Mutant Glu781→Ala of the Rat Kidney Na^+,K^+-ATPase Displays Low Cation Affinity and Catalyzes ATP Hydrolysis at a High Rate in the Absence of Potassium Ions†

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ABSTRACT: Site-specific mutagenesis was used to replace Glu329, Glu781, Asp806, Thr809, and Asp810 in the transmembrane domain of the ouabain-insensitive α_1-isoform of rat kidney Na^+,K^+-ATPase. cDNAs encoding any of the mutants Glu329→Ala, Glu781→Ala, Asp806→Asn, Thr809→Ala, and Asp810→Asn were transfected into COS cells, and transfectants were grown in the presence of ouabain to inhibit the endogenous COS cell Na^+,K^+-ATPase. Mutants Glu781→Ala and Thr809→Ala were functional as evidenced by their ability to confer ouabain resistance to the cells, whereas mutants Glu329→Ala, Asp806→Asn, and Asp810→Asn were inactive. The apparent Na^+ affinities determined by titrations of Na^+,K^+-ATPase activity, Na^+-ATPase activity, and phosphorylation from ATP in mutants Glu781→Ala and Thr809→Ala were strongly reduced relative to the affinity of the wild type (6−8-fold increase in $K_{0.5}$ for Na^+ in the phosphorylation assay for both mutants). The Glu781→Ala mutant displayed a 3−4-fold reduction in the apparent affinity for K^+ and was able to hydrolyze ATP at a high rate in the absence of K^+ (V_{max} for Na^+-ATPase activity 5-fold higher than that of the wild-type enzyme). The steady-state phosphoenzyme level formed by the Glu781→Ala mutant was increased 3-fold by addition of oligomycin, whereas only a slight effect of oligomycin was observed for mutant Thr809→Ala and the wild type. Using the steady-state phosphoenzyme level determined in the presence of oligomycin as a measure of the concentration of active enzyme sites, a maximum molecular turnover number for the Na^+,K^+-ATPase reaction was calculated to be slightly lower for mutant Glu781→Ala than the maximum turnover numbers of the wild type and mutant Thr809→Ala.

The Na^+,K^+-ATPase[1] is a membrane-spanning protein which actively transports three Na^+ out of eukaryotic cells in exchange for two K^+ at the expense of one molecule of ATP being hydrolyzed (Glynn, 1985). This enzyme together with the H^+,K^+-ATPase of gastric mucosa and the Ca^{2+}-ATPases of sarco(endo)plasmic reticulum and plasma membranes are members of a family of cation pumps termed P-type ATPases because they form a phosphorylated intermediate in the course of the reaction cycle (Pedersen & Carafoli, 1987). The catalytic subunits of the P-type ATPases consist of 2 major cytoplasmic domains that are attached to the membrane through 8 or 10 transmembrane segments, M1−M10, with a proposed α-helical structure (Lingrel et al., 1990; Green, 1989). Little is known about the structure of the cation binding sites of the Na^+,K^+-ATPase, but the amino acid residues making up the cavity for cation binding seem to be restricted to the transmembrane segments of the enzyme (Shani-Sekler et al., 1988; Karlish et al., 1990; Argüello & Kaplan, 1994). A plausible hypothesis is that amino acids with oxygen-containing side chains within or near the membrane are involved in coordination of the cations at the high-affinity Na^+- and/or K^+-transport sites (Eisenman & Dani, 1987; Toney et al., 1993; Larsen et al., 1994). Recently, this idea was supported by chemical modification studies with carbodiimides in which the glutamic acid residues in putative transmembrane segment M5 (Argüello & Kaplan, 1991, 1994) and in M9 (Goldshleger et al., 1992) were found to be labeled in a Na^+ and K^+ protectable way. The labeled enzymes were unable to occlude Na^+ and K^+. A problem is, however, whether the inactivation was due to a direct disruption of the cation binding sites or to an indirect effect of the bulky label on the access of the cations to the cation binding pocket or on transmembrane helix packing. The effect of bulkiness can be avoided by the use of site-directed mutagenesis. Results obtained in mutagenesis studies in which the glutamic acid residue in M4 was replaced point to a role for this residue in cation binding (Vilsen, 1993). Replacement of the carboxylic residues in M5 and M6 with leucine furthermore led to mutant Na^+,K^+ pumps that were incompatible with growth of HeLa cells, indicating that the amino acid substitutions were crucial to the normal function of the Na^+,K^+-ATPase (Jewell-Motz & Lingrel, 1993).

In this study, site-directed mutagenesis has been used to substitute alanines for either of the residues Glu781 in M5

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[1] Abbreviations: E1, conformation with high Na^+ and ATP affinities; E2, K^+-occluded conformation with low affinity for ATP; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; E1P, Na^+-occluded ADP-sensitive phosphoenzyme intermediate; E2P, K^+-sensitive and ADP-insensitive phosphoenzyme intermediate; $K_{0.5}$, concentration giving half-maximal activation; M1−M10, putative transmembrane helices numbered from the NH_2-terminal end of the peptide; Na^+,K^+-ATPase, (sodium plus potassium)-activated adenosinetriphosphatase (EC 3.6.1.37); TES, *N*-[tris(hydroxymethyl)methyl]2-aminoethanesulfonic acid; V_{max}, maximal activity.

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or Thr809 in M6 of the α_1-isoform of the ouabain-resistant rat kidney Na^+,K^+-ATPase. The cDNAs carrying the corresponding base substitutions were introduced into COS cells which contain an endogenous Na^+,K^+-ATPase sensitive to ouabain. Both of the mutant Na^+,K^+-pumps were functional as evidenced by their ability to confer ouabain resistance to the cells. However, the mutant pumps displayed strongly reduced affinities for Na^+. Moreover, the Glu781→Ala mutant displayed a 3−4-fold reduction in the apparent affinity for K^+ and was able to hydrolyze ATP at a high rate in the absence of K^+, suggesting a role of Glu781 in determination of the normal cation selectivity. In this study, also the residue Glu329 in M4 was replaced by alanine, and the residues Asp806 and Asp810 in M6 by asparagine, but these Na^+,K^+-ATPase mutants were unable to confer ouabain resistance to the cells.

MATERIALS AND METHODS

Construction of the Mutant cDNAs. Oligonucleotide-directed site-specific mutagenesis was carried out according to Kunkel (1985) as described previously (Vilsen et al., 1989). Mutations were introduced into appropriate restriction fragments, which had been excised from the entire cDNA encoding the rat α_1-isoform of Na^+,K^+-ATPase (Vilsen, 1992) and inserted into the Bluescript vector (Stratagene, La Jolla, CA). To verify that mutations were correct, nucleotide sequencing was performed using the dideoxynucleotide chain termination method (Sanger et al., 1977) with Sequenase as described (Vilsen et al., 1989). The mutated fragments were then transferred back into the original position of the full-length Na^+,K^+-ATPase clone contained in a Bluescript vector modified by removal of the restriction fragment *Pvu*II−*Pvu*II containing the multiple cloning region, to ensure easy shuttling of the full-length clone between different vectors.

Expression of the Mutant cDNAs in COS-1 Cells. The full-length cDNAs encoding wild type or the mutants of the rat α_1-isoform of Na^+,K^+-ATPase, carrying *Eco*RI/*Not*I adaptors, were cloned into the *Eco*RI site of the expression vector pMT2 (Kaufman et al., 1989). To obtain cell lines with the Na^+,K^+-ATPase cDNA stably integrated in the chromosomes, the supercoiled plasmid DNA purified on a cesium chloride gradient was transfected into COS-1 cells (Gluzman, 1981) by the calcium phosphate procedure (Vilsen, 1992). Following 65 h incubation, ouabain was added to the medium at a final concentration of 5 μM. After approximately 3 weeks, individual ouabain-resistant colonies appeared in the case of cells transfected with rat cDNA carrying amino acid substitutions at positions Glu781 and Thr809. At least two colonies from each of six different master dishes were isolated by use of cloning cylinders and expanded into stable cell lines.

Isolation of Plasma Membranes and Assay of Na^+,K^+-ATPase. A crude plasma membrane fraction was prepared and the vesicles opened with deoxycholate as previously described (Vilsen, 1992). Protein concentration was determined by the dye binding method of Bradford (1976) using bovine serum albumin as standard.

Na^+,K^+-ATPase and Na^+-ATPase activities were measured on the leaky membrane preparation at 37 °C by determination of inorganic phosphate production (Vilsen, 1993) or by a NADH-coupled assay in the presence of 0.15 mM NADH, 1 mM phosphoenolpyruvate, lactate dehydrogenase (10 IU/mL), and pyruvate kinase (10 IU/ml) (Vilsen et al., 1991). Generally the assay medium contained 30 mM TES or histidine buffer (pH 7.4), 1 mM EGTA, 0.01−3.0 mM ATP, and 0.1−4.0 mM $MgCl_2$ (keeping $[Mg^{2+}]$ constant in each series of experiments). In the standard Na^+,K^+-ATPase assay, the concentrations of ATP, $MgCl_2$, NaCl, and KCl were 3 mM, 3 mM, 130 mM, and 20 mM, respectively. In experiments in which concentrations of NaCl and KCl varied, choline chloride was added to keep the ionic strength constant. The rate of ATP hydrolysis was found to be constant over the incubation time (10−20 min) at all concentrations of ouabain, NaCl, KCl, and ATP values tested. To determine the Na^+,K^+-ATPase or Na^+-ATPase activity contributed by the transfected wild type or mutant rat kidney enzymes, assays were carried out in the presence of 10 μM ouabain, which inhibits endogenous COS-1 cell Na^+,K^+-ATPase, and in the presence of 1 or 10 mM ouabain, which inhibits all Na^+,K^+-ATPase activity. The ouabain-resistant ATPase activity associated with the expressed exogenous enzyme was calculated by subtraction of the background ATPase activity measured at 10 mM ouabain from the ATPase activity measured at 10 μM ouabain. The background ATPase activity resistant to 10 mM ouabain, usually comprising 10−30% of the total ATPase activity in the crude membrane preparation, was independent of the Na^+ and K^+ concentrations but depended on the ATP concentration. Therefore, in ATP concentration dependence studies, the Na^+,K^+-ATPase activity was obtained by subtraction of the background activity measured at each individual ATP concentration.

In all titrations of Na^+,K^+-ATPase and Na^+-ATPase activities, at least three separate membrane preparations corresponding to different clonal isolates of cells transfected with the same cDNA were assayed in duplicate. There were no significant differences between the $K_{0.5}$ values for Na^+, K^+, and ATP obtained with the different clonal isolates.

Procedures for fitting data to curves to obtain the $K_{0.5}$ values were as previously described (Vilsen, 1993).

Phosphoenzyme Formation. In the standard experiment, 25 μL of the leaky membrane suspension (corresponding to a total of 10 μg of protein) was phosphorylated for 10 s at 0 °C in 100 μL of a reaction mixture containing 2−4 μM [γ-^{32}P]ATP (i.e., saturating conditions), 20 mM Tris (pH 7.4), 3 mM $MgCl_2$, 1 mM EGTA, 10 μM ouabain, and either 150 mM NaCl or 50 mM KCl, the latter condition representing the background level of phosphoenzyme. Time dependence studies of phosphorylation from ATP showed that the maximum steady-state phosphorylation level was reached within 5 s. In experiments in which the effect of oligomycin was studied, the leaky membrane suspension was preincubated with 20 μg of oligomycin/mL (a mixture of oligomycin A, B, and C components, from Sigma, dissolved in ethanol on the day of the experiment) in the presence of 150 mM NaCl and 3 mM $MgCl_2$ for 10 min at 20 °C, and the phosphorylation was carried out at the same oligomycin concentration.

In all cases, the reaction was quenched by addition of 1 mL of ice-cold 7% (w/v) trichloroacetic acid containing 1 mM phosphate. The denatured protein was washed twice by centrifugation and subjected to SDS−polyacrylamide gel electrophoresis in 7% acrylamide gels under acid conditions (Vilsen et al., 1989). After the gel was dried, autoradio-

Table 1: Effect of K^+ Concentration in Growth Medium on Expression Level[a]

	sp Na^+,K^+-ATPase act. [nmol of ATP hydrolyzed min^{-1} (mg of protein)$^{-1}$]			
	270	900	1300	5000
wild-type Na^+,K^+-ATPase	543	260	280	132
mutant Glu781→Ala	nd[b]	523	nd	235
mutant Thr809→Ala	nd	510	nd	138

[a] The same clonal isolate of transfected cells was grown at various K^+ concentrations as indicated (in μM). The Na^+,K^+-ATPase measurements were carried out at 37 °C in the presence of 3 mM ATP, 3 mM $MgCl_2$, 30 mM histidine (pH 7.4), 1 mM EGTA, 10 μM ouabain, 130 mM NaCl, and 20 mM KCl. The assays were carried out in duplicate, and the average values are presented, as calculated after subtraction of background ATPase activity measured at 10 mM ouabain. [b] nd, not determined.

graphs were produced by exposure at −70 °C. Quantitation of the phosphoenzyme was obtained by liquid scintillation counting of slices of the dried gels.

RESULTS

In this study, the five residues with oxygen-containing side chains (Glu329 in putative transmembrane segment M4, Glu781 in M5, and Asp806, Thr809, and Asp810 in M6) were altered. The residues Asp806 and Asp810 were each replaced by asparagine, whereas alanine was substituted for each of the residues Glu329, Glu781, and Thr809. The mutants Glu781→Ala and Thr809→Ala were able to confer ouabain resistance to the COS cells. By contrast, no ouabain-resistant colonies were observed when COS cells were transfected with rat cDNA carrying the amino acid substitutions at positions Glu329, Asp806, and Asp810. The inability of mutants Asp806→Asn and Asp810→Asn to confer ouabain resistance to the cells is similar to the findings in a recent mutagenesis study in which no cell growth was observed when each of the homologous residues in the rat α_2-isoform of Na^+,K^+-ATPase was substituted with leucine (Jewell-Motz & Lingrel, 1993). It appears that the negative charge and/or the side chain oxygens of residues Asp806 and Asp810 are crucial for the overall Na^+,K^+-ATPase activity. On the other hand, the special properties of the side chains of amino acids Glu781 and Thr809 are not absolutely required for the function of the Na^+,K^+-ATPase. It has previously been demonstrated that mutation of Glu329 to either glutamine or leucine (Vilsen, 1993; Jewell-Motz & Lingrel, 1993) is compatible with cell growth. Therefore, the lack of cell growth following transfection with the Glu329→Ala mutant suggests a crucial role for the size of this side chain.

Because mutations Glu781→Ala and Thr809→Ala were compatible with cell growth, it was feasible to further characterize the functional properties of these mutants as described below.

Optimization of Expression Levels. To be able to carry out a detailed functional characterization of the mutants, it was essential to obtain the highest possible expression level. Hence, it was examined whether the transformed COS cells would respond to a reduction of the extracellular concentration of K^+ by expressing more of the exogenous ouabain-resistant rat Na^+,K^+-pumps (Lescale-Matys et al., 1990). As can be seen from Table 1, a reduction of the concentration of K^+ in the growth medium did indeed lead to up-regulation of the exogenous expressed wild-type Na^+,K^+-pump as indicated by the increase in specific Na^+,K^+-ATPase activity. Although the mutants Glu781→Ala and Thr809→Ala were found to display a lower apparent affinity for either of the cations (see below), COS cells expressing these mutants were found to be able to grow in the presence of a concentration of K^+ as low as 900 μM. As seen from Table 1, mutants Glu781→Ala and Thr809→Ala were up-regulated like the wild-type enzyme at the lower K^+ concentrations. The functional characteristics displayed by the up-regulated rat wild-type Na^+,K^+-ATPase and the mutants Glu781→Ala and Thr809→Ala were found to be identical to those of their matching wild-type and mutant Na^+,K^+-ATPases grown at normal K^+ concentrations. Because the DNA encoding the exogenous α-subunit of the rat Na^+,K^+-ATPase originally was carried on a plasmid, it seems unlikely that transcriptional regulation of the α-subunit is responsible for the increased expression levels of the wild-type and mutant rat Na^+,K^+-ATPases in cells grown in potassium-depleted medium. Since the β-subunit assembling with this α-subunit to form the mature pumps (Geering, 1991) was endogenous, coded by the gene of the COS cells, a likely mechanism would be transcriptional regulation of the β-subunit (Lescale-Matys et al., 1990). Alternatively, posttranscriptional regulation of either the α-subunit or the β-subunit may be involved (Pressley, 1988).

Ouabain Sensitivity. The expressed wild type and mutants Glu781→Ala and Thr809→Ala of the rat kidney Na^+,K^+-ATPase were characterized with respect to their ouabain sensitivity. First of all, it was found that each of the mutants exhibited a profile for ouabain inhibition of Na^+,K^+-ATPase activity as a function of ouabain concentration which was identical to that of the wild-type rat kidney enzyme, being approximately 500-fold less sensitive to ouabain than the endogenous Na^+,K^+-ATPase of untransfected COS cells (not shown). Thus, it can be concluded that replacement of the amino acids Glu781 and Thr809 with alanine had no effect on the affinity for ouabain.

Na^+ and K^+ Dependencies of Na^+,K^+-ATPase Activities. Figure 1 shows the results of experiments in which the Na^+,K^+-ATPase activity was measured at various NaCl concentrations on membranes isolated from COS cells expressing either the wild type or the mutants Glu781→Ala and Thr809→Ala of the ouabain-resistant rat Na^+,K^+-ATPase. The measurements were carried out in the presence of 10 μM ouabain to inhibit the endogenous Na^+,K^+-ATPase, because it was previously demonstrated that the endogenous Na^+,K^+-ATPase contributed less than 5% of the activity under these conditions (Vilsen, 1992). Both of the mutants Glu781→Ala and Thr809→Ala displayed a rather low apparent affinity for Na^+ corresponding to 4−5-fold reduction relative to the wild-type Na^+,K^+-ATPase ($K_{0.5}$ values of 35.6 and 29.3 mM, respectively, for the mutants Glu781→Ala and Thr809→Ala, compared with 7.1 mM for the wild-type Na^+,K^+-ATPase).

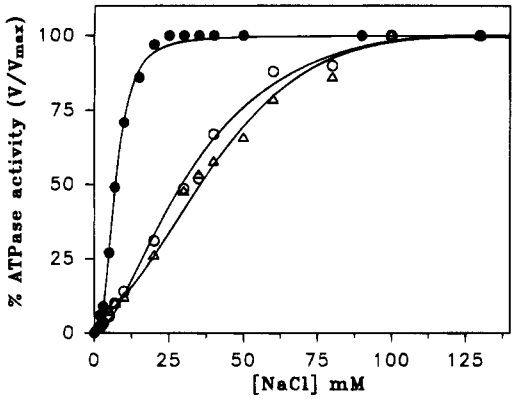

FIGURE 1: Na^+ dependence of Na^+,K^+-ATPase activity of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either the wild type or the mutants Glu781→Ala and Thr809→Ala. The Na^+,K^+-ATPase measurements were carried out at 37 °C, pH 7.4, in the presence of 20 mM KCl, 3 mM ATP, 3 mM $MgCl_2$, 1 mM EGTA, 10 μM ouabain, and various concentrations of NaCl and choline chloride (total concentration [KCl] + [NaCl] + [choline chloride] = 150 mM) as described under Materials and Methods. The average values resulting from duplicate measurements are presented as the percentage of the maximum Na^+,K^+-ATPase activity, as calculated after subtraction of background. The differences between the duplicate measurements were smaller than the size of the symbols. The respective values for the specific Na^+,K^+-ATPase activities corresponding to 100% and the $K_{0.5}(Na^+)$ values were the following: wild-type (●), 7.9 μmol h^{-1} mg^{-1}, 7.1 mM; Glu781→Ala mutant (△), 17.3 μmol h^{-1} mg^{-1}, 35.6 mM; Thr809→Ala mutant (○), 28.3 μmol h^{-1} mg^{-1}, 29.3 mM.

The KCl dependence properties were also analyzed for each of the mutants Glu781→Ala and Thr809→Ala and the wild-type Na^+,K^+-ATPase, and results of the KCl titrations of ATPase activity carried out in the presence of either 100 mM NaCl or 40 mM NaCl are shown in Figure 2A and Figure 2B, respectively. The following three features of the curves should be noted. First, in the presence of saturating K^+ concentrations, the activities of the mutants at 40 mM NaCl did not reach higher than 58−66% that of V_{max}. This is in accordance with the reduced affinity for Na^+ described above (Figure 1). Second, the ordinate intercept (the Na^+-ATPase activity measured in the absence of K^+) is much higher for the mutant Glu781→Ala compared to the wild type and mutant Thr809→Ala (13% vs 3% at 40 mM NaCl and 28% vs 5−6% at 100 mM NaCl), indicating an increased ability of the mutant to hydrolyze ATP in the presence of Na^+ without K^+. Third, the apparent affinity for K^+ was reduced as much as 3−4-fold in mutant Glu781→Ala relative to the wild-type enzyme (compare the $K_{0.5}$ value of 6.1 mM with 1.6 mM at 100 mM NaCl, and the $K_{0.5}$ value of 2.8 mM with 1.0 mM at 40 mM NaCl). In mutant Thr809→Ala, the apparent K^+ affinity was also reduced, but only 1.4-fold at 40 mM NaCl and 1.8-fold at 100 mM NaCl (compare the $K_{0.5}$ value of 2.9 mM with 1.6 mM at 100 mM NaCl, and the $K_{0.5}$ value of 1.4 mM with 1.0 mM at 40 mM NaCl).

Na^+-ATPase Activity. The ability of mutant Glu781→Ala to hydrolyze ATP at a high rate even in the absence of K^+ led to a more detailed investigation of the Na^+-ATPase activity. The Na^+-titrations are shown in Figure 3. In accordance with the literature, the low Na^+-ATPase activity exhibited by the wild type displayed a complex activation pattern reflecting activation of phosphorylation by Na^+ binding at intracellular high-affinity sites followed by a combination of inhibition and activation by Na^+ binding at

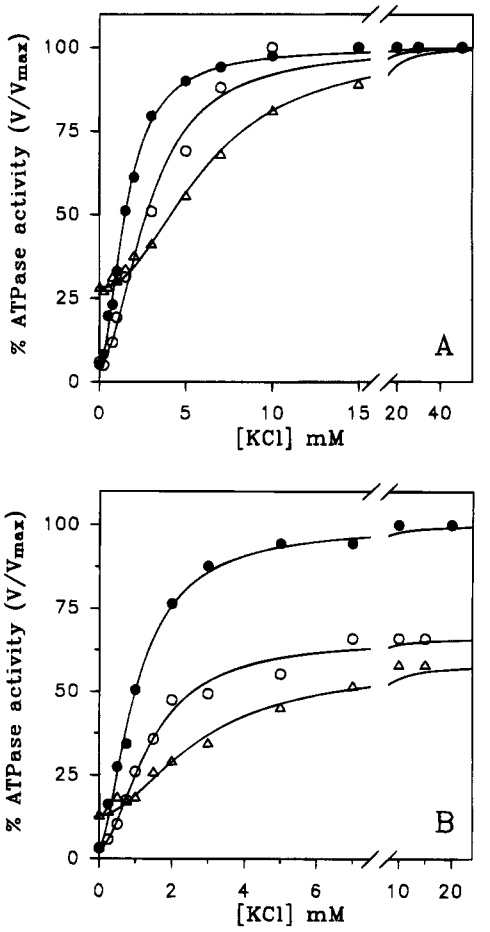

FIGURE 2: K^+ dependence of Na^+,K^+-ATPase activity of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either the wild type or the mutants Glu781→Ala and Thr809→Ala. The Na^+,K^+-ATPase measurements were carried out at 37 °C, pH 7.4, in the presence of 3 mM ATP, 3 mM $MgCl_2$, 1 mM EGTA, 10 μM ouabain, various concentrations of KCl and choline chloride (total concentration [KCl] + [NaCl] + [choline chloride] = 150 mM), and either (A) 100 mM NaCl or (B) 40 mM NaCl. The average values resulting from duplicate measurements are presented as the percentage of the maximum Na^+,K^+-ATPase activity, as calculated after subtraction of background. The differences between the duplicate measurements were smaller than the size of the symbols. The respective values for the specific Na^+,K^+-ATPase activities corresponding to 100% and the $K_{0.5}(K^+)$ values were the following: (A) wild type (●), 18.3 μmol h^{-1} mg^{-1}, 1.6 mM; Glu781→Ala mutant (△), 17.3 μmol h^{-1} mg^{-1}, 6.1 mM; Thr809→Ala mutant (○), 13.8 μmol h^{-1} mg^{-1}, 2.9 mM. (B) Wild type (●), 18.3 μmol h^{-1} mg^{-1}, 1.0 mM; Glu781→Ala mutant (△), 17.3 μmol h^{-1} mg^{-1}, 2.8 mM; Thr809→Ala mutant (○), 15.8 μmol h^{-1} mg^{-1}, 1.4 mM.

low-affinity extracellular sites. The latter may or may not encompass the sites involved in K^+ activation of dephosphorylation (Post et al., 1972; Glynn & Karlish, 1976; Glynn, 1985; Cornelius, 1991).

For mutants Thr809→Ala and Glu781→Ala, the high-affinity activation phase was displaced toward higher Na^+ concentrations. This is likely to reflect the reduced Na^+ affinity of the cytoplasmically facing activation sites also observed in the Na^+,K^+-ATPase assay (Figure 1). From Figure 3 it appears that the apparent affinity of either mutant for Na^+ at the cytoplasmically facing activation sites may have been reduced as much as 10-fold relative to the wild type, but the complex activation pattern precludes an accurate determination of $K_{0.5}$ values. As also seen in Figure 3,

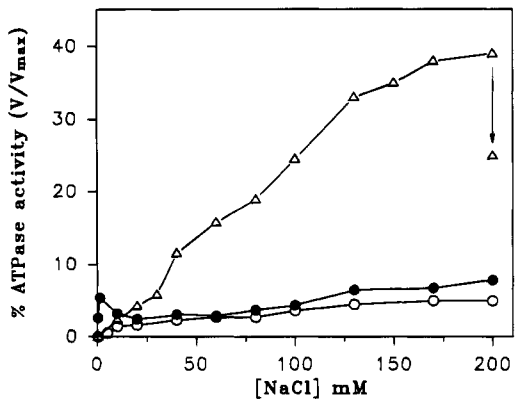

FIGURE 3: Na^+ dependence of Na^+-ATPase activity of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either the wild type or the mutants Glu781→Ala and Thr809→Ala. The Na^+-ATPase measurements were carried out at 37 °C, pH 7.4, in the absence of K^+ and presence of 3 mM ATP, 3 mM $MgCl_2$, 1 mM EGTA, 10 μM ouabain, and various concentrations of NaCl and choline chloride (total concentration [NaCl] + [choline chloride] = 200 mM). The arrow indicates the effect of preincubation with oligomycin. The average values resulting from duplicate measurements are presented as the percentage of the maximum Na^+,K^+-ATPase activity, as calculated after subtraction of background. The differences between the duplicate measurements were smaller than the size of the symbols. The respective values for the specific Na^+,K^+-ATPase activities corresponding to 100% were the following: wild type (●), 24.3 μmol h^{-1} mg^{-1}; Glu781→Ala mutant (△), 30.3 μmol h^{-1} mg^{-1}; Thr809→Ala mutant (○), 28.3 μmol h^{-1} mg^{-1}.

mutant Glu781→Ala displayed a V_{max} for Na^+-ATPase activity as high as 38% of the maximal Na^+,K^+-ATPase activity. This V_{max} was reached at a Na^+ concentration of 170−200 mM, which is comparable to the concentration range at which the wild-type enzyme reaches its maximal Na^+-ATPase activity due to saturation of the extracellularly facing activation sites (Post et al., 1972; Glynn & Karlish, 1976; Cornelius, 1991). The Na^+-ATPase activity of mutant Glu781→Ala measured at 200 mM Na^+ was found to be inhibited down to 25% of V_{max} by inclusion of oligomycin (20 μg/mL) in the reaction mixture (arrow in Figure 3). This is similar to the inhibitory effect of oligomycin on the wild type (Blostein, 1983). Oligomycin is known to promote occlusion of Na^+ in the E1 form of the enzyme, and to decrease the rate of release of Na^+ from the phosphoenzyme, thereby inhibiting the E1P→E2P interconversion (Glynn, 1985; Skou, 1990).

ATP Dependence. Figure 4A shows the ATP dependence of Na^+,K^+-ATPase activity measured in wild type and the mutants Glu781→Ala and Thr809→Ala, whereas Figure 4B shows the ATP dependence of the high Na^+-ATPase activity of mutant Glu781→Ala. In the Na^+,K^+-ATPase assay, either of the mutants displayed an increase in the apparent affinity for ATP, corresponding to 3−4-fold for the Glu781→Ala mutant and 2-fold for the Thr809→Ala mutant ($K_{0.5}$ values of 80 μM and 140 μM, respectively, for the mutants Glu781→Ala and Thr809→Ala, compared with 280 μM for the wild-type Na^+,K^+-ATPase). The $K_{0.5}$ value for ATP determined in the Na^+-ATPase assay for the mutant Glu781→Ala was even lower, corresponding to about 14 μM. This is comparable to the very high affinity for ATP corresponding to $K_{0.5}$ values below 10 μM reported in the literature for the wild-type enzyme in the absence of K^+ (Glynn, 1985). Unfortunately, it was not possible to measure

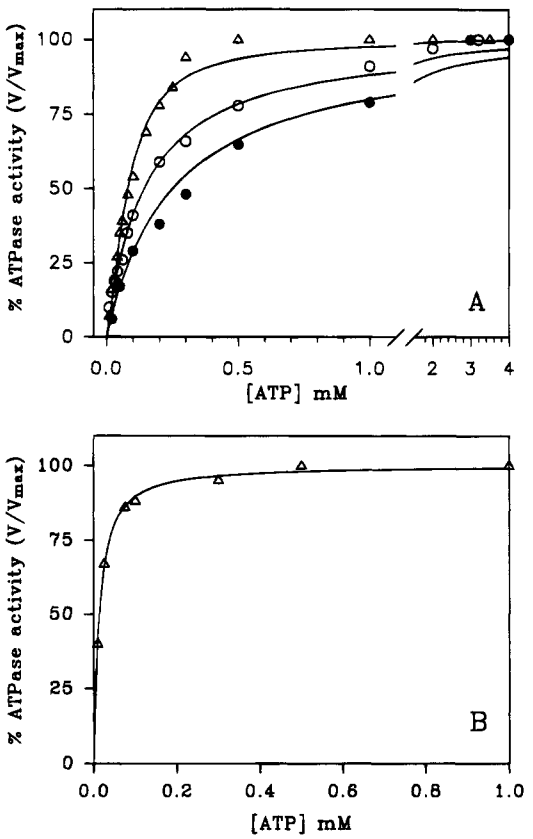

FIGURE 4: ATP dependence of Na^+,K^+-ATPase activity and Na^+-ATPase activity of plasma membranes isolated from COS-1 cells transfected with cDNA encoding either the wild type or the mutants Glu781→Ala and Thr809→Ala. (A) Na^+,K^+-ATPase measurements were carried out at 37 °C, pH 7.4, in the presence of 130 mM NaCl and 20 mM KCl. (B) Na^+-ATPase measurements were carried out at 37 °C, pH 7.4, in the presence of 200 mM NaCl and in the absence of K^+. $MgCl_2$ (0.1−4.0 mM, keeping $[Mg^{2+}]$ constant), 1 mM EGTA, 10 μM ouabain, and various concentrations of ATP were added in (A) as well as (B). The average values resulting from duplicate measurements are presented as the percentage of the maximum Na^+,K^+-ATPase activity (A) or the maximum Na^+-ATPase activity (B), as calculated after subtraction of background ATPase activity measured at the same ATP concentration. The differences between the duplicate measurements were smaller than the size of the symbols. The respective values for the specific ATPase activities corresponding to 100% and the $K_{0.5}$(ATP) values were the following: (A) Wild type (●), 7.6 μmol h^{-1} mg^{-1}, 0.28 mM; Glu781→Ala mutant (△), 30.3 μmol h^{-1} mg^{-1}, 0.08 mM; Thr809→Ala mutant (○), 28.3 μmol h^{-1} mg^{-1}, 0.14 mM. (B) Glu781→Ala mutant (△), 11.5 μmol h^{-1} mg^{-1}, 0.014 mM.

the $K_{0.5}$(ATP) value for the low Na^+-ATPase activity of the wild-type enzyme accurately in the present study.

Steady-State Phosphorylation and Turnover. To study the phosphoenzyme intermediate, the previously described procedures (Vilsen, 1993) were applied, involving SDS−polyacrylamide gel electrophoresis at acid pH to separate the Na^+,K^+-ATPase phosphoenzyme from the free [γ-^{32}P]-ATP and other phosphorylated proteins present in the crude plasma membrane preparation. In preliminary experiments, it was found that the maximal steady-state phosphorylation level obtained with mutant Glu781→Ala in the presence of Na^+ without K^+ was considerably lower than that of wild-type enzyme with a comparable Na^+,K^+-ATPase activity. Examination of a number of different phosphorylation conditions led to the finding that this difference disappeared when oligomycin was added. As seen in Figure 5 and Table 2, the inclusion of oligomycin in the phosphorylation assay

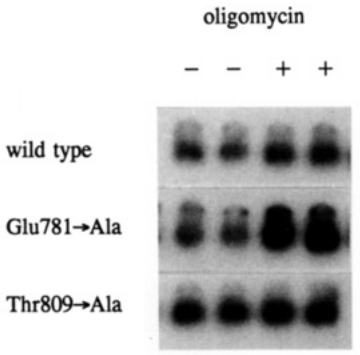

FIGURE 5: Phosphorylation from ATP of plasma membranes isolated from COS-1 cells transfected with the expression vector containing cDNA encoding either the wild type or the mutants Glu781→Ala and Thr809→Ala. Phosphorylation was carried out at 0 °C, pH 7.4, for 10 s in the presence of 3 mM $MgCl_2$, 1 mM EGTA, 4 μM [γ-^{32}P]ATP, 10 μM ouabain, and 150 mM NaCl without K^+. The acid-quenched samples were subjected to SDS–polyacrylamide gel electrophoresis at pH 6.0, and the autoradiograms of the dried gels are shown. Each lane was loaded with 7 μg of plasma membrane protein. Prior to phosphorylation the samples were preincubated for 10 min at 20 °C without (−) or with (+) oligomycin as described under Materials and Methods. Duplicate measurements in each condition are shown. The maximum specific Na^+,K^+-ATPase activities of the samples used for phosphorylation were the following: wild type, 8.0 μmol h^{-1} mg^{-1}; Glu781→Ala mutant, 30.3 μmol h^{-1} mg^{-1}; Thr809→Ala mutant, 13.8 μmol h^{-1} mg^{-1}.

led to a more than 3-fold increase in the steady-state level of phosphoenzyme in mutant Glu781→Ala. By contrast, the addition of oligomycin increased the steady-state phosphorylation levels of the wild-type enzyme and mutant Thr809→Ala by no more than 10−15%. Using the phosphorylation levels obtained in the presence of oligomycin as a measure of the concentration of active enzyme sites, a maximum molecular turnover number for the Na^+,K^+-ATPase reaction could be calculated for mutant Glu781→Ala. This was slightly lower than the maximum turnover numbers of the wild-type enzyme and mutant Thr809→Ala (see Table 2).

To obtain additional information about the affinity for Na^+, the phosphorylation from ATP was measured at various Na^+ concentrations in the absence of K^+. Figure 6 shows results of such phosphorylation experiments carried out in the presence of oligomycin. Each of the mutants exhibited a reduced affinity for Na^+ corresponding to approximately 8-fold for mutant Glu781→Ala and 6−7 fold for mutant Thr809→Ala ($K_{0.5}$ values of 6.5 and 5.0 mM, respectively, for the mutants Glu781→Ala and Thr809→Ala, compare with 0.8 mM for the wild-type Na^+,K^+-ATPase). Similar phosphorylation experiments were also carried out in the absence of oligomycin (not shown). The $K_{0.5}$ values for Na^+ determined for the wild-type Na^+,K^+-ATPase and mutant Thr809→Ala in the absence of oligomycin did not differ significantly from the respective values determined in the presence of oligomycin. Due to the lower steady-state phosphorylation level obtained with mutant Glu781→Ala in the absence of oligomycin, the $K_{0.5}$ value for Na^+ obtained in this condition was less reliable. It was nevertheless clear that half-maximum phosphorylation was attained in the range between 5 and 8 mM Na^+, i.e., rather close to the $K_{0.5}$ value determined in the presence of oligomycin.

DISCUSSION

To be able to understand the molecular mechanism of active cation transport by the Na^+,K^+-ATPase, it is essential to identify the amino acid residues that are responsible for the specific cation selectivity and coordination of the sodium and potassium ions in the cation binding/occlusion steps of the transport cycle. The ability of mutants Glu781→Ala and Thr809→Ala to confer ouabain resistance to COS cells demonstrated that these mutants were able to transport Na^+ and K^+ at rates sufficiently high to be compatible with normal cell function, and permitted the functional consequences of the mutations to be characterized.

First, it was found that each of the mutants Glu781→Ala and Thr809→Ala exhibited a reduced apparent affinity for Na^+. This was observed consistently in three different assays. In the Na^+,K^+-ATPase assay, a 4−5-fold increase in the $K_{0.5}$ value for Na^+ activation was observed for either mutant. The $K_{0.5}$ values for Na^+ activation of phosphorylation from ATP were even more increased, 6−8-fold relative to the wild-type enzyme. In the Na^+-ATPase assay, the first activation phase in the complex Na^+ activation pattern was similarly displaced toward higher Na^+ concentrations. The normal high-affinity activation by Na^+ of the phosphorylation

Table 2: Phosphorylation and Turnover Numbers of Different Clonal Isolates Expressing Wild Type of Exogenous Rat Na^+,K^+-ATPase or the Mutants Glu781→Ala and Thr809→Ala[a]

clonal isolate	sp Na^+,K^+-ATPase act. [nmol of ATP hydrolyzed min^{-1} (mg of crude membrane protein)$^{-1}$]	phosphorylation (pmol/mg of crude membrane protein)		turnover no. (min^{-1})
		+oligomycin	−oligomycin	
wild type				
1	277	11.4	9.9	24300
2	112	4.8	4.4	23300
3	535	23.7	21.2	22600
Glu781→Ala				
1	263	14.3	4.5	18400
2	107	6.3	2.0	17100
3	505	26.9	8.1	18800
Thr809→Ala				
1	203	7.5	6.8	27200
2	472	20.4	18.1	23150

[a] The Na^+,K^+-ATPase measurements were carried out at 37 °C in the presence of 3 mM ATP, 3 mM $MgCl_2$, 30 mM histidine (pH 7.4), 1 mM EGTA, 10 μM ouabain, 130 mM NaCl, and 20 mM KCl. The assays were carried out in duplicate, and the average values are presented, as calculated after subtraction of background ATPase activity measured at 10 mM ouabain. The phosphorylation was carried out at 150 mM Na^+, 4 μM ATP, and 10 μM ouabain, with and without oligomycin as described under Materials and Methods. The turnover number was calculated as the ratio between the specific Na^+,K^+-ATPase activity and the phosphorylation measured in the presence of oligomycin.

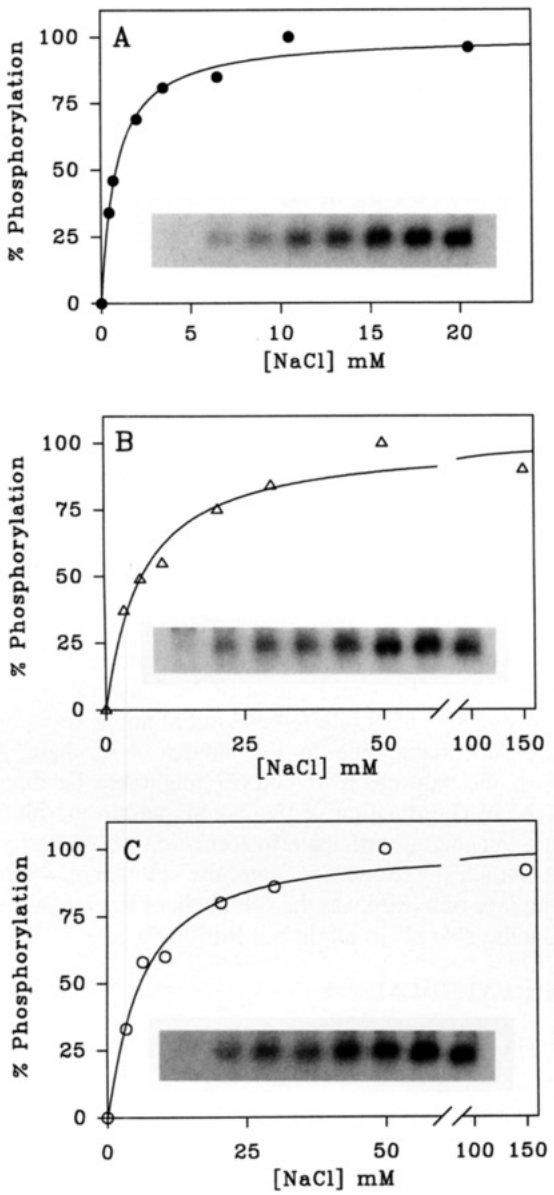

FIGURE 6: Na^+ dependence of phosphorylation from ATP of plasma membranes isolated from COS cells expressing either the wild type or the mutants Glu781→Ala and Thr809→Ala of the exogenous ouabain-resistant rat Na^+,K^+-ATPase. Phosphorylation was carried out as described in the legend to Figure 5, in the presence of oligomycin and various concentrations of Na^+ as indicated on the abscissa. The ionic strength was kept constant by addition of choline chloride. Quantitation of the phosphoenzyme was obtained by liquid scintillation counting of slices of the dried gels. The data are averages of duplicate measurements and are presented as the percentage of the maximum steady-state phosphorylation level. The insets show examples of autoradiographs of the dried SDS–polyacrylamide gels from which the quantitations were derived. Each lane was loaded with 7 μg of plasma membrane protein. The $K_{0.5}(Na^+)$ values were the following: wild-type (●), 0.8 mM; Glu781→Ala mutant (△), 6.5 mM; Thr809→Ala mutant (○), 5.0 mM.

from ATP and the Na^+,K^+- and Na^+-ATPase activities is associated with the binding of three Na^+ at cytoplasmically facing transport sites (Glynn, 1985; Skou, 1990; Cornelius, 1991). The present data, therefore, suggest that the binding of Na^+ at one or more of the cytoplasmically facing sites was disturbed in mutants Glu781→Ala and Thr809→Ala.

The mutant Glu781→Ala was furthermore found to exhibit a high Na^+-ATPase activity in the absence of K^+. This amounted to 38% of the maximal Na^+,K^+-ATPase activity, a V_{max} value for Na^+-ATPase activity at least 5-fold higher than observed for the wild-type enzyme. In the normal Na^+,K^+-ATPase reaction cycle, the binding of two K^+ to the E2P phosphoenzyme intermediate at sites facing the extracellular side of the membrane triggers dephosphorylation. The very modest Na^+-ATPase activity that can be observed with the wild-type enzyme in the presence of Na^+ on both sides of the membrane and in the absence of K^+ is thought to be associated with exchange of two extracellular Na^+ for three cytoplasmic Na^+, the two extracellular Na^+ substituting for two K^+ in activation of dephosphorylation (Glynn, 1985; Blostein, 1983; Cornelius, 1991). Thus, even in the normal enzyme, Na^+ has a slight K^+-like effect on the extracellular transport sites. With the nonsided enzyme preparation used here, it is not possible to distinguish with certainty the orientation of the sites involved in Na^+ activation of the enhanced Na^+-ATPase activity observed with mutant Glu781→Ala. Na^+ binding at the extracellularly facing sites may have contributed considerably, particularly at the highest Na^+ concentrations where the cytoplasmically facing sites must have been saturated as judged from the phosphorylation assay. There are various ways in which the enhanced Na^+-ATPase activity of mutant Glu781→Ala could be explained. One possible hypothesis is that the structure of a site binding extracellular Na^+, or the signal transmission from such a site to the catalytic domain, was altered in the mutant. Because the binding of extracellular Na^+ normally occurs at two or more distinct sites with inhibitory as well as activation potentials, respectively (Post et al., 1972; Glynn & Karlish, 1976; Glynn, 1985; Cornelius, 1991), the effect of the Glu781→Ala mutation could be either to disrupt the function of the inhibitory site(s) or to increase the efficiency of interaction of Na^+ with the activation site(s). The finding that in mutant Glu781→Ala the apparent affinity for K^+ detected in the Na^+,K^+-ATPase assay was reduced 3–4 fold would be in accordance with the notion that Glu781 is important for the ability to discriminate between extracellular Na^+ and K^+. This decrease in the apparent affinity for K^+ might arise from an increased efficacy of Na^+ as activator of dephosphorylation.

Alternatively, the Glu781→Ala mutation may have altered the catalytic mechanism more fundamentally, so that the mutant catalyzed an uncoupled Na^+-ATPase activity by a mechanism differing from the classic Post–Albers route through E1P and E2P (Glynn, 1985). This could possibly be a consequence of the defective binding of Na^+ at the cytoplasmically facing sites.

The steady-state phosphoenzyme level formed by the Glu781→Ala mutant was found to be low as compared to that of the wild-type enzyme, but the presence of oligomycin increased the phosphoenzyme level by at least a factor of 3. By contrast, only a slight effect of oligomycin was noticed with the wild type as well as with the mutant Thr809→Ala. The effect of oligomycin on the phosphorylation level is likely to be connected with the high Na^+-ATPase activity of mutant Glu781→Ala, which was reduced in the presence of oligomycin. Oligomycin promotes occlusion of Na^+ in the E1 form of Na^+,K^+-ATPase and stabilizes the Na^+-occluded E1P form of the phosphoenzyme, thereby reducing the rate of dephosphorylation (Glynn, 1985; Skou, 1990). This effect can explain the increase in phosphorylation level of the mutant. A question is whether in addition to an increased dephosphorylation rate also a reduced rate of

phosphorylation contributed to lower the steady-state phosphorylation level of mutant Glu781→Ala in the absence of oligomycin. This would be in accordance with the slight reduction of the maximum turnover rate of this mutant measured in the Na^+,K^+-ATPase assay (Table 2). Possibly, such an effect could arise from a defective occlusion of Na^+ in the E1 form (Skou, 1991). It should be noted, however, that the apparent Na^+ affinities measured in the phosphorylation assays with mutants Glu781→Ala and Thr809→Ala were close to being equally reduced. Thus, there was no clear evidence that occlusion of Na^+ was more defective in mutant Glu781→Ala as compared with mutant Thr809→Ala.

Both of the mutants Glu781→Ala and Thr809→Ala exhibited an increased apparent affinity for ATP in the Na^+,K^+-ATPase reaction, and this effect was most pronounced in mutant Glu781→Ala. Because the E2 form exhibits low affinity for ATP relative to the E1 form, and ATP binding to E2 accelerates the E2→E1 interconversion, the apparent affinity for ATP as substrate for the ATPase reaction depends on whether the E2→E1 interconversion is the rate-limiting step or not. In the wild-type enzyme, the deocclusion of K^+ from the E2 form is sufficiently slow for the low-affinity ATP effect to show up in the ATP titration of the overall Na^+,K^+-ATPase reaction (Figure 4A). The change in apparent affinity for ATP observed with the mutants could arise from enhancement of the E2→E1 interconversion rate (destabilization of the K^+-occluded E2 form?), or from inhibition of another step (the Na^+ occlusion in E1?), which would then contribute to rate limitation.

All of the residues examined in the present study are highly conserved among the various species and isoforms of the Na^+,K^+-ATPase and also among some of the other P-type ATPases with different cation specificities, such as the H^+,K^+-ATPases and the Ca^{2+}-ATPases of sarco(endo)-plasmic reticulum. It is interesting to compare the present and previous results obtained in studies of Na^+,K^+-ATPase mutants with the mutagenesis analysis carried out on the sarcoplasmic reticulum Ca^{2+}-ATPase. Thus, Glu329, Glu781, Asp806, Thr809, and Asp810 in the Na^+,K^+-ATPase have homologous counterparts in the Ca^{2+}-ATPase residues Glu309, Glu771, Asn796, Thr799, and Asp800, which previously were shown to be crucial to Ca^{2+} occlusion (Vilsen & Andersen, 1992a; Andersen & Vilsen, 1994). All mutations to the Ca^{2+}-ATPase residues Asn796 and Asp800 carried out so far have resulted in enzymes that were unable to phosphorylate from ATP at any Ca^{2+} concentration tested (Clarke et al., 1990; Andersen & Vilsen, 1992, 1994). By analogy, the residues Asp806 and Asp810 in the Na^+,K^+-ATPase could not be replaced by asparagine (present study) or leucine (Jewell-Motz & Lingrel, 1993) without loss of the ability to confer ouabain resistance to the cells. Some of the mutants with alterations to Glu309, Glu771, and Thr799 of the Ca^{2+}-ATPase have been shown to be able to phosphorylate from ATP, albeit with a reduced apparent Ca^{2+} affinity in activation (Andersen & Vilsen, 1992, 1994; Vilsen & Andersen, 1992b). The homologous substitutions in Na^+,K^+-ATPase Glu329→Gln or Leu (Vilsen, 1993; Jewell-Motz & Lingrel, 1993) and Glu781→Ala and Thr809→Ala (present study) were compatible with cell growth, while the substitutions Glu329→Ala (present study) and Glu781→Leu (Jewell-Motz & Lingrel, 1993) were not. The low apparent Na^+ affinity found for the mutants Glu781→Ala and Thr809→Ala in the present study is comparable to the reduced apparent Ca^{2+} affinity and lack of Ca^{2+} occlusion in Ca^{2+}-ATPase mutants with alterations to Glu771 and Thr799. On the basis of studies of dephosphorylation of the E2P phosphoenzyme intermediate, it was proposed that Glu771 but not Thr799 is important for countertransport of H^+ by the Ca^{2+}-ATPase (Andersen & Vilsen, 1992, 1994). This is analogous to the hypothesis that the Na^+,K^+-ATPase residue Glu781 might play a role in the binding of extracellular K^+ and Na^+. A significant difference between the functions of Glu771 in Ca^{2+}-ATPase and Glu781 in Na^+,K^+-ATPase is, however, that the dephosphorylation was blocked following substitution of Glu771 in Ca^{2+}-ATPase, whereas a destabilization of the phosphoenzyme appeared to result from substitution of Glu781 in the Na^+,K^+-ATPase. The reasons for this difference remain to be elucidated.

One may wonder why the substitutions Glu329→Leu and Glu781→Ala were compatible with cell growth, whereas the Glu329→Ala and Glu781→Leu mutants were inactive. A possible explanation is that Glu781 is located inside the cation binding pocket, so that the bulky side chain of the leucine substituent would interfere not only with Na^+ and K^+ binding to the oxygens of the substituted residue side chain but also with coordination of Na^+ and K^+ by other oxygen ligands. This interference might not arise with the alanine substituent, due to the smaller side chain. By contrast, the side chain of Glu329 might not be directly involved in coordination of the cations within the binding pocket, but might participate in formation of a gate to the occlusion sites. To serve as gate, the side chain would at least have to be as bulky as the side chain of leucine, whereas the alanine side chain might not fulfill this role.

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REFERENCES

Andersen, J. P., & Vilsen, B. (1992) *J. Biol. Chem. 267*, 19383−19387.

Andersen, J. P., & Vilsen, B. (1994) *J. Biol. Chem. 269*, 15931−15936.

Argüello, J. M., & Kaplan, J. H. (1991) *J. Biol. Chem. 266*, 14627−14635.

Argüello, J. M., & Kaplan, J. H. (1994) *J. Biol. Chem. 269*, 6892−6899.

Blostein, R. (1983) *J. Biol. Chem. 258*, 7948−7953.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Clarke, D. M., Loo, T. W., & MacLennan, D. H. (1990) *J. Biol. Chem. 265*, 6262−6267.

Cornelius, F. (1991) *Biochim. Biophys. Acta 1071*, 19−66.

Eisenman, G., & Dani, J. A. (1987) *Annu. Rev. Biophys. Biophys.Chem. 16*, 205−226.

Geering, K. (1991) *FEBS Lett. 285*, 189−193.

Gluzman, Y. (1981) *Cell 23*, 175−182.

Glynn, I. M. (1985) in *The Enzymes of Biological Membranes: The Na^+,K^+-transporting adenosine triphosphatase* (Martonosi, A. N., Ed.) pp 35−114, Plenum Publishing Corp., New York and London.

Glynn, I. M., & Karlish, S. J. D. (1976) *J. Physiol. 256*, 465−496.

Goldshleger, R., Tal, D. M., Moorman, J., Stein, W. D., & Karlish, S. J. D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6911−6915.

Green, N. M. (1989) *Biochem. Soc. Trans. 17*, 970−972.

Jewell-Motz, E. A., & Lingrel, J. B. (1993) *Biochemistry 32*, 13523−13530.
Karlish, S. J. D., Goldshleger, R., & Stein, W. D. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4566−4570.
Kaufman, R. J., Davies, M. V., Pathak, V. K., & Hershey, J. W. B. (1989) *Mol. Cell. Biol. 9*, 946−958.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 85*, 3314−3318.
Larsen, T. M., Laughlin, L. T., Holden, H. M., Rayment, I., & Reed, G. H. (1994) *Biochemistry 33*, 6301−6309.
Lescale-Matys, L., Hensley, C. B., Crnkovic-Markovic, R., Putnam, D. S., & McDonough, A. A. (1990) *J. Biol. Chem. 265*, 17935−17940.
Lingrel, J. B., Orlowski, M. M., Shull, G. E., & Price, E. M. (1990) *Prog. Nucleic Acid Res. Mol. Biol. 38*, 37−89.
Pedersen, P. L., & Carafoli, E. (1987) *Trends Biochem. Sci. 12*, 146−150.
Post, R. L., Hegyvary, C., & Kume S. (1972) *J. Biol. Chem. 247*, 6530−6540.
Pressley, T. A. (1988) *J. Membr. Biol. 105*, 187−195.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Shani-Sekler, M., Goldshleger, R., Tal, D., & Karlish, S. J. D. (1988) *J. Biol. Chem. 263*, 19331−19342.
Skou, J. C. (1990) *FEBS Lett. 268*, 314−324.
Skou, J. C. (1991) in *The Sodium Pump: Recent Developments* (Kaplan, J. H., & De Weer, P., Eds.) pp 317−319, The Rockefeller University Press, New York.
Toney, M. D., Hohenester, E., Cowan, S. W., & Jansonius, J. M. (1993) *Science 261*, 756−759.
Vilsen, B. (1992) *FEBS Lett. 314*, 301−307.
Vilsen, B. (1993) *Biochemistry 32*, 13340−13349.
Vilsen, B., & Andersen, J. P. (1992a) *J. Biol. Chem. 267*, 25739−25743.
Vilsen, B., & Andersen, J. P. (1992b) *FEBS Lett. 306*, 247−250.
Vilsen, B., Andersen, J. P., Clarke, D. M., & MacLennan, D. H. (1989) *J. Biol. Chem. 264*, 21024−21030.
Vilsen, B., Andersen, J. P., & MacLennan, D. H. (1991) *J. Biol. Chem. 266*, 16157−16164.

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