild-type Na^+,K^+-ATPase were preincubated for at least 10 min at 21 °C in the presence of 50 mM Tris−acetic acid (pH 6.0), 1.2 mM $MgCl_2$, 0.2 mM EDTA, and the indicated NaCl concentrations with (○) and without (●) 1 mM ouabain. After phosphorylation for 10 s at 21 °C with 0.1 μM [γ-^{32}P]ATP in the presence of 0.5 mg/mL $C_{12}E_8$, the phosphorylation level was determined and corrected for that of the mock-infected cells.

tion level of the D804Q mutant was 4.4 pmol of EP (mg of protein)$^{-1}$ in the absence of Na^+. However, with this mutant the phosphorylation level did not decrease but slightly increased upon addition of Na^+.

In the presence of 1 mM ouabain the dose−response curve for the effect of Na^+ on the phosphorylation of the wild-type enzyme was slightly lowered and shifted to the right (Figure 6A). The phosphorylation process was only partially inhibited, because of the use of rat Na^+,K^+-ATPase α_1 subunit, which is rather insensitive toward ouabain (*21*). The D804A mutant gave already a high steady-state phosphorylation level in the absence of added Na^+, and the steady-state phosphorylation level was dose-dependently lowered by this cation. This Na^+-dependent decrease did not occur in the presence of 1 mM ouabain (Figure 6B). The most likely explanation for this effect is the formation of a relatively stable E_2−P·ouabain complex (see Figure 1). Whereas Na^+ can stimulate the hydrolysis of E_2−P (*22*), although with a much lower affinity than K^+, it is hardly able to dephosphorylate the E_2−P·ouabain complex.

The dephosphorylation of the labeled phosphoenzymes was studied at pH 6.0 and 21 °C, in the presence of 1 mM unlabeled ATP to prevent rephosphorylation with labeled ATP (*13*). Next to the spontaneous dephosphorylation, the effects of Na^+ (10 and 100 mM) and K^+ (1 and 10 mM) were investigated. In the absence of added cations the residual phosphorylation level of the wild-type enzyme decreased to 75% of the original level in 3 s (Figure 7). K^+ already maximally increased the dephosphorylation rate at 1 mM, resulting in a residual phosphorylation level of less than 5%. Na^+ slightly stimulated the dephosphorylation rate of the wild-type enzyme, but only at a concentration of 100 mM. Compared to the wild-type enzyme, the D804A mutant displayed a rather similar basal dephosphorylation rate, while K^+ was less potent and Na^+ was more potent. The D804E, D804G, and D804S mutants behaved rather similarly as the D804A mutant. The D804Q mutant, however, was hardly dephosphorylated under the different conditions. This explains why this mutant did not show any ATPase activity (Figure 2).

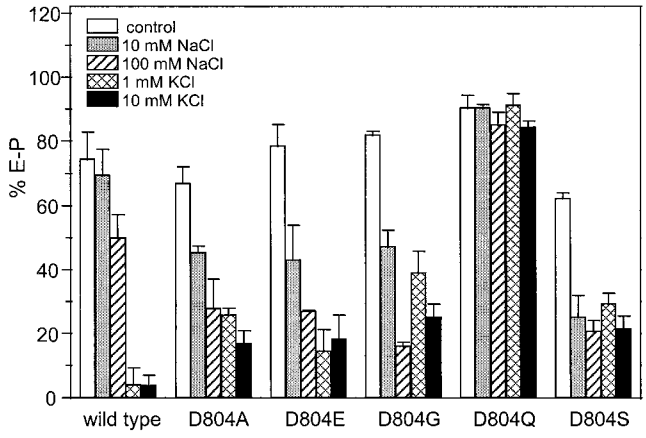

FIGURE 7: Dephosphorylation of the phosphorylated intermediate of the wild-type enzyme and the D804 mutants. The membrane preparations were preincubated for at least 10 min at 21 °C in the presence of 10 mM NaCl, 50 mM Tris−acetic acid (pH 6.0), 1.2 mM $MgCl_2$, and 0.2 mM EDTA. Next they were phosphorylated for 10 s with 0.1 μM [γ-^{32}P]ATP in the presence of 0.5 mg/mL $C_{12}E_8$. In the dephosphorylation reaction the initial NaCl concentration was diluted to 1 mM. Dephosphorylation was started by addition of excess (1 mM) nonradioactive ATP without added cations (open bars), or with 10 mM NaCl (gray bars), 100 mM NaCl (hatched bars), 1 mM KCl (crosshatched bars), or 10 mM KCl (solid bars). Samples were taken immediately before dephosphorylation and 3 s after the start of the dephosphorylation step. The results obtained were corrected for those of mock-infected cells. The following phosphorylation levels were obtained after 10 s of phosphorylation in the presence of 10 mM NaCl: Wild type, 2.9 pmol (mg of protein)$^{-1}$; D804A, 7.9 pmol (mg of protein)$^{-1}$; D804E, 1.3 pmol (mg of protein)$^{-1}$; D804G, 3.0 pmol (mg of protein)$^{-1}$; D804Q, 5.6 pmol (mg of protein)$^{-1}$; D804S, 2.8 pmol (mg of protein)$^{-1}$. The residual phosphorylation level was expressed as the percentage of the phosphorylation level before the start of the dephosphorylation step and is given as mean ± SE of two to three enzyme preparations. The addition of NaCl or KCl significantly ($p < 0.05$) lowered the spontaneous dephosphorylation level of all preparations, except for the wild type with NaCl and the D804Q with NaCl and KCl.

Since the D804A mutant showed a very high phosphorylation level in the absence of added Na^+, we investigated the phosphorylation characteristics of this mutant in more

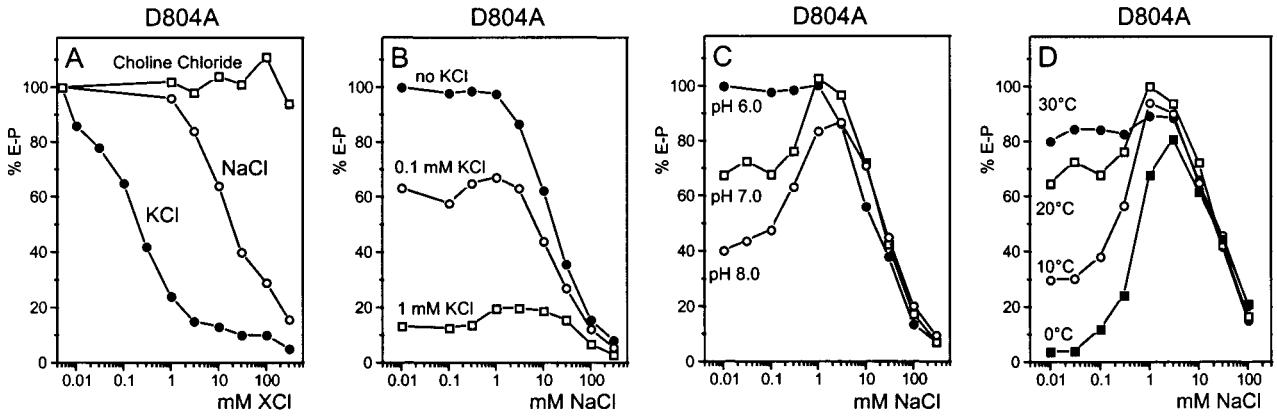

FIGURE 8: Phosphorylation capacity of the D804A mutant with different concentrations of K^+ and Na^+ at various temperature and pH values. Membrane preparations of the mutant Na^+,K^+-ATPase were preincubated for at least 10 min at 21 °C, unless indicated otherwise, in the presence of 50 mM Tris−acetic acid (pH 6.0, unless indicated otherwise), 1.2 mM $MgCl_2$, 0.2 mM EDTA, and the indicated choline chloride, KCl, and NaCl concentrations. After phosphorylation for 10 s at 21 °C (unless indicated otherwise) with 0.1 μM ATP, the phosphorylation level was determined and corrected for that of mock-infected cells (panel A) or for that in the presence of 10 mM KCl (panels B−D). Panel A demonstrates the effect of NaCl (○), KCl (●), and choline chloride (□) on the phosphorylation level of the D804A mutant at pH 6.0 and 21 °C. Panel B demonstrates the effect of NaCl on the phosphorylation level of D804A in the presence of 0 mM KCl (●), 0.1 mM KCl (○), and 1 mM KCl (□) at pH 6.0 and 21 °C. Panel C demonstrates the effect of NaCl on the phosphorylation level at pH values of 6.0 (●), 7.0 (□), and 8.0 (○) at 21 °C. Panel D demonstrates the effect of NaCl on the phosphorylation level at pH 7.0 and 30 °C (●), 20 °C (□), 10 °C (○), and 0 °C (■).

detail. Figure 8A shows the effect of monovalent cations on the steady-state phosphorylation level of the D804A mutant. Choline$^+$ [$HOCH_2CH_2N^+(CH_3)_3$] had no effect, while K^+ decreased this level at low concentrations ($IC_{50} = 0.2$ mM), probably both by prevention of the phosphorylation reaction and by stimulation of the dephosphorylation rate. Na^+ decreased the steady-state phosphorylation level, but at much higher concentrations than K^+ ($IC_{50} = 20$ mM). Since Na^+ increases the phosphorylation level of the wild-type enzyme, the decrease of the phosphorylation level of the D804A mutant by Na^+ is likely due to stimulation of the dephosphorylation rate (*23*), as is also shown in Figure 7. The decrease of the steady-state phosphorylation level by K^+ could not be antagonized by Na^+ (Figure 8B). Low concentrations of Na^+ did not increase the phosphorylation level, while high Na^+ concentrations, like K^+, decreased the phosphorylation level.

All phosphorylation experiments were performed at pH 6.0 and 21 °C. Upon increasing the pH to 7.0 and 8.0, the steady-state phosphorylation level in the absence of Na^+ decreased (Figure 8C). Under these conditions, however, 1 mM Na^+ could increase the phosphorylation level to about the same level as obtained at pH 6.0. A similar effect was observed when the incubation temperature was decreased (Figure 8D). At 0 °C and pH 7.0 apparently no phosphorylated intermediate was formed in the absence of Na^+. Only in the presence of Na^+ did the phosphorylation level increase, reaching a maximal level at 3 mM Na^+, which was virtually similar to the phosphorylation level at 30 °C in the absence of added Na^+. Eventually, at high Na^+ concentrations the phosphorylation level decreased again. At 10 °C and 20 °C the results were intermediate between those at 0 °C and 30 °C. Similar temperature dependence was seen at pH 6.0 and 8.0 (data not shown).

DISCUSSION

The purpose of the present study was to investigate whether site-directed mutagenesis of Asp804 in the α-subunit of Na^+,K^+-ATPase could yield mutants with a constitutive ATPase activity. Similar mutations in gastric H^+,K^+-ATPase on the corresponding residue Glu820 have given such results (*3*). Constitutive ATPase activity for H^+,K^+-ATPase was defined as the ATPase activity without the addition of monovalent cations. Because H^+ is always present in the reaction mixture, constitutive activity for H^+,K^+-ATPase is comparable with Na^+-ATPase activity for Na^+,K^+-ATPase. Indeed all of the Asp804 mutants (except D804Q) showed a high Na^+-ATPase activity, thereby confirming that the empty K^+-binding pocket, in which Asp804 plays a crucial role, inhibits the dephosphorylation process (inhibited dephosphorylation hypothesis).

The Sf9 insect cells used in the present study possess a minor amount of endogenous Na^+,K^+-ATPase [specific activity ~ 0.1 μmol of P_i (mg of protein)$^{-1}$ h^{-1}). The viral infection of the insect cells leads to a blockage of synthesis of endogenous proteins and thus of Na^+,K^+-ATPase 12 h postinfection (*24*), whereas the production of recombinant Na^+,K^+-ATPase starts very late (after 12 h) in the viral infection cycle. If oligomerization between two α-subunits occurs, it is unlikely that it would occur between α-subunits, which are synthesized at different time points. We expressed the D804A and D804S α-subunits without β-subunit and did not find any ATPase activity above that of the mock experiment (data not shown). Furthermore, Martin et al. (*25*) demonstrated that in microsomes of duct salt glands Na^+,K^+-ATPase is present as a monomer and that oligomer formation has no significant effect on the enzyme reaction mechanism. Therefore, we conclude that, in the baculovirus expression system, interaction between endogenous and recombinant Na^+,K^+-ATPase is not likely to occur.

The present study yielded interesting and in part surprising results. Whereas for maximal phosphorylation of the wild-type Na^+,K^+-ATPase 100 mM Na^+ was required, most of the produced mutants (D804A, D804E, D804S, D804G, and D804Q) phosphorylated in the absence of added Na^+. Addition of this cation even resulted in a lowering of the

phosphorylation level for most of these mutants. This reduction of the phosphorylation level can be explained by a K^+-like effect of Na^+, since similar Na^+ concentrations stimulate the dephosphorylation of the phosphorylated intermediate (this study and ref *22*).

The high phosphorylation level of the D804A mutant in the absence of added Na^+ can in principle be explained in three ways. First, H^+ may substitute for Na^+, since at low pH values the phosphorylation of the mutant enzyme is stimulated. On the other hand the mutated binding pocket may have such a high affinity for Na^+ that it could not be measured under the assay conditions (the incubation medium contained 5−10 μM Na^+). By increasing the pH or decreasing the temperature, the activation by Na^+ became apparent. A final possibility is that the mutated Na^+ binding pocket is in a constitutively active conformation, which can be influenced by a change in pH or temperature. The presented data do not favor one of these possibilities.

The basal ATPase activity (without added K^+ and Na^+) of the D804A mutant is probably initiated by the spontaneous phosphorylation, assuming that the rates of the spontaneous dephosphorylation and the $E_2 \rightarrow E_1$ conversion are sufficiently high. This constitutive activity is enhanced by increasing the ATP concentration, suggesting that the $E_2 \rightarrow E_1$ conversion is via the E_2·ATP intermediate (Figure 1, steps 1c and 1d). The basal ATPase activity of the D804A mutant was hardly increased by K^+, indicating that the dephosphorylation step was not rate-limiting under these conditions.

The ATPase reaction rate of this mutant, however, was stimulated by the addition of Na^+ at all ATP concentrations. This stimulatory effect of Na^+ has no direct relation to the phosphorylation reaction, since under these conditions submicromolar amounts of Na^+ were sufficient for phosphorylation of the D804A mutant. The stimulatory effect of Na^+ can also not be explained by a K^+-like effect of Na^+ on the dephosphorylation reaction alone, since with K^+ alone the ATPase activity was not stimulated. It is most likely that the main effect of Na^+ is a combination of two processes. First Na^+, like K^+, stimulates the dephosphorylation; second K^+, unlike Na^+, inhibits the $E_2 \rightarrow E_1$·ATP conversion through E_1 (steps 1a and 1b). This also explains why the rate of the ATPase reaction is already maximal at 100 μM ATP. Addition of K^+ in the presence of 100 mM Na^+ increases the ATPase activity independently of the ATP concentration used. This suggests that the dephosphorylation reaction is further stimulated, which under these conditions (high $E_2 \rightarrow E_1$ rate) contributes to the overall reaction rate.

ATP concentrations between 10 μM and 3 mM did not increase the ATPase activity of the D804A mutant with either Na^+ alone or with the combination of Na^+ and K^+. This suggests that in the presence of these ions for this mutant the pathway via E_2·ATP (Figure 1, steps 1c and 1d), even at high ATP concentrations, is much slower than via E_1 (steps 1a and 1b). Due to a change in the Na^+ binding pocket, step 1a is preferred over step 1c, leading to an enzyme in which the low-affinity ATP binding site is apparently absent. In the wild-type enzyme the situation is opposite: the rate of the route through E_1 is very slow and ATP can enhance the reaction rate by speeding up the reaction rate through E_2·ATP.

Although we only investigated the D804A mutant in detail, the qualitative behavior of most other mutants in the ATPase reaction is similar to that of D804A. All these mutants have no or very low (D804S) ATPase activity in the absence of added Na^+. Most of them have high Na^+-ATPase activity that cannot or can only slightly be stimulated by additional K^+ or ATP. Quantitatively there is a large difference in the sense that the ATPase activity of all other mutants is much lower than that of the D804A mutant. The D804Q mutant does not show significant ATPase activity at any condition but could be phosphorylated by ATP. The phosphorylation level even increased in the presence of 10 mM Na^+. The phosphorylated intermediate of this mutant is very stable and neither K^+ nor Na^+ can stimulate the dephosphorylation process. This suggests that the mutation has modified the K^+ binding site such that cation binding can no longer occur.

Some of the mutants used in this study have been investigated before in other systems. The Na^+,K^+-ATPase D804A, D804E, and D804N mutants transfected in ouabain-sensitive HeLa cells were not able to confer ouabain resistance to these cells, suggesting that they were not active. When the mutants were expressed in a mouse cell line, where ouabain binding was utilized to probe the exogenous protein, K^+ was no longer able to compete with ouabain binding (*6*). In the present study we also demonstrate that the K^+-ouabain antagonism is less. Pedersen et al. (*7*) found similar effects of K^+ on ouabain binding for the D804N and D804E mutants expressed in yeast (*7*). High-affinity ATP and ouabain binding, as well as E_1-E_2 transitions, were preserved when Asp804 was mutated (*7*). Studies of K^+ ion-dependent displacement of ATP or ouabain are consistent with the postulate that Asp804-mutated enzyme does not occlude K^+ in the E2(2K) conformation and also suggest that Asp804 may contribute to coordination of Na^+ as well as K^+ (*7*). The D804N and D804E mutants were also not able to show high-affinity occlusion of Tl^+ (*8*). In contrast to what was found in the present study, the D804N mutant showed Na^+-stimulated ATP phosphorylation to a level of 70% that of the wild type, although the Na^+ affinity was reduced 26-fold (*7*). Probably due to this low Na^+ affinity, we were not able to measure any phosphorylated intermediate.

Our results indicate that replacement of Asp804 by an alanine residue changes the Na^+ binding pocket considerably. Without knowledge of the three-dimensional structure of the Na^+ binding pocket, it is difficult to understand how this might occur. Obviously, Na^+ can substitute for K^+ in the dephosphorylation reaction. In the wild-type enzyme K^+ is released from the binding pocket, after which Na^+ is bound and the conformation of the enzyme is shifted to the E_1 conformation. In the D804A mutant Na^+ might already be present in the cation-binding pocket after the dephosphorylation step. Therefore the enzyme can shift quickly to the E_1 conformation. This hypothesis is supported by the fact that all the other mutants (D804E, D804G, and D804S) that have high Na^+-ATPase activity also have a high Na^+-stimulated dephosphorylation reaction. A similar effect has been described for the E779A mutant, which is probably also part of the cation-binding pocket (*26*). Similarly to what we have observed for Na^+,K^+-ATPase, most of the H^+,K^+-ATPase Glu820 mutants possess high ATPase activity in the absence of K^+ (*3*), indicating that H^+ may have taken over the role of K^+. The findings reported here suggest a direct role of the Na^+,K^+-ATPase aspartate residue 804 and the H^+,K^+-ATPase glutamate residue 820 in the selectivity for

cations. When the Na^+,K^+-ATPase 804 residue is mutated, the dephosphorylation reaction becomes relatively less K^+-sensitive and more Na^+-sensitive as compared to the wild-type enzyme, resulting in a high preference for the E_1 conformation.

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