Residues within Transmembrane Domains 4 and 6 of the Na,K-ATPase α Subunit Are Important for Na^+ Selectivity†

Gladis Sánchez and Gustavo Blanco*

Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas 66160

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ABSTRACT: The Na,K- and H,K-ATPases are plasma membrane enzymes responsible for the active exchange of extracellular K^+ for cytoplasmic Na^+ or H^+, respectively. At present, the structural determinants for the specific function of these ATPases remain poorly understood. To investigate the cation selectivity of these ATPases, we constructed a series of Na,K-ATPase mutants in which residues in the membrane spanning segments of the α subunit were changed to the corresponding residues common to gastric H,K-ATPases. Thus, mutants were created with substitutions in transmembrane domains TM1, TM4, TM5, TM6, TM7, and TM8 independently or together (designated TMAll). The function of each mutant was assessed after coexpression with the *β* subunit in *Sf*-9 cells using baculoviruses. The enzymatic properties of TM1, TM7, and TM8 mutants were similar to the wild-type Na,K-ATPase, and while TM5 showed modest changes in apparent affinity for Na^+, TM4, TM6, and TMAll displayed an abnormal activity. This resulted in a Na^+-independent hydrolysis of ATP, a 2-fold higher $K_{0.5}$ for Na^+ activation, and the ability to function at low pH. These results suggest a loss of discrimination for Na^+ over H^+ for the enzymes. In addition, TM4, TM6, and TMAll mutants exhibited a 1.5-fold lower affinity for K^+ and a 4−5-fold decreased sensitivity to vanadate. Altogether, these results provide evidence that residues in transmembrane domains 4 and 6 of the α subunit of the Na,K-ATPase play an important role in determining the specific cation selectivity of the enzyme and also its E1/E2 conformational equilibrium.

P-type ATPases form a group of enzymes that use the energy from the hydrolysis of ATP to drive the transport of a variety of cations against their transmembrane electrochemical gradients (reviewed in refs *1* and *2*). Within the members of this family of ATPases, two of them, the Na,K- and the H,K-ATPase, are the only ones capable of transporting one type of cation (extracellular K^+) in exchange for another (cytoplasmic Na^+ or H^+, respectively). The Na,K-ATPase or Na pump is ubiquitously expressed, and its function is critical for the control of osmotic cell balance, cell pH, and the resting membrane potential of most tissues. In addition, the enzyme plays a primary role in driving secondary Na^+-coupled transport systems and the reabsorption of salt and water in many epithelia (*3*, *4*). The H,K-ATPase on the other hand is essential for acid secretion by the gastric mucosa, kidney, and colon (*5*−*8*). Both the Na,K- and H,K-ATPase share common biochemical reaction mechanisms and undergo E1/E2 conformational changes coupled to the binding, occlusion, and translocation of ions (reviewed in refs *9* and *10*). Structurally, both transporters are heterodimers composed of stoichiometric amounts of a catalytic α and a glycosylated *β* subunit. The α subunit is a multispanning membrane protein with a molecular mass of approximately 110 kDa. It is composed of an N-terminal segment with four transmembrane spanning domains, a large cytoplasmic region and a carboxy-terminal segment containing another six transmembrane domains (reviewed in refs *11*−*13*). In contrast, the *β* subunit is a 40−60 kDa polypeptide that crosses the plasma membrane just once (reviewed in refs *14*−*16*). The α subunit is responsible for the catalytic and transport properties of the Na,K- and H,K-ATPases, while in vertebrates, the *β* subunit is important for the proper folding and delivery of the holoenzyme to the plasma membrane (*11*−*15*). The α polypeptide contains the binding sites for the respective cations, ATP, and the preferred inhibitors, ouabain for the Na,K-ATPase and omeprazole or Scheering 28080 for the H,K-ATPase (reviewed in refs *3*, *4*, *10*, and *17*). The Na,K- and H,K-ATPase α subunits share approximately 60% amino acid sequence identity. The major similarities between these P-type ATPases occur around the phosphorylated aspartate, the TGES/A motif between transmembrane domains 2 and 3, and several cytoplasmic regions involved in ATP binding (*1*). Most likely, these conserved regions participate in the common mechanism of operation of the transporters. On the other hand, the differences may contain the structural determinants that give each ATPase the ability to select the type of cation preferentially translocated out of the cell. A considerable amount of work has been devoted to identifying the amino acids in the Na,K- and H,K-ATPase α subunits involved in the binding occlusion and transport of the cations. Several different approaches have been utilized. These include the use of carboxyl-modifying agents (*18*), site-directed mutagenesis of specific

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* To whom correspondence should be addressed. Mailing address: Department of Molecular and Integrative Physiology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160. Phone (913) 588-7405. Fax: (913) 588-7430. E-mail: gblanco@kumc.edu.

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residues (*19*−*29*), and extensive trypsin digestion of the enzyme (*30*). Another essential piece of information was the elucidation of the structure of the Ca-ATPase of skeletal muscle sarcoplasmic reticulum (SERCA1a) through crystallization of the enzyme in its Ca^{2+}-bound (*31*) and unbound (*32*) states. Homology modeling of other P-type ATPases to the Ca-ATPase has aided in the identification of the putative cation binding sites in the Na,K-ATPase (*33*, *34*).

Despite all the progress made in discerning the structure and function of the H,K- and the Na,K-ATPase, the precise structural differences within the individual α subunits that govern their specific functional properties are not completely understood. To gain insight into the structural determinants that specify the activity of the Na,K-ATPase, Canfield and Levenson (*35*) studied the function of chimeric transporters in which segments of the ouabain-resistant Na,K-ATPase α subunit from rat were replaced by the corresponding regions of the gastric H,K-ATPase. The ability of the resulting chimeras to sustain Na,K-ATPase function were assayed by the ability of the polypeptides to prevent ouabain from killing transfected, ouabain-sensitive cells. With the use of this approach, it was found that 75% of the segments within the Na pump α polypeptide could be interchanged with proton pump sequences without loss of activity. Within the remaining 25% of the Na,K-ATPase however, four regions were found that, when substituted with the corresponding regions of the H,K-ATPase, could not confer ouabain resistance to the cells. These segments span residues 63−117, 320−413, 736−861, and 899−952 and include nonmembrane regions, as well as transmembrane domains 1, 4, 5, 6, 7, and 8 (TM1, TM4, TM5, TM6, TM7, and TM8) of the α polypeptide. In this manner, residues contained within these regions may be responsible for the cation selectivity exhibited by each transporter. The work by Blostein et al. confirmed that one of those domains, the N-terminal half of the M4−M5 loop of the α subunit has an important role in cation selectivity (*36*). Subsequent studies by the same group showed that three amino acids within TM4 (Leu319, Asn326, and Thr340), as well as the ectodomain contained between transmembrane domains 3 and 4, are important for Na^+ recognition by the Na,K-ATPase (*37*, *38*).

To further investigate the structural determinants involved in the cation selectivity of the Na,K-ATPase, we explored the role of several residues contained within the α subunit transmembrane domains. For this, we engineered a series of Na,K-ATPase mutants targeting residues at transmembrane regions contained within the protein segments found to be functionally noninterchangeable between the Na,K- and H,K-ATPases. In this manner, we substituted residues in the TM1, TM4, TM5, TM6, TM7, and TM8 domains of the Na,K-ATPase α subunit with the corresponding residues of the H,K-ATPase, choosing only different amino acids that are highly conserved for the respective transporters across species. The functional properties of the resulting Na,K-ATPase α mutants were then studied after coexpression with the β subunit in *Sf*-9[1] insect cells using baculoviruses. As previously reported, this system has been a valuable tool for the analysis of the Na,K- and H,K-ATPases since it allows for expression of catalytically competent enzymes in a cell environment almost free of these ATPases (*39*, *40*). Our results show that residues contained within spanning membrane segments 4 and 6 of the Na,K-ATPase α subunit play an important role in cation selectivity of the enzyme, as well as in the conformational equilibrium of the transporter.

EXPERIMENTAL PROCEDURES

DNA and Viral Constructions. To gain insight into the sites involved in cation specificity of the Na,K-ATPase, we used site-directed mutagenesis focusing on residues in transmembrane domains of the α subunit of the enzyme. Our mutagenesis approach assumed that residues that determine the Na^+ specificity of the enzyme are probably conserved in all Na,K-ATPases and may differ from the corresponding ones common to H,K-ATPases. To select the specific amino acids, we aligned the sequences of the Na,K-ATPase α1, α2, α3, and α4 isoforms from different species available and compared them with those of the α subunit of gastric proton pumps. The alignment included Na,K-ATPases from brine shrimp, hydra, *Caenorhabditis elegans*, *Drosophila*, *Xenopus*, shark, rat, mouse, chicken, dog, pig and human. For the gastric H,K-ATPase, the sequences from rat, mouse, rabbit, pig, dog, and human were included. Comparisons were performed using DNAstar and Megalign software (Madison, WI). We focused our attention on residues in the transmembrane domains of the α subunit of the Na,K-ATPase, since, being closely involved with the particular type of cation transported across the membrane, they may play a role in its discrimination. The transmembrane helices targeted were those included within regions of the α subunit previously shown to be required for the specific activity of the Na,K-ATPase (*35*). These include transmembrane regions TM1, TM4, TM5, TM6, TM7, and TM8. From the primary sequence comparison, a total of 24 amino acids that are conserved in all Na,K-ATPases and vary from residues conserved in the H,K-ATPases were identified. These comprise residues with both polar and nonpolar side chains. Charged amino acids have a more obvious impact in cation coordination; however, neutral residues were also included in our analysis since they have also been found to participate in ion binding (reviewed in refs *19*−*21*). Figure 1A lists the substitutions performed in each transmembrane region and their corresponding positions in the Na,K- and H,K-ATPase. The situation of the residues selected within the transmembrane helices is shown in Figure 1B,C on the predicted structure of the Na,K-ATPase α subunit obtained by homology modeling to the SERCA1a Ca-ATPase. We mutageneized groups of amino acids, switching those residues contained within each of the selected transmembrane domains one at a time. In this manner, we created a total of six mutants, which included four substitutions in TM1, four in TM4, two in TM5, seven in TM6, three in TM7, and four in TM8. The mutants were, respectively, named TM1, TM4, TM5, TM6, TM7, and TM8. In addition, we made another mutant that simultaneously carried all 24 substitutions (TMAll). Site-directed mutagenesis was performed on the cDNA corresponding to the rat Na,K-ATPase α1 isoform cloned into the baculovirus transfer vector, pBlueBac (Invitrogen Corp., Carlsbad, CA). Substitutions were obtained using the QuickChange XL mutagenesis kit following the

[1] Abbreviations: *Sf*-9, *Spodoptera frugiperda* insect cell line; SCH-28080, 2-methyl-8-(phenylmethoxy) imidazo[1,2]pyridine-3-acetonotrile; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS).

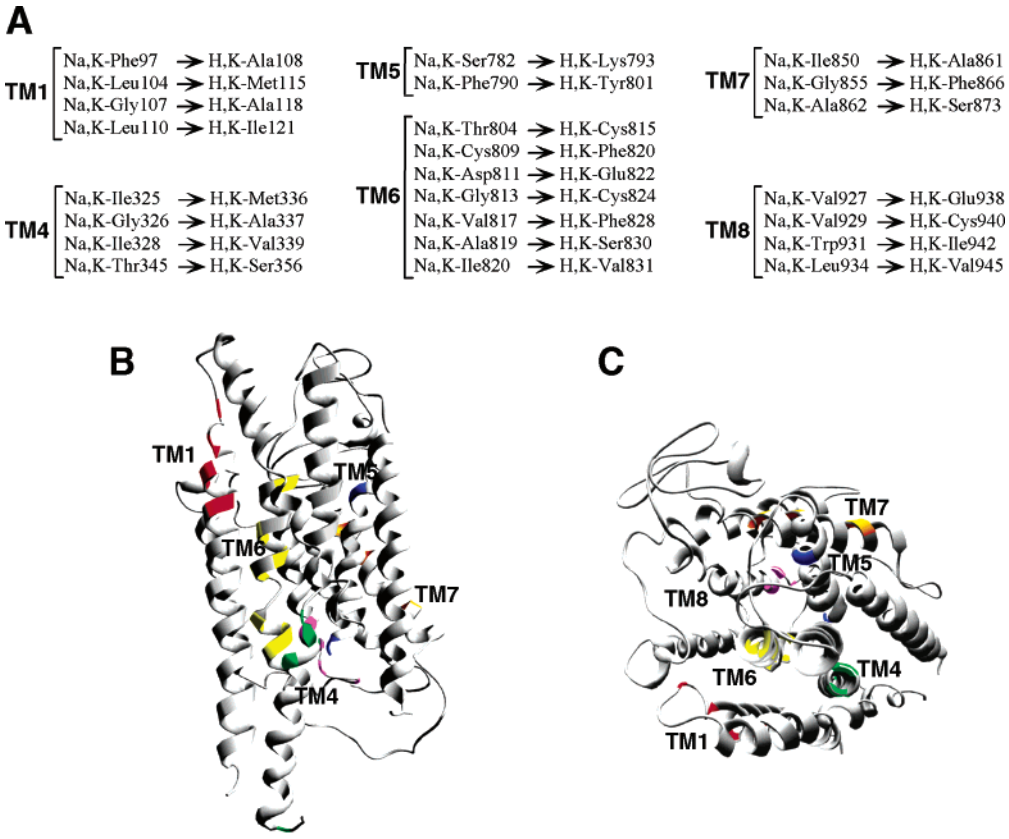

FIGURE 1: Amino acid substitutions in the Na,K-ATPase. Panel A shows mutations performed in the Na,K-ATPase and the corresponding replacements from the H,K-ATPase. Residues are listed according to the transmembrane spanning domain in which they are contained. This generated the TM1, TM4, TM5, TM6, TM7, and TM8 mutants. An additional mutant, TMAll, that simultaneously contained all mutations listed under panel A was prepared. Amino acid positions for the Na,K-ATPase correspond to those of the α1 isoform of the rat enzyme. For the H,K-ATPase, amino acid position is that of the gastric form of the rabbit enzyme. Panels B and C show homology models of Na,K-ATPase transmembrane helices from a lateral and cytoplasmic view, respectively. The model was obtained based on the high-resolution structure of the sarcoplasmic reticulum Ca-ATPase as described under Experimental Procedures. Colored residues depict the mutations performed: red (TM1), green (TM4), blue (TM5), yellow (TM6), orange (TM7), and magenta (TM8).

protocols suggested by the supplier (Stratagene, Cedar Creek, TX). After confirming the substitutions by sequencing, we used the obtained constructs to make the corresponding recombinant baculoviruses. Virus preparation and selection was performed according to the procedures recommended by the supplier (Invitrogen Corp., Carlsbad, CA). Both wild-type and mutated forms of the α subunit of the Na,K-ATPase were coexpressed with the rat β1 subunit, using for this a baculovirus described previously (*39*). Also, baculoviruses for the α and β subunits of the rabbit gastric H,K-ATPase were made and used as a control.

Cells and Viral Infections. *Sf*-9 cells were grown in TNM/FH medium (JRH Biosciences, Lenexa, KS, defined in ref *41*) supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL Fungizone (complete medium). Infections were performed in 150 mm Petri dishes in serum-free medium for 1 h. After addition of complete medium, cultures were maintained for 72 h. Cells were harvested and centrifuged (5000 × *g*) for 10 min and resuspended at a concentration of 5×10^6 cells/ml in 0.32 M sucrose, 1 mM EDTA, 30 mM imidazole HCl, pH 7.4. Then, total cell membrane preparations were obtained as described previously (*42*). Briefly, *Sf*-9 cells were homogenized on ice using a glass homogenizer, and the lysate was centrifuged for 10 min at 1000 × *g*. The supernatant was removed, and the pellet was homogenized and centrifuged as before. Both supernatants of the first and second centrifugation steps were combined and centrifuged for 30 min at 100 000 × *g*. Final pellet was resuspended in the homogenization buffer and used for assays after permeabilization with the ionophore alamethicin at a concentration of 0.01 mg/mg of protein (*43*).

PAGE and Immunoblot Analysis. Protein expression was analyzed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and immunoblotting. Proteins were separated in a 7.5% gel (*44*), transferred to nitrocellulose, and immunoblotted as described previously (*42*). The wild-type and mutated Na,K-ATPase α subunits were detected with a monoclonal antibody hybridoma supernatant specific for the α polypeptide, C464/6B, provided by Michael Caplan (Yale University School of Medicine). The β1 polypeptide was identified using an anti-αβ antiserum raised against rat kidney Na,K-ATPase (*42*, *45*). Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence.

Immunoprecipitations. Uninfected and 72 h infected *Sf*-9 cells grown in 6-well tissue culture plates were lysed with 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in 150 mM NaCl, 25 mM HEPES (pH 7.4). After removal of the insoluble material (10 min at 15 000 × *g*), samples were subjected to immunoprecipitation. To precipitate the wild-type and mutant α polypeptides, 50 μL of monoclonal antibody C464/6B and 100 μL (1 mg/mL) of goat anti-mouse coated magnetic beads (BioMag;

Qiagen, Inc., Stanford, CA) 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 DTT) and incubating for 15 min at 65 °C. Eluted proteins were separated by SDS−PAGE (7.5% gel), transferred to nitrocellulose, and probed with the polyclonal antibody Poly αA that recognizes the β subunit.

Immunocytochemistry and Confocal Microscopy. Sf-9 cells were plated in 24-well culture plates on 11 mm glass cover slips and infected with the wild-type or mutant rat Na,K-ATPase α and the β baculoviruses. Forty-eight hours after infection, cells were treated with 100 μg/mL cycloheximide, an inhibitor of protein synthesis. This treatment clears the biosynthetic pathway of newly synthesized proteins, allowing the detection of the expressed polypeptides at their final cellular destination. Samples were then processed for immunocytochemistry as described (*46*). As the primary antibodies, the monoclonal C464/6B hybridoma supernatant was used to detect the wild-type and mutated α subunits and a polyclonal affinity-purified antiserum (Upstate Biotechnology, Lake Placid, NY) was employed to identify the β polypeptide. A rhodamine (Rho)-conjugated goat anti-mouse and a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit were used as the secondary antibodies. Fluorescent digital images were obtained using a Zeiss LSM510 confocal microscope. Images were acquired in Multitrack channel mode (sequential excitation/emmision) with LSM510 (v 3.0) software and a Plan-NEOFLUAR 40×/1.3 oil DIC objective with a zoom factor of 3 (field size of 0.487 mm × 0.487 mm) and frame size of 1024 pixels × 1024 pixels.

Biochemical Assays. Protein assays were performed using the dye-binding assay based on the method of Bradford from Bio-Rad (Hercules, CA). Na,K-ATPase activity was assayed through determination of the initial rate of release of $^{32}P_i$ from γ[^{32}P]-ATP as described previously (*45*). The ATPase activity of 30-μg total protein samples was measured in a final volume of 0.25 mL. Maximal Na,K-ATPase activity was determined in medium containing 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 30 mM Tris-HCl (pH 7.4) with or without 1 mM ouabain. For maximal H,K-ATPase activity, the medium consisted of 20 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM Tris-HCl (pH 7.0). The assay was started by the addition of ATP with 0.2 μCi of [γ-^{32}P]ATP (3 mM final concentration). Following a 30 min incubation at 37 °C, the tubes were placed on ice, and the reaction was terminated by the addition of 25 μL of 55% trichloroacetic acid. Released [^{32}P]-P_i was converted to phosphomolybdate and extracted with isobutanol. Radioactivity of 190 μL of the organic phase was measured by liquid scintillation counting. The ATP hydrolyzed never exceeded 15% of the total ATP present in the sample, and hydrolysis was linear over the incubation time. Specific ATPase activity was determined as the difference in ATP hydrolysis in the absence and presence of 1 mM ouabain. Activity of the wild-type H,K-ATPase was determined in the presence and absence of SCH-28080 at a concentration of 100 μM. This inhibitor was also used to determine whether the introduced mutations in the Na,K-ATPase α subunit had made the resulting enzymes sensitive to the compound. In all cases, SCH-28080 did not affect the catalytic function of the mutants (data not shown). For the analysis of activation by Na^+ and K^+, incubation media was that used for Na,K-ATPase activity except that for Na^+ dependency, the Na^+ concentration was varied from 0 to 120 mM. For K^+ stimulation, the K^+ concentration was varied from 0 to 30 mM. Choline chloride was added so that the final concentration of Na^+ or K^+ plus choline totaled 150 mM.

Data Analysis. Curve fitting of the experimental data was performed using a Marquardt least-squares nonlinear regression computing program (Sigma Plot, Jandel Scientific, San Rafael, CA). Na^+ and K^+ activation curves were best fitted according to a cooperative model for ligand binding, represented by the equation

$$v = (V_m[S]^n)/(K + [S]^n) \quad (1)$$

where [S] is the concentration of the activating cation (Na^+ or K^+) and n is the Hill coefficient (n_H). The apparent affinity, $K_{0.5} = K^{1/n}$.

Dose−response relations for the inhibition of Na,K-ATPase by orthovanadate were fitted by

$$v = 100[1/(1 + [O]/K_i)] \quad (2)$$

where v is the Na,K-ATPase corresponding to a certain concentration of orthovanadate, [O], expressed as a fraction of activity in the absence of the inhibitor, and K_i is the concentration of orthovanadate that gives the half-maximal inhibition.

Statistical analysis of the concentration−response curves for each enzyme was done applying Snedecor's F test described previously (*45*).

Molecular Modeling. The homology modeling of wild-type and mutant versions of the α1-subunit of Na,K-ATPase from rat was generated using the DeepView/Swiss-Pdb-Viewer program, version 3.7 (SP5) (*47*). We used the crystallographic structure of rabbit sarcoplasmic reticulum Ca-ATPase, SERCA1a (PDB ID 1EUL) as a template (*31*, *32*) and a sequence alignment between the rat Na,K-ATPase (GenBank accession number AAA40775) and the rabbit SERCA1a identical to that used by Sweadner and Donnet (*48*). Based on the initial model of the wild-type Na,K-ATPase, we built the models of the different mutants by applying the *mutate* tool of DeepView and selecting the best available rotamer for the side chain of each substituted residue. Finally, we refined the initial models by energy minimizations consisting of 1000 steps of steepest descent, followed by conjugate gradient minimization until the energy difference between two consecutive steps was below 0.05 kJ/mol.

RESULTS

Recombinant baculoviruses encoding Na,K-ATPase α subunit mutants containing substitutions in each individual transmembrane domain, TM1, TM4, TM5, TM6, TM7, and TM8 (Figure 1), or simultaneously in all (TMAll) were used to infect *Sf*-9 insect cells. These viruses, as well as the wild-type Na,K-ATPase α subunit, were applied in combination with that of the rat Na,K-ATPase β1 subunit. To determine expression of the corresponding virally induced polypeptides,

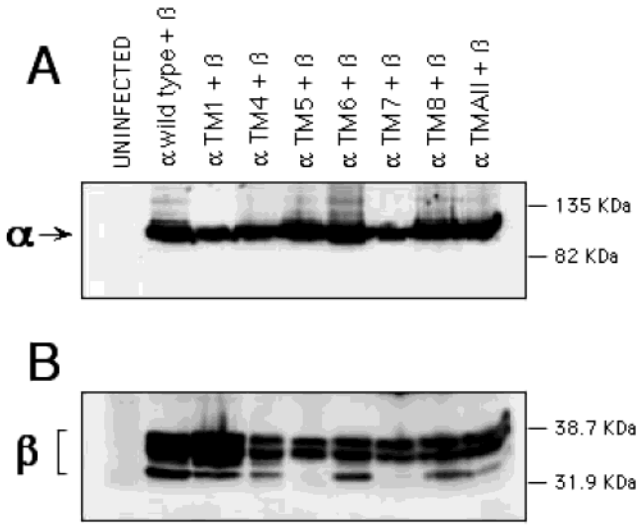

FIGURE 2: Immunoblot analysis of wild-type and mutated forms of the α subunit (A) and the *β* polypeptide (B) of the Na,K-ATPase in *Sf*-9 insect cells. Recombinant baculoviruses containing the cDNAs of the wild-type α and *β* subunits of the Na,K-ATPase and the indicated mutants were used to infect *Sf*-9 insect cells. After 72 h, *Sf*-9 proteins (30 *μ*g) were separated by SDS−PAGE (7.5% gel) and transferred to nitrocellulose. The α polypeptide was detected with monoclonal antibody C464-6B; the *β* subunit was identified with an anti-*β* antiserum raised against purified Na,K-ATPase from dog kidney (Poly αA). Detection of the primary antibodies was performed using horseradish peroxidase conjugated secondary antibodies and chemiluminescence.

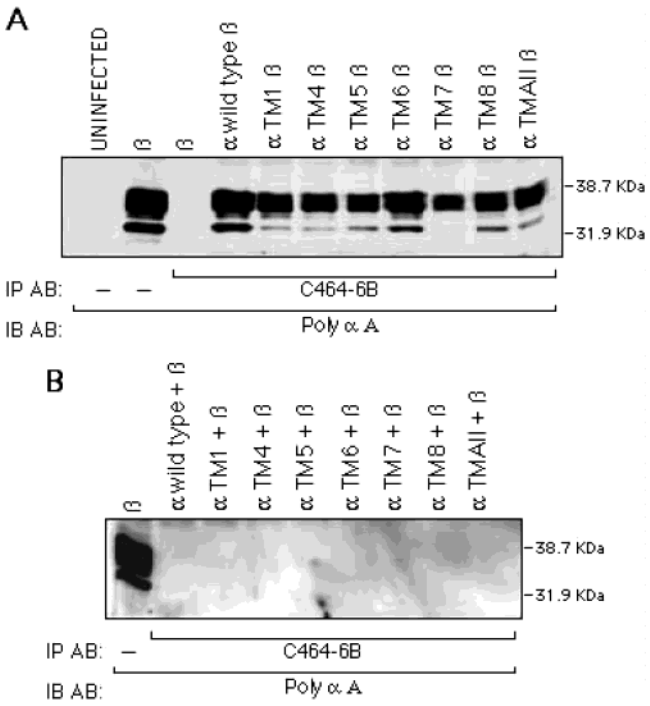

FIGURE 3: Association of Na,K-ATPase α mutants and *β* polypeptides in insect cells. In panel A, membrane proteins from cells coinfected with wild-type or mutated α forms and the *β* subunit were immunoprecipitated with the C464-6B antibody (IP AB). The precipitated proteins were separated by SDS−PAGE, transferred to nitrocellulose, and immunoblotted using Poly αA antiserum that recognizes the *β* subunit (IB AB). Cells infected with the *β* subunit alone and subjected to immunoprecipitation are shown as a negative control. Lack of reactivity of the antibody used with uninfected cells is shown in lane 1. In panel B, combined proteins from cells individually expressing the wild-type or mutated α subunits and the *β* polypeptide were immunoprecipitated as described in panel A. In all cases, horseradish peroxidase conjugated antibodies and chemiluminescence was used for detection. In both panels A and B, the *β* subunit expressed in insect cells (15 *μ*g) is shown as standard.

72 h after infection cells were harvested and proteins were subjected to SDS−PAGE and immunoblotting. As shown in Figure 2A,B, antibodies specific to the Na,K-ATPase α and *β* polypeptides detected high amounts of the corresponding proteins only in the infected cells. All Na,K-ATPase α mutants comigrated with the wild-type subunit at the expected relative molecular weight. This indicates that the insect cells are able to express the Na,K-ATPase mutants at levels that are approximately the same as those of the wild-type enzyme.

To confirm that the introduced mutations did not alter the ability of the modified Na,K-ATPase α subunits to associate with the *β* polypeptide, immunoprecipitation assays were performed. Thus, *Sf*-9 cells were infected with the corresponding Na,K-ATPase α mutants and the *β* subunit. Seventy-two hours after infection, cells were solubilized with 1% CHAPS and immunoprecipitated with an α-specific monoclonal antibody. Immunoprecipitated proteins were then separated by SDS−PAGE, transferred to nitrocellulose, and probed with a *β*-specific antiserum (Poly αA). In this manner, if the mutated α and the *β* subunits are produced in a stable detergent-resistant association, then the *β* polypeptide should be pulled down along with the corresponding α mutant. As shown in Figure 3A, the *β* subunit can be identified in the immunoprecipitates indicating that all α mutants and the *β* subunit can assemble in the insect cells. In contrast, the *β* polypeptide does not coimmunoprecipitate in cells in which the α subunit is absent (Figure 3A, lane 3) or when cells separately infected with the α and *β* polypeptides are mixed and then subjected to immunoprecipitation (Figure 3B). Thus, the α*β* assembly only occurs in coinfected cells. This result shows that the site-directed changes performed in the α subunit do not alter the ability of the mutants to form stable α*β* complexes.

To assess whether the residues substituted could affect the proper routing of the α subunit to the plasma membrane, we analyzed the distribution of the various mutants in the infected cells by immunocytochemistry and confocal microscopy. For this, cells coinfected with the wild-type Na,K-ATPase α subunit or each of the mutants and the *β* subunit were grown for 48 h, treated with cycloheximide, and fixed. Cells were probed with the anti-α and -*β* antisera. The α antibody was identified using a rhodamine (Rho)-conjugated goat anti-mouse secondary antibody, while the *β* antiserum was detected with a FITC-conjugated goat anti-rabbit secondary antibody. As shown in Figure 4, the antisera only recognize the Na,K-ATPase polypeptides in the baculovirus infected cells. The top and middle panels, respectively, show the coexpression of the corresponding α and *β* subunits in a representative cell, while the bottom panels are the merge of both images. Similar to the wild-type enzyme, the Na,K-ATPase α mutants are coexpressed with the *β* polypeptide at the plasma membrane of the cells. This indicates that none of the modifications introduced in the α subunit impair the ability of the enzyme to reach its final destination in the cell.

Altogether the results indicate that the Na,K-ATPase α mutants are structurally competent as judged by their ability to assemble and be delivered with the *β* subunit to the surface of the cells. To determine whether the α polypeptides are

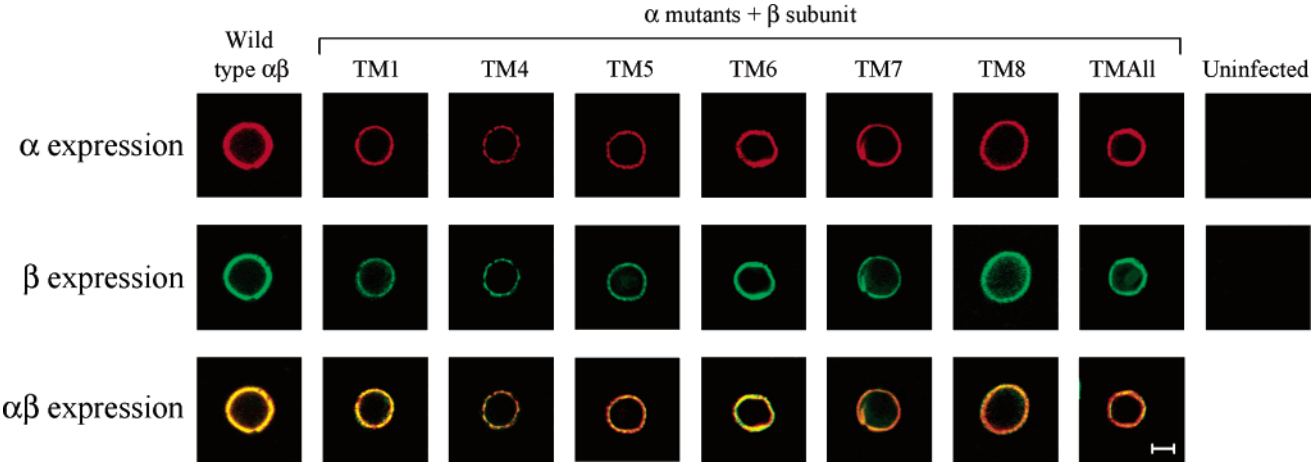

FIGURE 4: Immunolocalization of wild-type and mutated α subunit of the Na,K-ATPase and *β* polypeptide expressed in insect cells. *Sf*-9 cells coinfected with the indicated α and *β* baculoviruses were grown for 48 h in 24 well plates on glass cover slips. After treatment with cycloheximide for 1 h, cells were fixed with paraformaldehyde and subjected to immunofluorescence. The C464-6B antibody and a polyclonal affinity purified anti-*β* antiserum were used to detect the Na,K-ATPase α and *β* polypeptides, respectively. As secondary antibodies, a rhodamine (Rho)-conjugated goat anti-mouse and a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit were used. Scale bars = 10 *μ*m.

also catalytically competent, activity assays were performed at pH 7.4 in a medium containing Na^+ and K^+ at concentrations optimal for Na,K-ATPase activity. As shown in Figure 5A, all Na,K-ATPase mutants exhibited a ouabain-sensitive Na^+- and K^+-dependent hydrolysis of ATP higher than the uninfected cells or cells infected with only the *β* polypeptide. The activity levels of the mutated proteins were approximately the same as that of the wild-type Na,K-ATPase with the exception of TM4, TM5, TM6, and TMAll, which showed slightly lower values. In all cases, the ATP hydrolysis catalyzed by the mutants was sensitive to 1 mM ouabain, and none of them were inhibited by the specific H,K-inhibitor SCH-28080 (data not shown). This indicates that the replaced amino acid did not affect the normal preference of the original Na,K-ATPase for its natural inhibitor. In addition, the ouabain-insensitive hydrolysis of ATP was similar for the wild-type Na,K-ATPase and the mutants indicating that 1 mM ouabain inhibited all enzymes to a similar extent (data not shown).

Next, we tested the ability of the mutants to hydrolyze ATP in medium without Na^+ and at pH 7.0, optimal for H,K-ATPase activity (Figure. 5B). In this medium, cells infected with the TM1, TM5, TM7, and TM8 showed activities similar to or lower than that of the wild-type Na,K-ATPase. This was not significantly different from the values obtained for cells that were not infected. In contrast, cells expressing TM4, TM6, and TMAll mutants exhibited ATPase activities, that were 2−3-fold above the Na,K-ATPase wild-type control. Although functionally competent in H,K-ATPase medium, these mutants did not reach the ATP hydrolysis levels of the wild-type rabbit gastric H,K-ATPase also expressed in *Sf*-9 cells. These results suggest that the substitutions performed in tramsmembrane domains 4 and 6 of the Na,K-ATPase α subunit as well as the simultaneous substitutions in all transmembrane domains alter the normal function of the enzyme. Thus, these subunits are catalytically competent not only in medium with Na^+ but also in the absence of the cation and at increased concentration of protons.

To further explore the response of the mutants to Na^+, activation curves of Na,K-ATPase activity by the cation were performed. The Na^+ dependence of Na,K-ATPase activity was determined at varying concentrations of Na^+ and constant saturating K^+ (20 mM). Figure 6A−F shows the corresponding curves; while the calculated apparent affinities ($K_{0.5}$) and Hill coefficient (n_H) values are tabulated in Table 1. The TM1, TM4, TM5, TM6, TM7, and TM8 mutants displayed a sigmoidal dependence on the cation, which is reflected by Hill coefficients higher than one (*49*). This is consistent with the existence of more than one interacting site for Na^+ in the enzymes (*3*). The apparent affinity for Na^+ varied depending on the mutant considered. Thus, TM1, TM7, and TM8 showed $K_{0.5}$ values similar to that of the wild-type enzyme. TM5 exhibited a $K_{0.5}$ value slightly higher, and TM4 and TM6 displayed apparent affinities approximately 2-fold higher than that of the wild-type Na,K-ATPase. In addition, TM4 and TM6 showed a ouabain-sensitive hydrolysis of ATP in the absence of Na^+, which corresponded to approximately 23% and 28%, respectively, of their maximal activities (Figure 6B,D). For these enzymes, the apparent affinity for Na^+ was calculated by subtracting the Na^+-insensitive ATPase and expressing the remaining Na^+-dependent activity as a percentage of the maximal Na,K-ATPase. In the case of the mutant containing all substitutions, TMAll, the activity in the absence of Na^+ was the highest (approximately 80% of the total hydrolysis of ATP) and showed a poor dependency on Na^+ (Figure 6F). This characteristic did not allow an accurate calculation of the apparent Na^+ affinity of this mutant. The Na^+-dependent relations suggest that single substitutions in transmembrane domains 1, 7, and 8 do not affect the Na^+ affinity of the enzyme, and those of TM5 have a modest effect, while the changes at membrane spanning domains 4 and 6 or the ones contained in the TMAll mutant can significantly influence the interaction of the enzyme with the cation, reducing its apparent affinity for Na^+.

The partial insensitivity of the TM4 and TM6 mutants for Na^+, as well as their lower apparent affinity for the cation, suggests that those enzymes have lost their selectivity for Na^+. We then evaluated the possibility that protons could substitute for Na^+ in the catalytic cycle of the mutants. For this, we determined the ouabain-sensitive ATPase activity

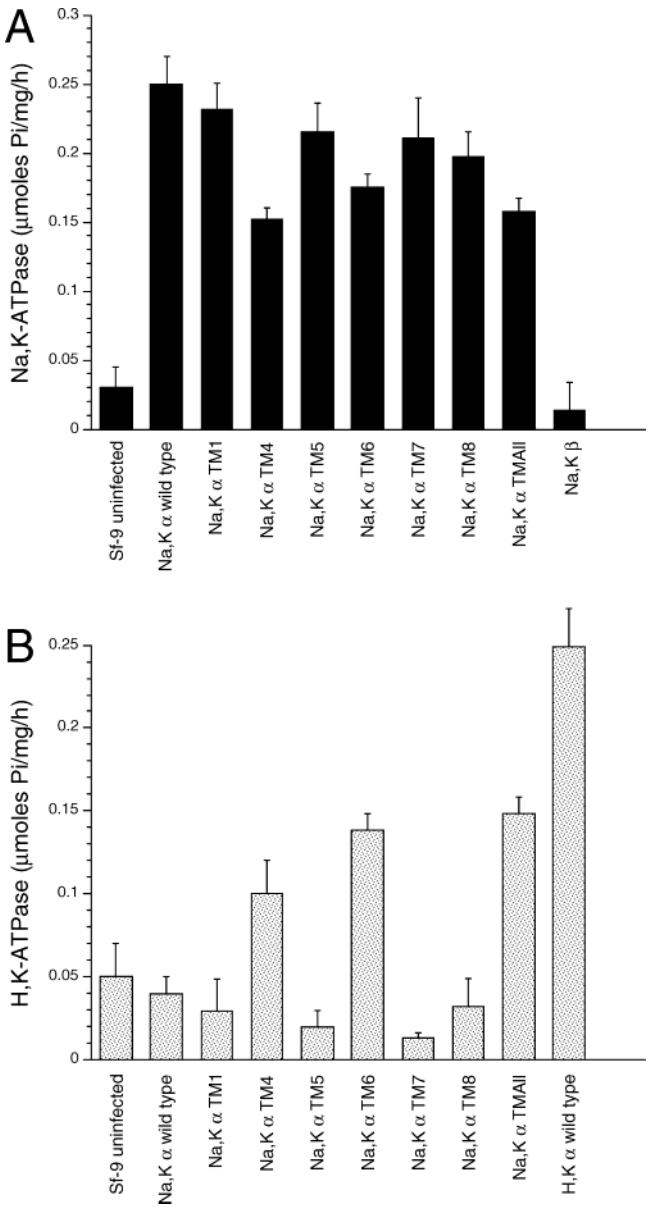

FIGURE 5: ATPase activity of wild-type and mutated forms of the α subunit of the Na,K-ATPase. Panel A shows activity levels in medium optimal for Na,K-ATPase activity. Ouabain-sensitive hydrolysis of ATP was measured as described in Experimental Procedures in medium containing 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 3 mM [γ-^{32}P]ATP−cold ATP, and 30 mM Tris-HCl (pH 7.4) in the absence or presence of 1 mM ouabain. Panel B shows ATPase activity in medium optimal for H,K-ATPase. The incubation medium contained 20 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, and 100 mM Tris-HCl (pH 7.0). Activity of the wild-type and mutant forms of the Na,K-ATPase was sensitive to 1 mM ouabain, whereas activity of the H,K-ATPase was sensitive to SCH-28080. Each value is the mean, and error bars represent the standard errors of the mean of three to seven experiments performed in triplicate on samples obtained from different infections.

of all mutants at different pHs and under two concentrations of Na^+, 1 and 50 mM. Because both these concentrations of Na^+ are not saturating for the ATPase reaction (see Figure 6), this allowed the determination of the competition between Na^+ and protons. Figure 7A,B shows the activities of the TM1, TM5, TM7, and TM8 mutants at both concentrations of Na^+, while those of TM4, TM6, and TMAll are depicted in Figure 7C,D. The wild-type Na,K- and H,K-ATPases have been included for comparison. As shown, TM1, TM5, TM7, and TM8 have a behavior similar to that of the wild-type Na,K-ATPase with negligible levels of ATP hydrolysis at 1 mM Na^+ (Figure 7A) and a Na^+ activation that reaches maximal activity at pH 7.4 (Figure 7B). In contrast, the TM4, TM6, and TMAll mutants exhibited a ouabain-sensitive hydrolysis of ATP that was independent of Na^+ and is stimulated by a decrease in pH (Figure 7C). The properties of the TM4, TM6, and TMAll mutants are significantly different from those of the wild-type Na,K-ATPase and resemble more those of the wild-type H,K-ATPase (Figure 7C). In addition, at 50 mM Na^+, these mutants display high activity over the pH range tested, and they are not limited by the decrease in proton concentration seen at 1 mM of Na^+ (Figure 7D). These observations support the idea that the mutated residues in TM4, TM6, and TMAll impair the capacity of the enzyme to recognize Na^+ over H^+, making it able to use both cations for ATP hydrolysis.

To characterize the kinetics of the mutants to K^+, Na,K-ATPase activity was measured at varying concentrations of K^+ (0−30 mM) with Na^+ fixed at 120 mM. The obtained curves are presented in Figure 8A−F, and values describing the kinetic parameters are depicted in Table 1. As shown, the half-activation constant for K^+ of the TM1, TM7, and TM8 is similar to that of the wild-type enzyme, while TM4, TM5, TM6, and TMAll exhibited a modest decrease in the apparent affinity for the cation with $K_{0.5}$ values approximately 1.5-fold higher. The computed Hill coefficients for K^+ reflect positive cooperativity at more than one ligand binding site for all enzymes. Different from the dependency curves to Na^+, none of the mutants showed an ATP hydrolysis independent of K^+, demonstrating that the selectivity of the enzymes for this cation is retained.

Shifts in apparent affinity for K^+ in the Na,K-ATPase are commonly associated with changes in the steady-state E1/E2 conformational equilibrium of the enzyme in favor of the E1 conformation (*3*, *4*). To explore this possibility, we measured the sensitivity of the Na,K-ATPase activity of TM4, TM5, TM6, and TMAll mutants to orthovanadate. Orthovanadate acts as a Na,K-ATPase inhibitor competing with inorganic phosphate and binding to the E2 conformation of the enzyme (*50*). Thus, vanadate can be used as a tool to obtain information on the E1/E2 conformational state of the Na,K-ATPase (*36*, *51*). A displacement of the conformational equilibrium in favor of E1 is expected to result in a reduced sensitivity of the Na,K-ATPase to orthovanadate. Figure 9 shows the dose response curves of Na,K-ATPase activity to orthovanadate, while the calculated K_i and Hill coefficient values are depicted in Table 1. As shown, the TM4, TM5, TM6, and TMAll mutants displayed affinities that are 4−5-fold lower than that of the wild-type enzyme. Also, in all cases, and consistent with the existence of a single binding site in the enzyme (*3*), the Hill coefficients indicated values lower than 1. This indicates that the TM4, TM5, TM6, and TMAll mutants have become more resistant to orthovanadate, suggesting they exist mainly in the E1 state.

DISCUSSION

Although experimental evidence and homology modeling based on the crystal structure of the Ca-ATPase is providing new insights into the structure and function of P-type

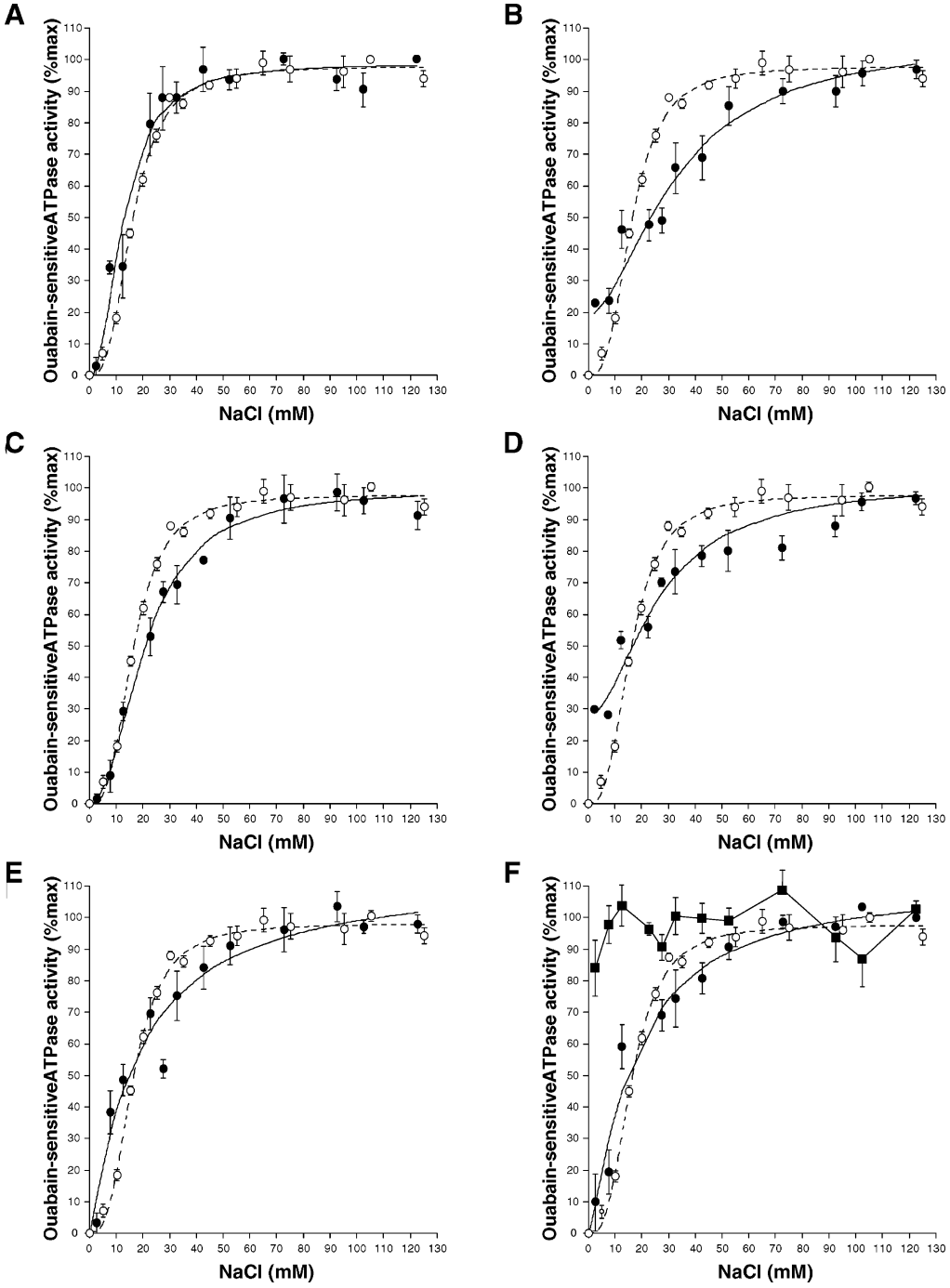

FIGURE 6: Na^+ activation of wild-type and α mutated forms of the Na,K-ATPase: (A) TM1 mutant; (B) TM4 mutant; (C) TM5 mutant; (D) TM6 mutant; (E) TM7 mutant; (F) TM8 and TMAll mutants. Na,K-ATPase activity of membrane preparations from *Sf*-9 cells coinfected with the indicated α and the β subunits was determined in a reaction medium containing 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 3 mM [γ-^{32}P]ATP−cold ATP, 30 mM Tris-HCl (pH 7.4), and NaCl as indicated in the absence or presence of 1 mM ouabain. Ionic strength was kept constant with choline chloride. Data are expressed as percent of the maximal Na,K-ATPase activity obtained. Curves are the best fit of the data to eq 1 shown in Experimental Procedures. Each value is the mean, and error bars represent the standard errors of the mean of three experiments performed in quadruplicate on samples obtained from different infections. In all cases, the wild-type Na,K-ATPase is shown as a control (open circles and dotted lines). Mutated enzymes are shown in filled circles and solid lines, with the exception of TMAll, which is represented by filled squares and solid lines. The statistical significance of the differences in Na^+ requirement between the wild-type Na,K-ATPase and the TM5, TM4, or TM6 mutants ($p < 0.01$) was confirmed by using an *F* test (*45*).

ATPases, it is not fully understood how ion motive ATPases can selectively discern the ion they transport. To gain insight into the structural basis for the Na^+ selectivity of the Na,K-ATPase, we studied the functional properties of a series of mutants of the catalytic subunit of the Na,K-ATPase. Mutations were directed to transmembrane domains contained within regions of the polypeptide known to be specific for Na,K-ATPase activity (*35*). Amino acids highly conserved for the polypeptide across species and isoforms were targeted and changed to the corresponding ones common to gastric H,K-ATPases. We reasoned that the divergent amino acids contained within the cation path across the plasma membrane could be responsible for restricting the type of ion crossing the lipid bilayer and thus be important in defining the selective ion-dependent hydrolytic properties of these closely related enzymes. The mutants generated carried

Table 1: Kinetic Characteristics of Na,K-ATPase Mutants Expressed in *Sf*-9 Insect Cells[a]

	Na^+ activation		K^+ activation		vanadate	
enzyme	$K_{0.5}$ (mM)	n_H	$K_{0.5}$ (mM)	n_H	K_i (M)	n_H
wild-type α/β	16.4 ± 0.7	2.89 ± 0.20	1.9 ± 0.2	1.42 ± 0.15	$(0.9 \pm 0.1) \times 10^{-7}$	0.6 ± 0.03
TM1 α/β	14.0 ± 1.6	2.21 ± 0.43	2.1 ± 0.2	1.48 ± 0.20		
TM4 α/β	33.1 ± 6.0	1.79 ± 0.46	3.0 ± 0.4	1.75 ± 0.38	$(5.5 \pm 0.5) \times 10^{-6}$	0.6 ± 0.03
TM5 α/β	20.5 ± 1.3	2.11 ± 0.26	3.0 ± 0.2	1.98 ± 0.20	$(4.2 \pm 0.5) \times 10^{-6}$	0.6 ± 0.04
TM6 α/β	27.4 ± 1.9	3.10 ± 0.79	2.5 ± 0.4	1.10 ± 0.18	$(4.0 \pm 0.3) \times 10^{-6}$	0.7 ± 0.03
TM7 α/β	15.1 ± 4.9	1.12 ± 0.27	2.1 ± 0.5	1.10 ± 0.25		
TM8 α/β	16.4 ± 3.1	1.30 ± 0.26	1.8 ± 0.4	1.29 ± 0.54		
TMAll α/β			3.4 ± 0.7	1.39 ± 0.38	$(4.0 \pm 0.6) \times 10^{-6}$	0.7 ± 0.05

[a] Apparent affinities ($K_{0.5}$), inhibition constants (K_i), and Hill coefficients (n_H) were calculated from dose-response curves for the activity of Na,K-ATPase for the indicated ligands. Values represent the mean ± standard error of the mean.

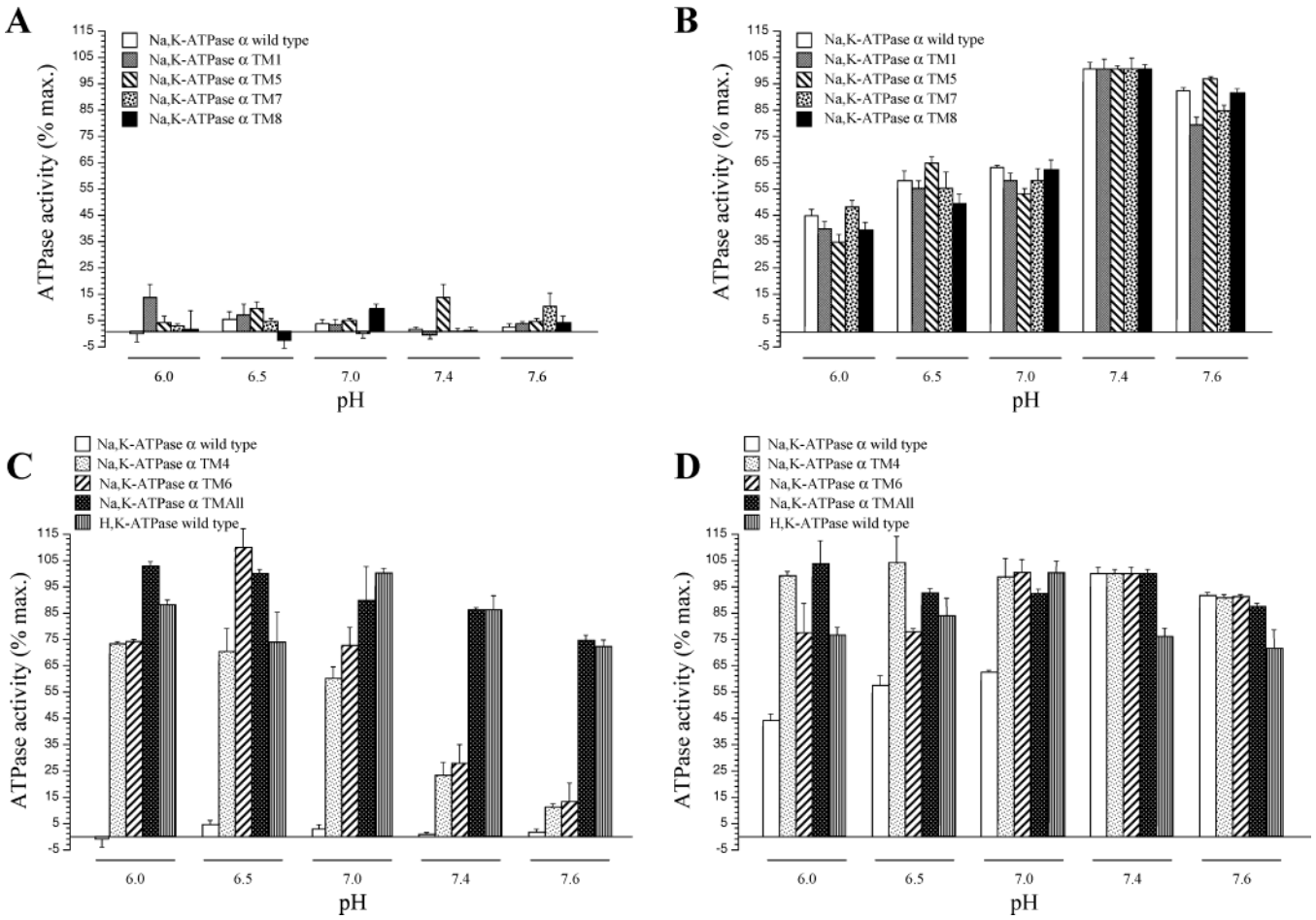

FIGURE 7: ATPase activity of wild-type and α mutated forms of the Na,K-ATPase under different pHs and Na^+ concentrations. Activity was measured in medium containing 20 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 3 mM [γ-^{32}P]ATP−cold ATP, 30 mM Tris-HCl (at the indicated pHs), and 1 mM (A,C) or 50 mM (B,D) NaCl in the absence or presence of 1 mM ouabain. Ionic strength was kept constant with choline chloride. All data are expressed as percent of the ATPase activity obtained at 50 mM Na^+ and at pH 7.4. Each value is the mean, and error bars represent the standard errors of the mean of two experiments performed in quadruplicate.

multiple substitutions in transmembrane segments TM1, TM4, TM5, TM6, TM7, and TM8 individually or simultaneously (TMAll mutant). Analysis of the modified α subunits in *Sf*-9 insect cells indicated that, similar to the wild-type enzyme, the mutants are properly routed to the plasma membrane of the cells and are assembled with the β subunit of the Na,K-ATPase. In addition, the resulting enzymes are catalytically competent and retain the ability to bind ouabain exhibiting a ouabain-sensitive ATPase activity. However, while the TM1, TM5, TM7, and TM8 show maximal function in the presence of Na^+ and K^+ and properties similar to the wild-type Na,K-ATPase, TM4, TM6, and TMAll exhibit different properties. These mutants show a ouabain-sensitive activity in the absence of Na^+ and a hydrolysis of ATP that is dependent on protons (see Figures 5 and 7). Although the Na,K-ATPase has been shown to be able to utilize protons as a substitute for Na^+, this only occurs under particular situations (*52*). This property is exacerbated in the TM4, TM6, and TMAll mutants, which are able to efficiently utilize H^+ instead of Na^+ to support their catalytic cycles. This becomes more apparent from the ability of protons to maintain high levels of ATPase activity of TM4, TM6, and TMAll in media with relatively high Na^+ concentration (see Figure 7D). The altered use of Na^+ by TM4, TM6, and TMAll is further supported by the dose-dependent curves for the cation. In a medium at pH 7.4, where H^+ is limiting,

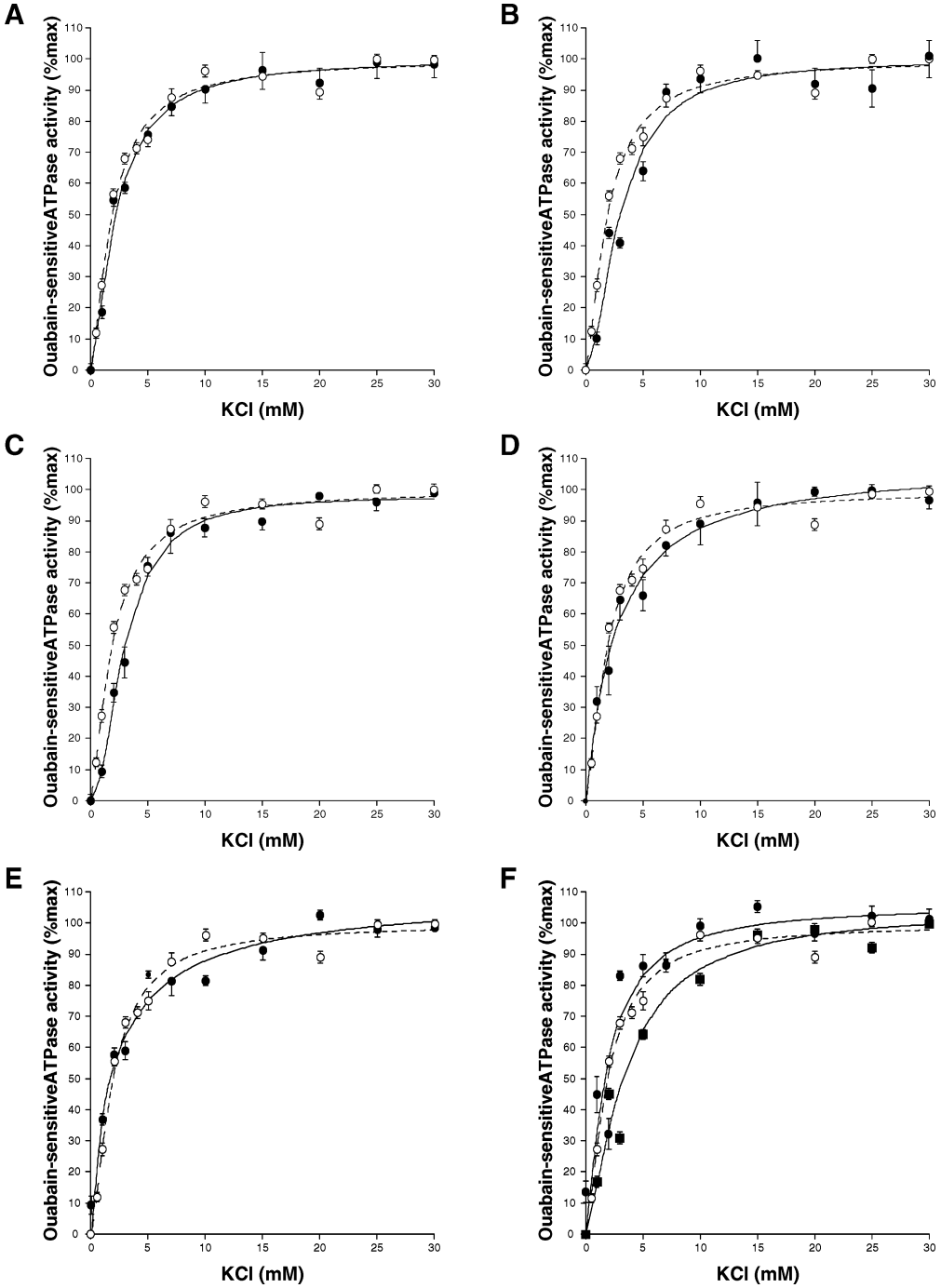

FIGURE 8: K^+ dependence of wild-type and α mutated forms of the Na,K-ATPase: (A) TM1 mutant; (B) TM4 mutant; (C) TM5 mutant; (D) TM6 mutant; (E) TM7 mutant; (F) TM8 and TMAll mutants. Na,K-ATPase activity of membrane preparations from *Sf*-9 cells coinfected with the indicated α and the *β* subunit was determined as described under Experimental Procedures. The reaction medium contained 120 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 3 mM [γ-^{32}P]ATP−cold ATP, 30 mM Tris-HCl (pH 7.4), and KCl (from 0 to 30 mM) with or without 1 mM ouabain. Ionic strength was kept constant with choline chloride. Data are expressed as percent of the maximal Na,K-ATPase activity obtained. Curves are the best fit of the data to eq 1 shown in Experimental Procedures. Each value is the mean, and error bars represent the standard errors of the mean of three experiments performed in quadruplicate on samples obtained from different infections. In all cases, the wild-type Na,K-ATPase is shown as a control (open circles and dotted lines). Mutated enzymes are shown in filled circles and solid lines, with the exception of TMAll, which is represented by filled squares and solid lines. The statistical significance of the differences in K^+ requirement between the wild-type Na,K-ATPase and the TM5, TM4, TM6, or TMAll mutants ($p < 0.01$) was confirmed by using an *F* test (*45*).

the mutants exhibit a slower activation by Na^+ with an approximate 2-fold increase in the $K_{0.5}$ values for the cation. The possibility that K^+ is also replacing Na^+ in the catalytic cycle of these enzymes is unlikely, because at low Na^+ and at constant saturating K^+ (20 mM), activity of the TM4, TM6, and TMAll mutants diminishes with the increase of pH (see Figure 7C). Altogether these results indicate that the modified enzymes can drive a ouabain-sensitive Na^+- as well as a H^+-dependent ATPase activity. This can be interpreted as a loss of selectivity of the TM4, TM6, and TMAll mutants for Na^+ over H^+.

Our results indicating that residues in TM4 are important for Na^+ selectivity of the enzyme agree with other studies showing that point mutations within this transmembrane

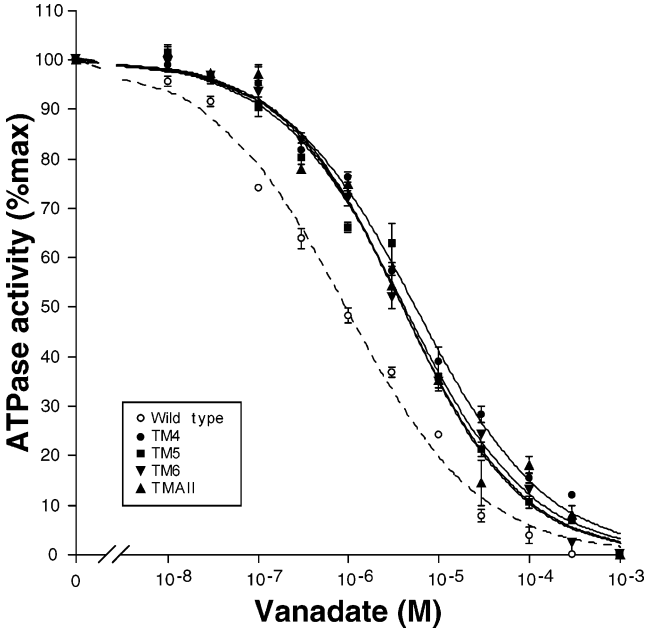

FIGURE 9: Dose−response curves for the inhibition of wild-type and mutant α subunits of the Na,K-ATPase by vanadate. Na,K-ATPase activity of membrane preparations from *Sf*-9 cells coinfected with the indicated α and the *β* subunit was determined as described under Experimental Procedures. The reaction mixture contained 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 30 mM Tris-HCl, and the indicated vanadate concentrations. Values are expressed as percentage of maximal activity in the absence of the inhibitor. Curves represent the best fit of the data using eq 2 shown in Experimental Procedures. Each value is the mean, and error bars represent the standard errors of the mean of triplicate determinations. The statistical significance of the differences between the wild-type Na,K-ATPase and the TM5, TM4, TM6, or TMAll mutant dose−response curves ($p < 0.01$) was confirmed by using an *F* test (*45*).

domain affect binding and occlusion of cations (*20*, *21*, *36*, *37*, *53*−*56*). In addition, our results support homology modeling data of the Na,K-ATPase that predict several residues in TM4 as being involved in forming the Na^+ binding pocket in the enzyme. The four substitutions that we performed in TM4 (Ile325Met, Gly326Ala, Ile328Val, and Thr345Ser) are in the vicinity of the predicted binding site for the second Na^+ ion. All the substitutions performed in TM4 are conservative; however, Gly326 is of particular interest. Glycine appears to have a structural role in membrane proteins that is distinct from that of soluble proteins. The residue facilitates packing of transmembrane helices and helps to stabilize membrane protein structure (*57*). Therefore, the functional change of the mutant containing the Gly326Ala replacement could be a result of the altered packing of helix 4 with regions in helices 5 and 6, all predicted to be involved in forming the Na^+ binding site (*33*, *34*). By comparing the models of the TM4 mutant and the wild-type Na,K-ATPase, we only found minimal changes in the backbone of these proteins. However, this is not surprising, since the modeling of the mutants is heavily governed by the overall structure of the wild-type enzyme template. Another important replacement in TM4 is Thr345Ser. Similar to our results, Mense et al. (*37*) found this substitution to be responsible for conferring the Na,K-ATPase with a Na-independent ATPase activity and a dependency toward protons. In that work, however, Thr345 alone was important but not sufficient to induce the enzymatic changes mentioned and substitutions in the Na,K-ATPase of two additional residues (Leu319 and Thr340), as well as the TM3−TM4 ectodomain of the H,K-ATPase were also required (*38*). Our results show that substitutions confined to transmembrane domain 4 are able to alter Na^+ selectivity of the enzyme. However, this is not sufficient to convert the Na,K-ATPase into a H,K-ATPase. It is possible that cation selectivity in the enzyme requires different recognition steps, which involve residues contained within as well as outside the plasma membrane.

In the case of the TM5 mutant, the two substitutions we performed, Ser782Lys and Phe790Tyr gave only modest changes in the apparent affinities for both Na^+ and K^+ without a gain in proton affinity by the enzyme. Several reports have shown that Ser782 is important for cation binding. For example, substitution of Ser782 for alanine in the pig Na,K-ATPase rendered in yeast an enzyme with a much higher $K_{0.5}$ for Na^+ in the Na-dependent ATP phosphorylation by the cation and a lower capacity of Tl^+ (K^+) binding (*58*). Similarly, Blostein et al. found the same substitution to significantly affect Na^+ binding (*59*). Substitution of Ser782 for arginine in the *Buffo marinus* Na,K-ATPase affects the electrogenicity and, thus, the cation transport stoichiometry of the enzyme (*60*). Exchanging Ser782 for alanine, cysteine, or tyrosine, Arguello and Lingrel found the resulting enzyme to be altered mainly in its apparent affinity for K^+ (*61*). The functional consequence of Ser782 replacements highlights the importance of the residue and the polarized hydroxyl group it brings to that position. Accordingly, homology modeling studies predict Ser782 to intervene in coordination of the second K ion in the Na,K-ATPase (*33*). In addition, in the H,K-ATPase, the lysine residue corresponding to the Na,K-ATPase Ser782 has been shown to be important in stabilizing the geometry of the putative K^+ site of the enzyme (*22*). In general, the studies focused on Ser782 substitutions of the Na,K-ATPase report a more drastic effect for the interaction of the enzyme with the cations than the one that we encountered. The difference may depend in the concomitant replacement, Phe790Tyr, that we performed in transmembrane spanning domain 5. With the addition of this second mutation, we introduced back to the TM5 domain the hydroxyl group that was lost with the Ser782Lys substitution. It is conceivable that the polarized hydroxyl group of the added tyrosine may act as a compensatory mechanism to coordinate Na^+ or K^+ ions in this mutant. The slightly lower apparent cation affinity of TM5 suggests that such compensation is not complete and it cannot support the properties of the wild-type Na,K-ATPase. On the other hand, it is tempting to propose that the hydroxyl group of Tyr801 in the H,K-ATPase may be equivalent to that of Ser872 in the Na,K-ATPase and may serve in cation coordination of the enzyme.

In analysis of the replacements that we introduced in transmembrane domain 6, one of the residues that we targeted was Asp811. Both experimental evidence and data coming from homology modeling of the Na,K-ATPase suggest that the ionized carboxyl group of this amino acid is important in coordination of Na^+ and K^+ and forms part of the predicted ion binding site that may alternatively engage in binding of the cations (*33*, *34*). Thus, replacement of Asp811 by neutral or positively or negatively charged residues renders an enzyme with impaired ability to bind Na^+. This

is reflected by a reduction in Na^+ affinity for phosphorylation of the enzyme by ATP (*62*, *63*). The decrease in the apparent Na^+ affinity that we observe in the TM6 mutant agrees well with those observations. The homology modeling that we performed on the TM6 mutant also supports the role of Asp811 in Na^+ binding. The carboxylic group of the glutamate that we introduced at that position is displaced approximately 2 Å away from the predicted binding site for Na^+. This would be expected to affect Na^+ coordination within the cation binding pocket. Therefore, Asp811 represents an essential residue for Na^+ coordination and recognition by the Na,K-ATPase. In addition, Asp811 has been shown to be involved in interaction with K^+. This is evidenced by a lower IC_{50} value for the antagonism of ouabain binding by K^+ (*64*, *65*), by a decrease in K^+ affinity for occlusion (*63*), and by a reduction in K^+ affinity for dephosphorylation of the enzyme (*63*). Replacement of the corresponding amino acid in the H,K-ATPase (Glu820) also results in a drastic loss of K^+ affinity (*66*). The changes that we find in the $K_{0.5}$ for K^+ of the TM6 mutant are much more modest. This reflects the influence of the additional substitutions performed. Among these, the polarized hydroxyl group provided by Ser819 in place of alanine could play a role in facilitating coordination of the cation. This suggests that the role of Asp811 in the Na,K-ATPase in K^+ coordination depends on the neighboring residues. It is conceivable that the replacement of Asp811Glu naturally occurring in the H,K-ATPase may provide cation specificity to the enzyme disfavoring Na^+ binding, while concomitant changes, such as that of Ser819 may help maintain the interaction with K^+. The other substitutions that we performed in TM6 that most probably affect the interaction of the enzyme with Na^+ are Gly813Cys and Val817Phe. Regarding Gly813, homology modeling studies predict the residue as being involved in the formation of the site for the third Na^+ ion (*33*, *34*). For the phenylalanine substituting for Val817, the side chain geometry of phenylalanine may impose local steric effects that could affect ion coordination through the neighboring Gly813, Thr814, and Asp815, all of which form part of the predicted Na^+ binding pocket (*33*).

The enzymatic changes described for the substitutions at TM4 and TM6 appear to be exacerbated in the TMAll mutant. This mutant displays the poorest dependency on Na^+ and the highest Na-independent ATPase activity under different pHs (see Figures 6F and 7C). This suggests a synergistic effect of both transmembrane domains in the cation selectivity of the TMAll enzyme. However, we cannot completely discard that the concurrent presence of substitutions in other transmembrane domains may be contributing to the larger effect. In this respect, transmembrane domain 5, along with domains 6 and 7, is thought to shape the cation binding pocket (*34*). Although the isolated mutations in TM5 show only a minor change in apparent affinity for Na^+ and in the E1/E2 conformational state with no activation of ATPase activity by H^+, they may play a more relevant role when combined with those of TM4 and TM6. Interestingly, TMAll shares some of the characteristics of the H,K-ATPase, such as its stimulation by protons. However, TMAll differs from the H,K-ATPase in its activation by Na^+ at pH 7.4 and 7.6. Thus, the substitutions in TMAll are not sufficient to convert the function of the enzyme to a H,K-ATPase, and instead, it behaves as a hybrid Na/H,K-ATPase. Coincidentally with this observation, some of the residues that we mutated (Thr345Ser in TM4 and Cys809Phe and Ala819Ser in TM6) naturally occur in the distal colon ATPase from rat and guinea pig. This may explain the ability of these enzymes to transport both Na^+ and H^+ and function as Na/H,K-ATPases (*67*, *68*). The function of the TMAll mutant indicates that transmembrane domains are not sufficient to confer Na^+ selectivity to the Na,K-ATPase and other domains are involved. Our observation confirms previous results by Mense at al., who show that cytoplasmic, as well as extracellular, domains interact to create the particular properties of P-type ATPases (*38*).

Our results also indicate that changes in certain transmembrane domains do not alter the enzymatic properties of the Na,K-ATPase. The normal behavior of TM1 and TM7 mutants suggests that the residues targeted in those helices are not involved in cation selectivity. This agrees with models predicting the absence of sites for cation coordination in those positions (*33*, *34*). Similarly, the TM8 mutant preserved the properties of the original Na,K-ATPase. In this spanning membrane domain, homology modeling predicts Gly920 as a putative cation binding site (*34*). This residue was not changed in the TM8 mutant, and comparison of the models of the TM8 and wild-type enzymes showed that the substitutions performed do not alter the backbone structure of the helices in the region. Therefore, the changes introduced in TM8 are not affecting the ability of Gly920 to coordinate the cations.

Besides the differences in the apparent affinity for Na^+ and the gain in reactivity to H^+, another consequence of mutations in TM4, TM5, TM6, and TMAll is a slight change in the $K_{0.5}$ for K^+. In this case however, a significant K^+-independent ATPase activity was not detected suggesting that the selectivity of the mutants to K^+ is preserved. The changes in apparent affinity for K^+ are accompanied by a decrease in sensitivity to vanadate. Differences in vanadate binding are commonly used as an indicator of Na,K-ATPase conformational equilibrium (*37*, *38*, *50*, *51*). Our finding that the mutants are 4−5-fold more resistant to vanadate suggests that the substitutions introduced affect the conformational equilibrium of the enzyme causing it to favor the E1 conformation. The possibility that the vanadate binding site has been altered with the substitutions introduced is unlikely. The proposed vanadate binding site is located within the cytoplasmic loop between domains TM4 and TM5, in a region highly conserved for all P-type ATPases and distant from the residues that we targeted (*1*−*3*). On the other hand, we cannot completely rule out the possibility that the sites that bind K^+ in the mutants have been secondarily affected by the changes introduced. However, the concomitant change in K^+ and vanadate affinities supports the idea of a displacement in the conformational equilibrium of the enzyme (*50*). Interestingly, it is known that in the H,K-ATPase, the E2K form of the enzyme is less stable than that of the Na,K-ATPase, that the E2K to E1K transition is faster, and that it can deocclude K^+ more rapidly (*69*). This is in agreement with the concept that the TM4, TM5, TM6, and TMAll mutants have adopted some of the properties of the H,K-ATPase. Supporting our data are the results from Mense et al., indicating that replacement of residues at Na,K-ATPase TM4 by the corresponding ones of the H,K-ATPase stabilizes the K^+-induced conformation and renders an enzyme with

properties of those of the H,K-ATPase (*38*). Likewise the H,K/Na,K chimeras engineered by Koenderink et al. show that introduction of H,K-ATPase sequences to the Na,K-ATPase results in a shift of the enzyme toward the E1 conformation (*70*).

In conclusion, our study shows that residues within TM4 and TM6 in the Na,K-ATPase play an important role in the cation selectivity of the Na,K-ATPase and participate in the conformational transitions associated with the binding of ions. Our results also indicate that the differences between the Na,K- and H,K-ATPase in the transmembrane domains are not sufficient to account for the ability of the enzyme to fully discriminate between cations. Future studies of the function of other regions along the structure of these ion pumps will provide additional information in understanding ion selectivity of P-type ATPases.

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