Substitutions of Glutamate 781 in the Na,K-ATPase α Subunit Demonstrate Reduced Cation Selectivity and an Increased Affinity for ATP

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The intramembrane Glu781 residue of the Na,K-ATPase α subunit has been postulated to have a role in the binding and/or occlusion of cations. To ascertain the role of Glu781, the residue was substituted with an aspartate, alanine, or lysine residue and the mutant Na,K-ATPases were coexpressed with the β1 subunit and produce catalytically competent Na,K-ATPase molecules with hydrolytic activities comparable to that of the wild-type enzyme. Analysis of the kinetic properties of the mutated enzymes showed a decrease in apparent affinity for K+ compared to wild-type Na,K-ATPase, with the lysine and alanine substitutions displaying the greatest reduction. All Na,K-ATPase mutants demonstrated a significant increase in apparent affinity for ATP compared to wild-type Na,K-ATPase, while the sensitivity to the cardiotonic inhibitor, ouabain, was unchanged. The dependence on Na+, however, differs among the mutant enzymes with both the Glu781→Asp and Glu781→Ala mutants displaying a decrease in the apparent affinity for the cation, while the Glu781→Lys mutant exhibits a modest increase. Furthermore, in the absence of K+, the Glu781→Ala mutant displays a Na+-ATPase activity and a cellular Na+ influx suggesting that Na+ is substituting for K+ at the extracellular binding sites. The observation that trypsin digestion of the Glu781→Ala mutant in Na+ medium produces a K+-stabilized tryptic fragment also intimates a decreased capacity of the mutant to discriminate between Na+ and K+ at the extracellular loading sites. All together, these data implicate Glu781 of the Na,K-ATPase α subunit as an important coordinate of cation selectivity and activation, although the modest effect of Glu781→Lys substitution seemingly precludes direct involvement of the residue in the cation binding process. In addition, the fifth membrane segment is proposed to represent an important communicative link between the extramembranous ATP binding domain and the cation transport regions of the Na,K-ATPase.

The Na,K-ATPase, or sodium pump, is a membrane-spanning, heterodimeric protein that uses the energy from the hydrolysis of ATP to maintain the low intracellular sodium concentration and high intracellular potassium concentration common to most animal cells. During enzyme turnover, three intracellular sodium ions and two extracellular potassium ions are countertransported across the plasma membrane for each molecule of ATP hydrolyzed. The ion-motive enzyme consists of a 110-kDa catalytic α subunit and a smaller noncovalently associated β subunit. The catalytic α subunit contains the binding sites for ATP and the cardiotonic inhibitor, ouabain; it is phosphorylated by ATP, and undergoes the cation-dependent E1→E2 conformational transitions associated with cation transport. The exact function of the glycosylated β subunit remains unknown, although, it has been implicated in the K+′-binding process of the enzyme (Lutsenko and Kaplan, 1993).

Understanding the molecular events associated with the Na,K-ATPase catalytic cycle requires a knowledge of the individual amino acids that coordinate the binding of the enzyme ligands. While data from site-directed mutagenesis and chemical modification studies have implicated specific residues within the α subunit in ATP binding and ouabain sensitivity (for review, see Lingrel and Kuntzweiler (1994)), comparatively little is known concerning the intramembrane amino acids that comprise the monovalent cation binding and occlusion sites. It is generally believed that movement of sodium and potassium through the low dielectric medium of the plasma membrane involves the neutralization and stabilization of the positively charged ions through coordination with carboxyl-bearing residues within or near the membrane. The use of hydrophobic, carboxyl-selective reagents to chemically modify and inactivate cation binding of the Na,K-ATPase has proven useful in identifying intramembrane residues in the α subunit that may be involved in cation transport function (Goldshleger et al., 1992; Argüello and Kaplan, 1994).

Recently, a fluorescent, carboxyl-reactive derivative of diazomethane, 4-(diazomethyl)-7-(diethylamino)coumarin (DEAC), has been shown to inactivate cation occlusion of the Na,K-ATPase in a K+ and Na+ preventable manner by covalently labeling Glu779, a residue in the fifth transmembrane segment (Argüello and Kaplan, 1994). DEAC inhibition appears to be selective for the cation binding site as the modified enzyme is still able to undergo the E1→E2 conformational transitions and displays normal levels of ATP/ADP binding. The importance of this residue in Na,K-ATPase function is further evidenced by the findings that (i) substitution of Glu779 with a leucine or aspartate residue inhibits Na,K-ATPase-dependent

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The abbreviations used are: DEAC, 4-(diazomethyl)-7-(diethylamino)coumarin; PAGE, polyacrylamidegel electrophoresis; CHAPS, 3-(3-cholamidopropyl)dimethyl ammonium)1-propanesulfonate.
cell growth in transfected HeLa cells (Jewell-Motz and Lingrel, 1993; Feng and Lingrel, 1995), (ii) replacement of the corresponding glutamate residue, Glu771, in the sarco(endo)plasmic reticulum Ca^2+-ATPase blocks Ca^2+-dependent functions of the enzyme (Clarke et al., 1989), and (iii) the glutamate residue is highly conserved among members of the P-type family of ATPases (Argüello and Kaplan, 1994). More recently, expression of Glu779 mutations in transfected mammalian cells have shown that this residue, although not required for activity, has an important role in cation interactions or selectivity (Vilsen, 1993; Feng and Lingrel, 1995), (ii) replacement of the corresponding glutamate residue, Glu771, in the sarco(endo)plasmic reticulum Ca^2+-ATPase blocks Ca^2+-dependent functions of the enzyme (Clarke et al., 1989), and (iii) the glutamate residue is highly conserved among members of the P-type family of ATPases (Argüello and Kaplan, 1994). More recently, expression of Glu779 mutations in transfected mammalian cells have shown that this residue, although not required for activity, has an important role in cation interactions or selectivity (Vilsen, 1993; Feng and Lingrel, 1995).

To further examine the role of the glutamate in Na,K-ATPase function, site-directed mutagenesis was used to replace Glu781 (the equivalent residue in the rat Na,K-ATPase) with an alanine, aspartate, or lysine residue, and the mutant Na,K-ATPase polypeptides were expressed in Spodoptera frugiperda (SF9) cells using the baculovirus expression system. All mutant sodium pumps are catalytically active, but display altered affinities for Na^+ and K^+. In addition, the Glu781 → Ala mutant displays an increased affinity for Cs^+, a close analog of K^+ in the reaction cycle. In the absence of potassium ions, the Glu781 → Ala mutant mediates a Na^+-dependent ATPase activity and a cellular Na^+ influx suggesting that Na^+ is acting at the extracellular binding sites as a surrogate for K^+. An identical Na^+-dependent ATPase activity was recently reported for the Glu781 → Ala mutant of the α subunit in transfected COS cells (Vilsen, 1995), but not in HeLa cells (Feng and Lingrel, 1995). The cause of this difference is unclear. In addition, tryptic digestion of the Glu781 → Ala mutant demonstrates that Na^+ alone is sufficient to stabilize the E2(K) conformational state of the enzyme. All together, these results suggest that in the Glu781 → Ala mutant, the Na^+ is acting at extracellular binding sites as a replacement for K^+ in the catalytic cycle. Moreover, these results suggest that Glu781 is an important coordinate of cation selectivity and activation, and may represent a link between the extramembrane ATP binding domain and the cation transport regions of the Na,K-ATPase.

**MATERIALS AND METHODS**

DNA and Viral Constructions—The cDNAs corresponding to the rat Na,K-ATPase α1 (Schneider et al., 1985) and β1 (Mercer et al., 1986) subunits were subcloned into the dual promoter baculovirus transfer vector p2Bac (Invitrogen Corp., San Diego, CA) to allow for simultaneous expression in the same vector. Transfection of linearized baculovirus DNA, and recombinant baculovirus preparation and selection were performed following the manufacturer’s procedures (Invitrogen Corp.). Amino acid substitutions in the α1 subunit were introduced using oligonucleotide-directed mutagenesis (Amersham Corp.). Prior to subcloning, mutant cassettes were sequenced to ensure that the desired mutation had been incorporated and that no other mutations were introduced. Mutant cassettes of the α1 subunit were subcloned as PFMl/Msd restriction fragments into the wild-type α1β1 p2Bac transfer vector.

Viral Infections and Membrane Preparation—SF9 cells were grown in suspension cultures (from 50 ml to 2 liters) in TNM-FH medium (defined in O'Reilly et al., 1992); J R Biosciences, Lenexa, KS), supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungzone (complete medium). Infections were performed in serum-free medium for 1 h using a viral multiplicity of infection ranging from 5 to 10. After addition of complete medium, cultures were maintained for 72 h. All experiments were performed using membrane preparations from the insect cells. For isolation of α1 subunits, SF9 cells were infected (5,000 m.o.i.) with the ΔGlu781 mutant and resuspended at a concentration of 5 × 10^6 cells/ml in 0.1 mM EDTA, 30 mM imidazole HCl, pH 7.4. The cells were homogenized on ice using a glass homogenizer and the lysate was centrifuged for 10 min at 1,000 × g. The supernatant was removed, centrifuged for an additional 10 min at 12,000 × g, and the final pellet was suspended in 250 mM sucrose, 0.1 mM EDTA, and 25 mM imidazole HCl, pH 7.4.

PAGE and Immunoblot Analysis—The expressed proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Proteins were separated by SDS-PAGE (Laemmli, 1970), transferred to nitrocellulose (Hybond C+, Amersham Corp.), and immunoblotted as described previously (Blanco et al., 1995). The α1 and β1 isoforms were identified with a polyclonal antibody (poly(A)) that recognizes both the rat α1 and β1 subunits (DeTomaso et al., 1993). In the immunoprecipitation experiments, the β1 subunit was detected using an anti-β1 antiseraum raised against purified β1 subunit from dog kidney (provided by Dr. Amir Askari, Medical College of Ohio, Toledo, OH).

Immunoprecipitations—Uninfected and 48 h infected SF9 cells grown in 6-well tissue culture plates were lysed with 1% CHAPS in 150 mM NaCl, 25 mM HEPES, pH 7.4. After removal of the insoluble material (10 min; 15,000 × g), samples were subjected to immunoprecipitation. The rat α1 isoform, 50 μl of a monoclonal antibody hybridoma supernatant that is specific for the α1 subunit (C646-68, provided by Dr. Michael Caplan, Yale University) and 100 μl (1 mg/ml) of goat anti-mouse coated magnetic beads (BioMag PerSeptive Diagnostics, Inc., Cambridge, MA) were used. After overnight incubation on a rocking table at 4°C, beads were isolated by holding the microcentrifuge tube to a magnet and aspirating the supernatant. The beads were washed 3 times in the lysis buffer. The precipitated protein was eluted by resuspending the beads in sample buffer (100 mM Tris-HCl, pH 6.8, 2% SDS, 33% glycerol, 100 mM diethiothreitol) and incubating for 15 min at 56°C. Eluted proteins were separated by SDS-PAGE (7.5%), transferred to nitrocellulose, and probed with the anti-β1 specific antisera.

Tryptic Digestion—Membrane proteins (200 μg) from α1β1 or α1Glu781 → Alaβ1 infected SF9 cells were preincubated in a medium containing 1 mM EDTA, 0.05% CHAPS, and 25 mM imidazole, pH 7.5, in the absence or presence of 150 mM NaCl. Membranes were incubated for 20 min at 37°C in the respective medium, then 1 mM trypsin (anti-α1 protein, anti-α1 protein (anti-α1, anti-β1 antibodies were obtained from Drs. Pressley, University of Texas, Houston, TX), that recognizes an amino acid sequence (KPNASEPKHSL) common to both the 77- and 58-kDa tryptic fragments of the α subunit.

Biochemical Assays—Protein assays were performed using the biocinchonic acid/1mgallic acid solution as described by the supplier (Pierce Chemical Co.).

Na,K-ATPase activity was assayed through determination of the initial rate of release of 32P, from [γ-32P]ATP as described previously (Beauchamp and Camps, 1983). Samples of 50–100 μg of total protein were assayed in a final volume of 0.2 ml. Following a 30-min incubation period at 37°C, the reaction was terminated by the addition of 5.5 mM ouabain. For the cations, incubation media were the same as above except that for Na^+ dependence, Na^+ concentration was varied from 2.5 to 122.5 mM. For K^+ and Cs^+ stimulations, the respective ionic concentrations on Na^+ were kept constant. In medium depleted of K^+, flame photometry demonstrated that the concentration of free K^+ was approximately 30 μM. Choline chloride was added so that the final concentration of Na^+ or K^+ plus choline totaled 150 mM. The ATP dependence was determined under saturating concentrations of all cations (120 mM Na^+, 30 mM KCl, 3 mM MgCl₂, 3 mM ATP, 0.2 mM EGTA, 2.5 mM sodium azide, 30 mM Tris-HCl, pH 7.4, ± 1 mM ouabain. For the cations, incubation media were the same as above except that for Na^+ dependence, Na^+ concentration was varied from 2.5 to 122.5 mM. For K^+ and Cs^+ stimulations, the respective ionic concentrations on Na^+ were kept constant. In medium depleted of K^+, flame photometry demonstrated that the concentration of free K^+ was approximately 30 μM. Choline chloride was added so that the final concentration of Na^+ or K^+ plus choline totaled 150 mM. The ATP dependence was determined under saturating concentrations of all cations (120 mM Na^+, 30 mM K^+, and 3 mM MgCl₂). To determine the effect of different Ouabain concentrations, samples were preincubated with the indicated concentrations of ouabain for 30 min at 37°C in the reaction medium; the reaction was started by the addition of ATP.

For 22Na transport assays, infected SF9 cells were harvested at 48 or 72 h post-infection. Cells were centrifuged (2,000 × g) and resuspended at a density of 1 × 10^6 cells/ml in preincubation medium (150 mM NaCl, 100 μM ouabain, 25 mM HEPES, and 2.5 mM MgCl₂, pH 7.4). After a 30-min incubation on ice, cells were centrifuged (2,000 × g) and resuspended at a density of 2 × 10^7 cells/ml in flux medium (50 mM Na^+).
Glu$^{781}$ of the Na,K-ATPase α Subunit Influences Ion Selectivity

**TABLE I**

| Cells | 0 mM NaCl, 0 mM KCl, 140 mM choline, 1.5 mM NaN$_3$ | 120 mM NaCl, 20 mM KCl, 2.5 mM NaN$_3$ | 120 mM NaCl, 20 mM KCl, 2.5 mM NaN$_3$ |
|-------|--------------------------------------------------|--------------------------------------|--------------------------------------|
| Uninfected Sf9 | | | |
| α1, β1 wt | 0.02 ± 0.05 | -0.02 ± 0.13 | 0.06 ± 0.02 |
| α1(Glu$^{781}$ → Lys), β1 | -0.04 ± 0.03 | 0.00 ± 0.09 | 0.61 ± 0.04 |
| α1(Glu$^{781}$ → Ala), β1 | 0.07 ± 0.04 | 0.23 ± 0.02 | 0.60 ± 0.03 |
| α1(Glu$^{781}$ → Asp), β1 | 0.06 ± 0.01 | 0.00 ± 0.02 | 0.44 ± 0.02 |

**RESULTS**

Expression and Assembly of Mutant Na,K-ATPase Polypeptides in Sf9 Insect Cells—To determine the role of the DEAC-modified glutamate in Na,K-ATPase function, site-directed mutagenesis was used to replace Glu$^{781}$ in the rodent Na,K-ATPase with an alanine, aspartate, or lysine residue. Single recombinant baculoviruses containing the cDNAs coding for both the mutant α1 (Glu$^{781}$ → Ala, Glu$^{781}$ → Asp, or Glu$^{781}$ → Lys) and native β subunits were used to infect Sf9 insect cells, a cell line derived from the ovary of the fall armyworm, S. frugiperda. As shown in Fig. 1A, insect cells infected with the respective αβ recombinant baculoviruses express stoichiometric levels of mutant α1 and native β polypeptides that are recognized by the anti-αβ polyclonal antisera, poly(αβ). The Na,K-ATPase from rat kidney is shown for comparison.

To determine if the individual α polypeptides can assemble with the β subunit infected Sf9 cells were solubilized in 1% CHAPS and immunoprecipitated with an α-specific monoclonal antibody. If the mutant α1 and β subunits are in a stable detergent-resistant association, then the β subunit should coimmunoprecipitate with the mutant α1 polypeptide. Using an anti-β antisera, the β subunit can be identified in the immunoprecipitate. As shown in Fig. 1B, the α mutants and the β1 polypeptide can form detergent-stable complexes when coexpressed in the cells. In contrast, when cells singly infected with either an α1 or β1 baculovirus are combined prior to solubilization, the β1 polypeptide is not identified in the immunoprecipitate (Fig. 1B, lane 2). Thus, the site-directed α mutants are expressed at high levels in the Sf9 cells and are structurally competent as judged by their ability to stably assemble with the β1 subunit in cells coexpressing both the α and β polypeptides.

Enzymatic Analysis of Glu$^{781}$ Mutants of the Na,K-ATPase—In order to determine whether the expressed mutant α polypeptides are functionally active, Na,K-ATPase activity was measured using saturating concentrations of ligands (120 mM NaCl, 20 mM KCl, and 3 mM MgATP). The corresponding activities are presented in Table I. Interestingly, replacement of Glu$^{781}$ with an alanine, aspartate, or a positively charged lysine does not diminish Na,K-ATPase activity, with the mutant αβ complexes displaying activities 7-10-fold higher than the uninfected cells or cells expressing the unrelated integrin associated protein (Lindberg et al., 1993). (It is assumed that the lysine residue side chain, pK$_a$ 10.8, displays a net positive charge in the reaction buffer, pH 7.4.) Thus, the mutant-devoid activities are comparable to the level of Na,K-ATPase observed with the wild-type enzyme. These results are consistent with previous studies using transfected mammalian cells demonstrating that the Glu$^{781}$ → Ala (Vilsen, 1995; Feng and Lingrel, 1995) and Glu$^{781}$ → Gln (Feng and Lingrel, 1995) mutants are functional. However, in transfected HeLa cells the substitution of Glu$^{781}$ with aspartate did not confer ouabain resistance, suggesting that the modification impaired enzymatic activity. The functional expression of the Glu$^{781}$ → Asp...
mutation in insect cells suggests that in mammalian cells the substitution may influence processing or assembly of the enzyme. Dose-response curves for the ouabain inhibition of Na,K-ATPase activity of the mutants were determined on membranes from infected Sf9 cells under non-limiting ligand concentrations (120 mM Na\(^+\), 20 mM K\(^+\), and 3 mM Mg\(^2+\)). All mutants displayed inhibition curves similar to the wild-type Na,K-ATPase (data not shown) with \(K_i\) values in the order of \(10^{-6}\) M. These values are in good agreement with the low ouabain sensitivity previously reported for the rodent \(\alpha\beta\) isozyme (Blanco et al., 1993; O'Brien et al., 1994) and suggest an unaltered ouabain-binding capacity for the Na,K-ATPase mutants.

To characterize the cation requirements of the mutant enzymes, ATPase assays were performed in medium free of both Na\(^+\) and K\(^+\), in K\(^+\)-free medium with Na\(^+\), or in medium containing both Na\(^+\) and K\(^+\). Interestingly, as shown in Table I, the Glu\(^{781}\) \(\rightarrow\) Ala mutant displays a significant ouabain-sensitive Na\(^+\)-ATPase activity in the absence of potassium ions. This Na\(^+\)-ATPase activity represents approximately 38% of the maximal activity and is not present at a sodium concentration of 1.5 mM. No ouabain-sensitive ATPase activity was observed with K\(^+\) alone (data not shown).

\(^{22}\)Na Uptake—One possible cause of the elevated Na\(^+\)-ATPase activity of the Glu\(^{781}\) \(\rightarrow\) Ala mutant is an increased efficacy of extracellular Na\(^+\) to act as a surrogate for K\(^+\) in stimulating enzyme turnover and transport. To analyze the cation transport properties of the mutant Na\(^+\)-ATPase activity at the extracellular surface, ouabain-sensitive uptake of extracellular Na\(^+\) was characterized in infected Sf9 cells expressing the wild-type enzyme (Fig. 2, inset) or in Sf9 cells infected with the \(\alpha\)Glu\(^{281}\) \(\rightarrow\) Ala\(\beta\) baculovirus as compared to the wild-type \(\alpha\beta\) baculovirus. Each value is the mean with error bars representing the standard error of triplicate determinations.

absence of K\(^+\), extracellular Na\(^+\) is transported as K\(^+\) to stimulate enzyme dephosphorylation (Blostein, 1983; Campos and Beaugé, 1994). As Na\(^+\) /Na\(^+\) exchange accompanied by ATP hydrolysis has been shown to represent approximately 6 to 10% of the maximal Na\(^+\)/K\(^+\) exchange in the wild-type enzyme (Cornelius, 1991; Mercer and Dunham, 1981) (undetectable in our assays), the elevated level observed with the Glu\(^{781}\) \(\rightarrow\) Ala mutant suggests a reduction in the ability of the enzyme to discriminate between Na\(^+\) and K\(^+\) at the extracellular loading sites.

Trypsin Digestion of the Na,K-ATPase—The Na,K-ATPase exhibits a characteristic pattern of cleavage by trypsin, which differs depending on the presence of Na\(^+\) or K\(^+\) in the medium.
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Moreover, this property is taken as evidence that the cations stabilize different conformational states of the enzyme $E_i$ (Na$^+$ versus E$_i$(K$^+$)) (Jørgensen, 1975, 1977). In the presence of Na$^+$, tryptic fragmentation produces one major fragment of $M_r = 77,000$, while digestion in K$^+$ medium results in two specific peptide fragments of $M_r = 58,000$ and $M_r = 41,000$ (Castro and Farley, 1979). As extracellular Na$^+$ exhibits an increased K$^+$-like effect on enzyme activation in the Glu$^{781}$ → Ala mutant, this effect might also be reflected in the proteolytic cleavage pattern of the enzyme resulting in the generation of the K$^+$-specific tryptic fragments in the presence of only Na$^+$. To analyze the tryptic digestion pattern of baculovirus-induced Na,K-ATPase, membrane proteins from $\alpha$1Glution $\alpha$1β1 or $\alpha$1(Glu$^{781}$ → Ala)$\beta$1 infected Sf9 cells were digested with trypsin, electrophoresed, and transferred to nitrocellulose. The immunoblot was probed with an anti-α antisera (anti-NASE) that recognizes a 12-amino acid sequence that is common to both the sodium ($M_r = 77,000$) and potassium ($M_r = 58,000$) tryptic fragments. As shown in Fig. 3, when digested in Na$^+$ medium, both the wild-type and mutant Na,K-ATPase generate the sodium tryptic fragment ($M_r = 77,000$). However, in contrast to the wild-type enzyme, digestion of the Glu$^{781}$ → Ala mutant produces the normally K$^+$-induced tryptic fragment (58 kDa). The tryptic fragmentation pattern of the mutant and wild-type Na,K-ATPase in Na$^+$ and K$^+$-free media is shown as a control (Fig. 3, lanes 1 and 3, respectively). As previously reported (Castro and Farley, 1979), trypsin digestion of the Na,K-ATPase in imidazole/EDTA buffer without salt generates a tryptic fragment of $M_r = 77,000$ (lanes 1 and 3). However, the level of the 77-kDa fragment produced is significantly increased when the tryptic digestion is performed in a Na$^+$ medium (lanes 2 and 4). Thus, this data suggests that in the mutant, Na$^+$ can stabilize the $E_i$(K) form of the enzyme and is consistent with an increased efficacy of Na$^+$ at the extracellular binding sites.

K$^+$, Na$^+$, and ATP Dependence of Na,K-ATPase Activities—To determine the affinity of the Glu$^{781}$ → Asp, Glu$^{781}$ → Lys, and Glu$^{781}$ → Ala mutants toward physiological ligands, activation curves of Na,K-ATPase activity by K$^+$, Na$^+$, and ATP were performed. For the K$^+$ dependence of Na,K-ATPase, enzyme activity was measured at varying concentrations of K$^+$ (0–30 mM) with Na$^+$ fixed at 120 mM. The obtained K$^+$-activation curves of the three mutants are presented in Panel A of Figs. 4, 5, and 6, where the corresponding activation curve for the wild-type enzyme has been included for comparison. Values describing the kinetic parameters of the different Na,K-ATPases are presented in Table II. As shown, all three mutants exhibited a modest decrease in the apparent affinity for K$^+$. While replacement of Glu$^{781}$ with an aspartate residue reduces the K$^+$ affinity of the enzyme around 1.5-fold ($K_{0.5}$ value of 2.8 mM compared to 1.9 mM for the wild-type enzyme), both the Glu$^{781}$ → Ala and Glu$^{781}$ → Lys mutants displayed lower apparent affinities for K$^+$ which correspond to a 2–3-fold reduction relative to the wild-type enzyme ($K_{0.5}$ values of 6.3 and 4.7 mM for the Glu$^{781}$ → Ala and Glu$^{781}$ → Lys mutants, respectively). For the Glu$^{781}$ → Ala mutant, the apparent K$^+$ affinity formed as described in A but with a reaction medium containing 30 mM KCl and varying concentrations of Na$^+$ from 2.5 to 122.5 mM. In the Na$^+$ and K$^+$-activation experiments, ionic strength was kept constant with choline chloride. C, ATP stimulation of the Glu$^{781}$ → Asp mutant enzyme. Ouabain-sensitive ATPase activity assays were performed in the reaction medium described in A but containing constant Na$^+$ and K$^+$ (30 mM KCl, 120 mM NaCl) and the indicated ATP concentrations. All data are expressed as percent of the maximal Na,K-ATPase activity obtained. Each value is the mean and error bars represent the standard errors of the mean of at least three experiments performed in triplicate on samples obtained from different infections.
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was calculated by subtracting the Na\(^+\)-ATPase component from the total ouabain-sensitive activity and expressing the remaining K\(^+\)-dependent activity as a percentage of the maximal Na,K-ATPase. As Na\(^+\) is postulated to compete more efficiently with K\(^+\) for binding at the extracellular activation sites in the mutant, the calculated apparent affinity for K\(^+\) in this case may be overestimated.

To determine the requirement of the Glu781 → Asp, Glu781 → Lys, and Glu781 → Ala mutants for sodium, Na,K-ATPase activity was measured at varying concentrations of Na\(^+\) (2.5–122.5 mM) with K\(^+\) fixed at 30 mM. In all cases the mutant Na,K-pumps display a shift in the apparent affinity for Na\(^+\) relative to the wild-type enzyme (Panel B, Figs. 4, 5, and 6). As listed in Table II, the conservative substitution of glutamate with an aspartate residue resulted in approximately a 1.5-fold reduction in Na\(^+\) affinity (compare the \(K_{0.5}\) value of 33.5 mM for the Glu781 → Ala mutant). Surprisingly, in contrast to the Glu781 → Ala and Glu781 → Asp mutants, the replacement of Glu781 with the positively charged lysine residue demonstrates a modest, 1.5-fold increase in the \(K_{0.5}\) value for Na\(^+\) activation (\(K_{0.5}\) value of 9.5 mM). As the lysine substitution exhibits opposing effects on cation affinity and an undiminished level of Na,K-ATPase activity over the wild-type enzyme (see Table I), a direct role of Glu781 in charge neutralization and cation binding is unlikely.

To characterize the kinetics at the low affinity ATP site, dose-response curves of Na,K-ATPase activity at millimolar concentrations of ATP were performed. The ATP dependence of the baculovirus-induced α mutants is presented in Panel C of Figs. 4, 5, and 6 and the \(K_m\) values are described in Table II. As shown, all three mutant pumps display a 3–9-fold increase in apparent affinity for ATP over the wild-type enzyme (\(K_m\) values of 0.11, 0.11, and 0.05 for the Glu781 → Ala, and Glu781 → Lys mutants, respectively, compared to 0.46 for the wild-type enzyme).

Cs\(^+\) Dependence of Na,K-ATPase Activity—At the extracellular K\(^+\) site, other alkali metal ions have been shown to replace K\(^+\) and activate ouabain-sensitive Na\(^+\) efflux with a sequence of affinities of Rb\(^+\) > K\(^+\) > Cs\(^+\) > Li\(^+\) (McConaghey and Maizels, 1962). If replacement of Glu781 with an alanine residue reduces the selectivity of the enzyme at the extracellular surface, a change in the Cs\(^+\) half-maximal activation of Na,K-ATPase activity over the wild-type enzyme should be observed. To determine the affinity of the Glu781 → Ala mutant for Cs\(^+\), ouabain-sensitive ATPase activity was measured with varying concentrations of Cs\(^+\) (0–30 mM) in the absence of K\(^+\) and with Na\(^+\) fixed at 120 mM. Fig. 7 shows that the Glu781 → Ala mutant exhibits a 2-fold increase in the apparent affinity for Cs\(^+\) compared to the wild-type enzyme (\(K_{0.5}\) values of 5.7 ± 0.7 mM for the Glu781 → Ala mutant compared to 13 ± 2 mM for the wild-type Na,K-ATPase). Interestingly, the affinity of the mutant enzyme for Cs\(^+\) is similar to that for K\(^+\) in supporting ATPase activity. The apparent Cs\(^+\) affinity of the mutant was calculated by subtracting the Na\(^+\)-ATPase activity and ex-

Fig. 5. K\(^+\), Na\(^+\), and ATP activation of the α1[Glu781 → Lys]β1 mutant enzyme. A, K\(^+\) activation of Glu781 → Lys mutant. Na,K-ATPase activity of Sf9 membranes expressing the mutant α1[Glu781 → Lys]β1 (■, ■, and ○) or wild-type α1[β1 (□, □, and ◼), was determined as described in the legend to Fig. 4. B, Na\(^+\) activation of Glu781 → Lys mutant enzyme. C, ATP stimulation of the Glu781 → Lys mutant enzyme. All data are expressed as percent of the maximal Na,K-ATPase activity obtained. Each value is the mean and error bars represent the standard errors of the mean of at least three experiments performed in triplicate on samples obtained from different infections.
pressing the remaining Cs⁺-dependent activity as a percentage of the maximal Na,Cs-ATPase. Thus, this data is consistent with a reduction in the ability of the mutant Na,K-ATPase to discriminate between the monovalent cations at the extracellular site.

**DISCUSSION**

Previous chemical modification studies using DEAC have implicated a glutamate residue of the Na,K-ATPase a-subunit in cation binding (Argüello and Kaplan, 1994). In the present study, this glutamate residue, which in the rat enzyme corresponds to Glu⁷⁸¹, was replaced with an aspartate, alanine, or lysine residue and the Na,K-ATPase mutants were coexposed with the β₁ subunit in Sf9 cells and characterized. All mutants are able to support ouabain-sensitive ATPase activity at levels comparable to that of the wild-type α₁β₁ enzyme. In addition, studies using transfected mammalian cells demonstrate that the Glu⁷⁸¹→Ala and Glu⁷⁸¹→Gln mutants have maximal turnover numbers similar to the wild-type enzyme (Vilsen, 1995; Feng and Lingrel, 1995). Analysis of the kinetic properties of the mutant Na,K-ATPases, however, revealed altered dependences for Na⁺, K⁺, and ATP as compared to the wild-type Na,K-ATPase. In the case of K⁺, all mutants display a decrease in the apparent affinity for the cation, while for Na⁺, the affinity is reduced in the Glu⁷⁸¹→Ala and Glu⁷⁸¹→Asp substitutions and, unexpectedly, is increased slightly in the Glu⁷⁸¹→Lys mutant. A similar decrease in the relative Na⁺ affinity was observed for the a(Glu⁷⁸¹→Ala)β₁ enzyme in transfected COS and HeLa cells (Vilsen, 1995; Feng and Lingrel, 1995). All three mutants exhibit a significantly higher affinity for ATP compared to the wild-type enzyme, while sensitivity to the cardiotoxic inhibitor, ouabain, remains relatively unaltered.

The observation that replacement of intramembrane Glu⁷⁸¹ with the positively charged lysine residue does not inactivate Na,K-ATPase function nor exhibit a greater effect on cation affinities, argues against a primary role for Glu⁷⁸¹ as a ligating residue within the cation binding domain. Rather, it seems likely that the glutamate residue participates indirectly in transport function by contributing to the overall structural integrity of the cation binding domain by possibly allowing the proper positioning of the ligating residues within the binding pocket. Hence, amino acid substitutions at position 781 that modify cation dependence are predicted to alter the proper active site architecture required for high-affinity binding. Furthermore, the observation that mutations of Glu⁷⁸¹ affect both Na⁺ and K⁺ affinities is consistent with the notion that the residues involved in Na⁺ binding and transport are also involved in binding and transport of K⁺. This is also supported by the finding that K⁺, and to a lesser extent Na⁺, can protect against modification by DEAC (Argüello and Kaplan, 1991). In addition, the ability of all three mutants to stably associate with the β subunit to form active enzymes, along with the unmodified ouabain sensitivities, demonstrates that mutation of Glu⁷⁸¹ does not introduce gross conformational changes in the enzyme. Rather, the effect of the Glu⁷⁸¹ mutations appears to be localized to the cation binding domain and, secondarily, to

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**Fig. 6.** K⁺, Na⁺, and ATP activation of the α₁(Glu⁷⁸¹→Ala)β₁ mutant enzyme. A, K⁺ activation of Glu⁷⁸¹→Ala mutant enzyme. Na,K-ATPase activity of Sf9 membranes expressing the mutant α₁(Glu⁷⁸¹→Ala)β₁ (●, ■, and □) or wild-type α₁β₁ (○, △, and □), was determined as described in the legend to Fig. 4. B, Na⁺ activation of Glu⁷⁸¹→Ala mutant enzyme. C, ATP stimulation of the Glu⁷⁸¹→Ala mutant enzyme. All data are expressed as percent of the maximal Na,K-ATPase activity obtained. Each value is the mean and error bars represent the standard errors of the mean of at least three experiments performed in triplicate on samples obtained from different infections.
the catalytic site.

Site-specific mutagenesis was recently used by Vilsen (1995) to replace Glu781 of the rat Na,K-ATPase α subunit with an alanine residue. When expressed in COS cells, the Glu781→Ala mutant was shown to exhibit a significant K⁺-independent Na⁺-ATPase activity, although the exact mechanism underlying the Na⁺-ATPase was not determined. A similar mutation in the sheep α1 subunit, however, did not exhibit a detectable K⁺-independent Na⁺-ATPase activity (Feng and Lingrel, 1995). In the present study, using the rat α1 subunit, we report a significant ouabain-sensitive Na⁺-ATPase activity in Sf9 cells expressing the α1(Glu781→Ala)β1 isozyme and provide evidence for a Na⁺/Na⁺ exchange mechanism, with Na⁺ acting as a surrogate for K⁺, to account for this aberrant activity. This conclusion is based on the finding that (i) Na⁺ in the absence of K⁺ stimulates the ATPase activity of the Na,K-ATPase mutant, (ii) in flux assays, the α1(Glu781→Ala)β1 mutant can mediate the inward transport of extracellular Na⁺, and (iii) trypsin digestion of the α1(Glu781→Ala)β1 mutant in Na⁺ medium produces a peptide fragment (Mr = 58,000) normally associated with the E2(K) conformation of the enzyme. These results preclude both the ADP-sensitive form of Na⁺/Na⁺ exchange, which is not accompanied by appreciable hydrolysis of ATP, and uncoupled Na⁺ efflux in which only intracellular sodium is bound and transported (reviewed in Glynn, 1985).

The elevated Na⁺ influx associated with the Glu781→Ala substitution can be readily explained by a decreased capacity of the enzyme to discriminate between Na⁺ and K⁺ at the extracellular loading sites. As a result, extracellular Na⁺ substitutes for K⁺, promoting its uptake and enzyme turnover. A rearrangement of the ligating residues within the cation binding pocket that alter the constants for K⁺ and Na⁺ binding could also account for the decreased capacity of the Glu781→Ala mutant enzyme to discriminate among alkali metal ions at the outer surface. Consistent with a reduction in cation selectivity is the finding that the affinity for Cs⁺, a weak congener of K⁺ in enzyme activation, is increased in the Glu781→Ala mutant as compared to the wild-type enzyme. This increase in Cs⁺ affinity gives the mutant enzyme a nearly identical affinity for Cs⁺ and K⁺. Interestingly, this effect appears to be limited to the extracellular loading sites, as K⁺ alone cannot substitute for Na⁺ in mutant enzyme activation. This indicates a greater cation selective capacity at the cytoplasmic surface, and is consistent with the inability of cytoplasmic K⁺ to substitute for Na⁺ in the reaction cycle of the wild-type enzyme (Dunham and Senyk, 1977).

During the reaction cycle, binding information is transmitted between the ATP domain in the major cytoplasmic loop and the cation sites. Communication between the two domains is critical for coordination of cation transport. For example, both ATP and Pi have been shown to stimulate deocclusion of K⁺, while binding of Na⁺ at the cytoplasmic activation site is required for the phosphorylation by ATP bound at the catalytic center. On the basis of the potential role of intramembrane Glu781 in cation binding and its proximity to the cytoplasmic ATP binding domain, it has been suggested that this residue may reside within a segment that actively links the two ligand binding domains (Argüello and Kaplan, 1994; Lutsenko et al., 1995). Consistent with this theory, we show that replacement of intramembrane Glu781 by an aspartate, lysine, or alanine residue results in an increase in the ATP affinity. Interestingly, substitution of Glu378 in the forth transmembrane segment has also been shown to modify the affinities for Na⁺, K⁺, and ATP (Vilsen, 1993). By analogy, this may also indicate a role for the fourth transmembrane segment in the transmission of information between the cation and ATP binding domains. Thus, ligand-induced conformational changes in one domain appear to be transmitted to the corresponding domain through the adjacent transmembrane segment. It should be noted that the ATP affinities reported here are for the low-affinity ATP site, as accurate calculation of the ATP dependence at the high-affinity site is not possible in our enzyme preparations.

Interestingly, our observations using infected Sf9 insect cells are similar to studies using site-directed mutagenesis and expression in mammalian cells (Vilsen, 1995; Feng and Lingrel, 1995). Substitutions of this glutamate in all three studies demonstrate a modified affinity for Na⁺ and an unaltered sensitivity to ouabain. Moreover, with the exception of the Glu779→Gln mutant expressed in HeLa cells (Feng and Lingrel, 1995), all glutamate mutants display a decreased apparent affinity for K⁺. However, there are significant differences between the

### Table II

| Isozyme       | Na⁺ activation | K⁺ activation | ATP activation, Km |
|---------------|----------------|---------------|--------------------|
|               | Kₐₛ nᵢ         | Kₐₛ nᵢ        |                    |
| α1β1          | 16.4 ± 0.7 2.90 ± 0.3 | 1.9 ± 0.2 1.43 ± 0.2 | 0.46 ± 0.10        |
| Glu781→Asp    | 23.2 ± 1.8 2.30 ± 0.5   | 2.8 ± 0.5 2.06 ± 0.2   | 0.12 ± 0.02       |
| Glu781→Ala    | 33.5 ± 3.4 1.95 ± 0.3   | 6.3 ± 0.4 2.06 ± 0.2   | 0.15 ± 0.02       |
| Glu781→Lys    | 9.5 ± 0.7 1.95 ± 0.3   | 4.7 ± 0.5 1.20 ± 1.1   | 0.05 ± 0.01       |

Where the Hill coefficient is not given, apparent affinity was calculated using the Michaelis-Menten equation where nᵢ is equal to 1.

*Fig. 7. Cs⁺ activation of α1(Glu781→Ala)β1 and native α1β1 enzymes. Na⁺-Cs⁺-ATPase activity of Sf9 membranes expressing the mutant α1(Glu781→Ala)β1 (●) or wild-type α1β1 (○) was determined in a reaction medium containing: 120 mM NaCl, 3 mM ATP, 3 mM MgCl₂, 0.2 mM EGTA, 30 mM Tris-HCl, pH 7.4, and CsCl as indicated, in the absence or presence of 1 mM ouabain. Apparent cesium affinity was calculated as a percent of the maximal Na⁺,Cs⁺-ATPase activity obtained after subtraction of the Na⁺-ATPase activity. Each value is the mean and error bars represent the standard errors of the mean of at least three experiments performed in triplicate on samples obtained from different infections. Total ionic strength was kept constant with choline chloride.
studies. For example, (i) while Glu781 mutants expressed in COS or Sf9 insect cells exhibit an increase in the apparent affinity for ATP at the low affinity site, no change in ATP activation is observed for the equivalent mutants expressed in HeLa cells, and (ii) the Glu→Ala mutant when expressed in HeLa cells does not exhibit a K⁺ independent, Na⁺-ATPase activity (Feng and Lingrel, 1995). It is possible that the modified sheep α1 subunit has different properties than the rodent subunit. This possibility will require further investigation. Finally, an advantage of the baculovirus expression system is that the mutant Na,K-ATPases do not have to confer ouabain resistance to be analyzed. Thus, although the Glu781→Asp substitution did not alter ouabain sensitivity in HeLa cells (Feng and Lingrel, 1995), it is catalytically competent.

In conclusion, Glu781 of the Na,K-ATPase α subunit is a critical coordinate of cation selectivity and activation, however, this residue does not appear to have a direct role in the binding and occlusion of cations. Similar conclusions have been obtained studying mutations in Glu781 using transfected mammalian cells (Vilsen, 1995; Feng and Lingrel, 1995). Additionally, the effect of Glu781 substitutions on the apparent ATP affinity suggests a role for the fifth membrane segment in the transmission of binding information between the ATP binding domain and the cation transport region of the α subunit.

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