We have generated protein chimeras to investigate the role of the fourth transmembrane segments (TM4) of the Na,K- and gastric H,K-ATPases in determining the distinct cation selectivities of these two pumps. Based on a helical wheel analysis, three residues of TM4 of the Na,K-ATPase were changed to their H,K-counterparts. A construct carrying three mutations in TM4 (L319F, N326Y, and T340S) and two control constructs were heterologously expressed in Xenopus laevis oocytes and in the pig kidney epithelial cell line LLC-PK1. Biochemical ATPase assays demonstrated a large sodium-independent ATPase activity at pH 6.0 for the pump carrying the TM4 substitutions, whereas the control constructs exhibited little or no activity in the absence of sodium. Furthermore, at pH 6.0 the $K_{1/2}$ (Na$^+$) shifted to 1.5 mM for the TM4 construct compared with 9.4 and 5.9 mM for the controls. In contrast, at pH 7.5 all three constructs had characteristics similar to wild type Na,K-ATPase. Large increases in $K_{1/2}$ (K$^+$) were observed for the TM4 construct compared with the control constructs both in two-electrode voltage clamp experiments in Xenopus oocytes and in ATPase assays. ATPase assays also revealed a 10-fold shift in vanadate sensitivity for the TM4 construct compared with the control constructs both in vitro and in vivo. Based on these findings, it appears that the three TM4 residues play an important role in determining both the specific cation selectivities and the E1/E2 conformational equilibria of the Na,K- and H,K-ATPases.

The P-type class of ion motive ATPases includes the Na,K-, H,K-, and Ca-ATPases. These molecules couple the energy liberated through ATP hydrolysis to the transmembrane translocation of cations. Much remains to be learned about the structural features of these pumps that determine their functional characteristics.

In this study we take advantage of the high mechanistic and functional homology relating the gastric H,K-ATPase and the Na,K-ATPase to investigate the molecular basis for their distinct cation selectivity properties. Both enzymes form functional heterodimers consisting of a larger $\alpha$-subunit ($\sim$110 kDa) and a highly glycosylated $\beta$-subunit ($\sim$35 kDa). For both enzymes, the structural determinants required for enzymatic function have been mapped to the $\alpha$-subunits, whereas the $\beta$-subunits are required to ensure the structural integrity of the protein and for delivery of the heterodimeric pump complex to the plasma membrane (for review see Ref. 1). The Na,K-ATPase is ubiquitous, whereas the gastric H,K-ATPase is primarily found in gastric parietal cells (2), though it has been detected in the kidney as well (3). The $\alpha$-subunits of these two P-type ATPases are 62% homologous, and their hydropathy plots are virtually identical. However, the proteins exhibit major functional differences. Whereas the Na,K-ATPase exports three Na$^+$ ions from the cytoplasm to the extracellular side in exchange for two K$^+$ ions, the H,K-ATPase secretes two protons in exchange for two potassium ions (1).

The high degree of similarity between the Na,K-ATPase and the gastric H,K-ATPase allows their functional determinants to be studied through the generation of chimeras. By exchanging sequence domains and analyzing the functional properties of the resultant chimeric proteins it is possible to identify protein segments that are critical for the different physiological properties of the pumps. We have previously reported that the N-terminal halves of the $\alpha$-subunits of the Na,K- and H,K-ATPases in part determine their distinct ion specificities (4). The N-terminal half of each $\alpha$-subunit includes the first four transmembrane domains (TM1-TM4) as well as half of the large cytoplasmic loop connecting TM4 and TM5. To analyze this region further, a chimera was prepared in which the ectodomain between TM3 and TM4, the fourth transmembrane domain, and the N-terminal half of the large cytoplasmic loop between TM4 and TM5 (amino acids 309–506) of the Na,K-ATPase $\alpha$-subunit were replaced by the complementary portions of the H,K-ATPase. The chimera was transfected into polarized LLC-PK1 pig kidney epithelial cells and was found to accumulate at the apical plasma membrane. When these cells were grown on permeable filter supports, an ouabain-sensitive acidification of the medium bathing their apical surfaces was observed. These data suggest that residues in TM4 and/or its flanking sequences confer the capacity to mediate proton transport. We were not able to analyze the kinetic properties of this chimera H85N/H356–519N (4).

The abbreviations used are: TM, transmembrane domain; NMDG, N-methyl-d-glucamine.

1 In previous publications on Na,K-/H,K-ATPase chimeras, we have used a numbering system referring to the positions of gastric H,K-ATPase residues. To facilitate the comparison of the present results to other mutagenesis studies of the Na,K-ATPase, we have employed a numbering system based on the Na,K-ATPase sequence in this manuscript. It should be noted that the Na,K-ATPase amino acid residues 343–506 correspond to amino acids 556–519 of the H,K-ATPase in the chimera H85N/H356–519N (4).

2 L. A. Dunbar, P. Aronson, and M. J. Caplan, submitted for publication.
chimera, because we could not reliably detect chimera-associated ATPase activity.

To define more precisely these amino residues that are critical for the cation selectivity, we have begun to characterize chimeras that have only portions of the Na,K-ATPase sequence between amino acids 309 and 506 replaced by their H,K-counterparts. Recently we have reported that a chimera comprising the Na,K-ATPase sequence in which the proximal half of the cytoplasmic loop (amino acids 343–506) is replaced by the corresponding H,K-ATPase sequence has an altered cation selectivity. In ATPase assays at pH 6.0 this chimera achieved 28% of its maximal activity without any added sodium in the assay solution, whereas there is no detectable sodium-independent activity for the wild type enzyme. This suggests that protons can substitute for Na⁺ ions in the catalytic cycle of the pump (4).

Extensive site-directed mutagenesis studies performed on the Na,K- and Ca-ATPase indicate that amino acid residues that reside in four of the predicted transmembrane domains (TM4, TM5, TM6, and TM8) make important contributions to cation binding. Each of these amphiphatic helices contains charged and polar residues whose side chains might be expected to participate in the formation of cation binding sites. Results from several labs indicate that mutations in the fourth transmembrane domain of the Ca-ATPase change this pump's apparent affinity for Ca²⁺ (for review see Ref. 6). Comparable results have been obtained for the Na,K-ATPase.

In the present study we dissect further the relevance to cation selectivity of selected TM4 residues together with the extracellular ectodomain (residues 309–342). The TM4s of the two pumps differ by only eight amino acid residues. We found that the replacement of only three Na,K-ATPase residues with their H,K-counterparts (L319F, N326Y, and T340S) has drastic effects on pump function. These three mutations in TM4 together with the exchange of the ectodomains between TM3 and TM4 yield an ATPase, which at pH 6.0 achieves more than 50% of its maximal Na,K-ATPase activity in the absence of sodium. A control construct that only incorporated the ectodomain replacement but not the mutations in TM4 showed only 13% sodium-independent ATPase activity at pH 6.0. Our data suggest, therefore, that these three amino residues play an important role in determining the distinct cation selectivity properties of the Na,K- and H,K-ATPase.

**Experimental Procedures**

**Construction and Expression of Chimeras**—The chimeras presented in this paper were constructed between the rat Na,K- and Ca-ATPase a-subunit (cDNA provided by E. Benz, Johns Hopkins University) and the rat gastric H,K-ATPase a-subunit (cDNA provided by G. Shull, University of Cincinnati). The Kunkel method of site-directed mutagenesis was used to silently introduce Apol, Accl, and BglII restriction sites into the two cDNAs. An Apol site was created in the Na,K-ATPase to match the site existing in the H,K-ATPase at base pair 465 corresponding to amino acid 85. The Accl and BglII sites corresponding to H,K-ATPase amino acids 329 and 356 were introduced into both cDNAs. Chimera H85N was generated by subcloning the small Apol fragment of the H,K-ATPase into the larger Apol fragment of the Na,K-ATPase.

The ecto and ecto + A chimera were constructed by ligating annealed synthetic oligonucleotides between the Accl and BglII sites of the H85N chimera. The oligonucleotide 5′-ATACCTCTGGCGGCTGC AGTCTTTCGA TTGGTATCAT CGTAGCCAAC GTGGCGGAAG GT TTGCTTGAG GGCCTGAG TGCTGGCTGCT GCTGGCCTG CTTGACCCACAC GTGGCGGAAG GTGGTGCTAC CCACCGTCAC GTATGTCTGA CGTT-3′ and its complementary strand were used for the ecto chimera. The oligonucleotide 5′-ATACCTCT TGGCGGCTGC AGTCTTTCGA TTGGTATCAT CGTAGCCAAC GTGGCGGAAG GT TTGCTTGAG GGCCTGAG TGCTGGCTGCT GCTGGCCTG CTTGACCCACAC GTGGCGGAAG GTGGTGCTAC CCACCGTCAC GTATGTCTGA CGTT-3′ and its complementary strand were used for the ecto + A chimera.

Construction of the chimeras took place in either the pBluescript or pSP72 vector (Promega Corp, Madison, WI). Once the chimeras were sequenced through their ligation points, they were subcloned into expression vectors suitable for the different expression systems.

For expression in *Xenopus* oocytes the cDNAs encoding the constructs were excised from pBluescript using the ClaI and XbaI restriction sites and ligated into a modified pBluescript vector using ClaI and SpeI sites. The modified pBluescript vector, enhanced for *Xenopus* oocyte expression, was kindly provided by William Joiner, Yale University. The polylinker of the vector is flanked by the 5′- and 3′-untranslated regions of *Xenopus* oocyte globin. This vector has been shown to boost mRNA stability and expression levels in *Xenopus* oocytes (7). The 5′-untranslated region is inserted between the KpnI and the Apal sites of the pBluescript-KS vector. The 3′-untranslated region plus a poly(A) sequence was ligated into the vector using the EcoRI site and the BamHI site. For *in vitro* transcription of the chimera cDNAs, the vector is linearized with XbaI, which cuts downstream from the poly(A) sequence. An Ambion T3 mMessage-Machine kit was used for the transcription reaction. Oocytes were obtained and treated according to standard methods (8). *Xenopus* oocytes were injected with 50 nl of cDNA solution containing a total of 16 ng of a-subunit and 12 ng of Na,K-ATPase β₂-subunit RNA. Oocytes were maintained in ND96 medium supplemented with 5 mM pyruvate and 50 µg/ml gentamycin (*Life Technologies, Inc.*). They were used for experiments on day 3 or 4 postinjection.

For expression in LLC-PK₁ cells, the chimeric cDNAs were subcloned behind a cytomegalovirus promoter in the mammalian expression vector pcB6 (kindly provided by M. Roth, University of Texas Southwestern), which carries resistance to the antibiotic G418 (*Life Technologies, Inc.*). Transfection, screening for expression, and oocyte selection were carried out as described in Ref. 4.

**Electrophysiology**—Electrophysiological measurements of pump currents were performed essentially as described by Horisberger et al. (9) with the following minor modifications. Prior to measurements, oocytes were loaded with sodium by incubating them for 2 h at room temperature in a K⁺- and Ca²⁺-free medium (97 mM Na⁺, 0.82 mM Mg²⁺, 0.5 mM EGTA, 22.5 mM Cl⁻, 76 mM gluconate, 10 mM Heps, pH 7.4). They were subsequently incubated for 15 min in potassium-free bath solution (97 mM Na⁺, 0.82 mM Mg²⁺, 0.42 mM Ca²⁺, 5 mM Ba²⁺, 22.5 mM Cl⁻, 76 mM gluconate, 2 µM ouabain, and 10 mM Heps, pH 7.4). This solution contains barium to block nonspecific potassium currents and 2 µM ouabain to inhibit endogenous Xenopus Na,K-ATPase. Finally, oocytes were transferred to the chamber and voltage-clamp setup, which initially contained the same potassium-free bath solution. Whole cell currents were measured at a holding potential of −50 mV in the following manner: after the baseline had stabilized with the oocyte in the potassium-free bath, the solution was exchanged for a potassium-containing solution, and the potassium-induced current was recorded. The potassium-free and potassium-containing solutions differed only by the replacement of a fraction of the Na⁺ ions with K⁺ ions. The total concentration of Na⁺ and K⁺ was always 97 mM. To ensure that the observed current was pump-mediated, it was suppressed by replacing the potassium-containing solution with an identical solution to which 5 mM ouabain had been added. The data were obtained using a Warner Instruments Oocyte-Clamp 725C (*Warner Instruments, Hamden, CT*) and analyzed with Pulse and Pulsifit software from HEKA (*Darmstadt, Germany*). The pump currents were plotted against the corresponding potassium concentrations and fitted to the Hill equation,

\[
I = I_{\text{max}} [K^+]^{n} / [K^+]^{n/2}
\]

where \(I_{\text{max}}\) is the maximal or saturated current, \(K^+\) is the half activation constant for the ion current, \([K^+]\) is the potassium concentration in the bath, and \(n\) is the Hill coefficient.

**Preparation of Plasma Membranes**—The following procedure was performed entirely on ice. It has been modified from the method published by Vilsen (10). After LLC-PK₁ cells were grown to confluence in 10-cm tissue culture dishes, they were washed twice with phosphate-buffered saline. They were harvested by scraping with a rubber policeman. They were then sonicated for 45 s with a solution containing 250 mM sucrose, 20 mM Tris-HCl, and 1 mM EDTA at pH 7.4. The cells were loaded with sodium by incubating them for 2 h at room temperature in a K⁺- and Ca²⁺-free medium (97 mM Na⁺, 0.82 mM Mg²⁺, 0.5 mM EGTA, 22.5 mM Cl⁻, 76 mM gluconate, 10 mM Heps, pH 7.4). They were subsequently incubated for 15 min in potassium-free bath solution (97 mM Na⁺, 0.82 mM Mg²⁺, 0.42 mM Ca²⁺, 5 mM Ba²⁺, 22.5 mM Cl⁻, 76 mM gluconate, 2 µM ouabain, and 10 mM Heps, pH 7.4). This solution contains barium to block nonspecific potassium currents and 2 µM ouabain to inhibit endogenous Xenopus Na,K-ATPase. Finally, the cells were loaded with sodium by incubating them for 2 h at room temperature in a K⁺- and Ca²⁺-free medium (97 mM Na⁺, 0.82 mM Mg²⁺, 0.5 mM EGTA, 22.5 mM Cl⁻, 76 mM gluconate, 2 µM ouabain, and 10 mM Heps, pH 7.4). This solution contains barium to block nonspecific potassium currents and 2 µM ouabain to inhibit endogenous Xenopus Na,K-ATPase. Finally, oocytes were transferred to the chamber and voltage-clamp setup, which initially contained the same potassium-free bath solution. Whole cell currents were measured at a holding potential of −50 mV in the following manner: after the baseline had stabilized with the oocyte in the potassium-free bath, the solution was exchanged for a potassium-containing solution, and the potassium-induced current was recorded. The potassium-free and potassium-containing solutions differed only by the replacement of a fraction of the Na⁺ ions with K⁺ ions. The total concentration of Na⁺ and K⁺ was always 97 mM. To ensure that the observed current was pump-mediated, it was suppressed by replacing the potassium-containing solution with an identical solution to which 5 mM ouabain had been added. The data were obtained using a Warner Instruments Oocyte-Clamp 725C (*Warner Instruments, Hamden, CT*) and analyzed with Pulse and Pulsifit software from HEKA (*Darmstadt, Germany*). The pump currents were plotted against the corresponding potassium concentrations and fitted to the Hill equation,

\[
I = I_{\text{max}} [K^+]^{n} / [K^+]^{n/2}
\]
Fig. 1. Helical wheel representation of the fourth transmembrane domains (TM4) of the Na,K- and gastric H,K-ATPase α-subunits. Of the 29 amino acids, which are thought to form TM4, only eight differ between Na,K- and H,K-ATPase. They are depicted in black bold face. A, mutational analysis of the Ca-ATPase indicates that the five positions marked with an asterisk play a role in cation translocation (6, 16). These residues reside on the same half of the helix, suggesting that this helical face might be oriented toward the putative cation translocation pore of the P-type ATPases. In contrast, the face on the opposite side of the dashed line might confront surrounding lipid and adjoining helices. In this study we have replaced the Na,K residues above the dashed line with their H,K-counterparts. The resulting mutations are L319F, N326Y, and T340S. B, the sequences shown in this alignment were used for the helical wheel analysis shown above.

Results

Chimera Design—As discussed in the introduction, the portion of the Na,K-ATPase α-subunit residing between TM3 and the fluorescein isothiocyanate binding site (residues 309–506) appears to be important in determining cation selectivity. We have previously shown that a portion of this selectivity is attributable to the sequence domain bounded by amino acids 343 and 506, which constitutes roughly half of the large cytoplasmic loop connecting transmembrane segments 4 and 5 (Fig. 2A). The goal of this study is to further elucidate the role of residues 309–342 of the Na,K-ATPase in determining the cation selectivity properties of the pump. These residues comprise the ectodomain between TM3 and TM4 and all of TM4. Initially, a chimera was generated that had amino acids 309–342 exchanged for the corresponding sequence from the H,K-ATPase α-subunit (Fig. 2A). Although this construct assembles with the β-subunit and is delivered to the cell surface, no enzymatic activity could be detected (data not shown). We chose, therefore, to generate new pump constructs incorporating more narrowly defined substitutions.

Extensive mutagenesis studies have demonstrated that specific residues of the Ca-ATPase, which is structurally and functionally homologous to both the Na,K- and H,K-ATPase α-subunits, play a role in cation binding. Fig. 1A depicts helical wheel representations of the TM4s of the Na,K- and H,K-ATPase. Residues marked with asterisks correspond to the
positions of the five Ca-ATPase amino acids (Ala-305, Ala-306, Glu-309, Gly-310, and Pro-312), which have been shown to play a role in calcium translocation (6, 16). It is interesting to note that all five residues are predicted to lie on the same helical face. It would appear, therefore, that TM4 has two domains or faces, one of which is likely to be directed toward the presumed ion translocation pore, whereas the other may face the surrounding lipid domain or adjoining helices. These two putative faces are separated by a dashed line (Fig. 1A).

Fig. 1B shows a sequence alignment of the TM4s of the Na,K- and H,K-ATPase α-subunits. The TM4s of Na,K- and H,K-ATPase differ at only eight amino acid positions marked by the solid black letters. Of these eight residues, only Leu-319, Asn-326, and Thr-340 of the sodium pump are located in the domain that faces the putative ion translocation pore (Fig. 1A). We have hypothesized, therefore, that these three residues might be intimately involved in establishing the distinct cation selectivities of the Na,K- and H,K-pumps. To test this possibility, construct ecto + A (Fig. 2B) was generated, which had these three TM4 residues mutated to their H,K-counterparts (L319F, N326Y, and T340S), and includes the ectodomain between TM3 and TM4. This ectodomain replacement resulted in two amino acid replacements compared with the sodium pump sequence, W312F, and E314R (Fig. 2C). To control for a contribution of the ectodomain the construct ecto was produced, which had only the ectodomain replaced with its H,K-ATPase counterpart (Fig. 2B).

Finally, for both constructs the first 72 amino acids of the Na,K-ATPase were replaced with the corresponding H,K-ATPase sequence, which consists of 85 amino acids. An antibody specific to this epitope allows us to distinguish the chimeric constructs, expressed in LLC-PK1 cells or Xenopus oocytes, from endogenous Na,K-ATPase. Previous studies demonstrated that this swap does not alter the functional properties of the Na,K-ATPase (4). Thus, construct H85N, which had only the N-terminal replacement, was used as the “wild type” control throughout the study (Fig. 2).

Measurements of Potassium-induced Ouabain-sensitive Pump Currents—A two-electrode voltage clamp of oocytes from Xenopus laevis was used to assess whether the chimeric constructs catalyze electrogenic ion fluxes. We took advantage of the species-dependent differences in ouabain sensitivity of the Na,K-ATPase (4). Thus, construct H85N, which had only the N-terminal replacement, was used as the “wild type” control throughout the study (Fig. 2).

For all of the following experiments crude plasma membranes from stably transfected LLC-PK1 cells were used. The LLC-PK1 cell line is derived from pig kidney. Pig Na,K-ATPase α1 has a Ki for ouabain of approximately 10^-7 M, whereas the rat-α1, which was used for our constructs, is only slightly affected by this low concentration of ouabain (K1 ~ 10^-4 M). Thus, at all times the assay solutions contained a low concentration of ouabain (10 μM), which is sufficient to inhibit the endogenous but not the exogenous Na,K-ATPase.
Sodium Dependence of the Ouabain-sensitive ATPase—We assessed the Na⁺ dependence of the ATPase activity of our constructs. The sodium titration experiments were performed both at pH 7.5 and at pH 6. At pH 7.5 the apparent sodium affinities for all three constructs were not significantly different from what had been reported for wild type enzyme. The $K_{1/2}$ for ecto + A was 1.5 mM, whereas $H85N$ and ecto yielded $K_{1/2}$ (Na⁺) values of 9.4 and 5.6 mM, respectively. Hill coefficients were between 1.1 and 1.5. It was especially interesting to note that the ATPase activity for ecto + A in the absence of sodium was 53% of $V_{\text{max}}$, whereas for $H85N$ the ouabain-sensitive ATPase activity with no sodium added to the incubation solution was only 5% of maximal. For ecto the ATPase activity under the same conditions was 13% of $V_{\text{max}}$ (Fig. 4). It would appear, therefore, that at low pH ecto + A can catalyze a substantial sodium-independent ATPase activity. This observation is consistent with the possibility that protons can effectively substitute for Na⁺ ions in the ecto + A chimera. According to this interpretation, the apparent decrease in $K_{1/2}$ (Na⁺) for ecto + A at low pH is attributable to protons replacing Na⁺ ions in the catalytic cycle of the pump, resulting in the loss of the requirement for cooperative sodium binding.

$pH$ Dependence of Ouabain-sensitive ATPase—If, in fact, protons can substitute for Na⁺ ions in the catalytic cycle of ecto + A, we would expect that an increased proton concentration would facilitate its sodium-independent enzymatic activity. To test this hypothesis, we measured the $pH$ dependence of the sodium-independent ATPase activity. The $pH$ of the assay solution was varied stepwise by 0.4 $pH$ units (corresponding to a 2.5-fold decrease in proton concentration). Within a range of $pH$ 6.0 to pH 8.0 for each $pH$ condition, assays were performed with either 0 or 60 mM Na⁺ in the incubation solution. We chose 60 mM for these experiments based on the results of the sodium titration experiments (Fig. 4), which demonstrated that this sodium concentration supports an enzymatic turnover rate close to $V_{\text{max}}$. Comparison of the fraction of sodium-independent ATPase activity (the ratio of ATPase activity at 0 mM sodium to activity at 60 mM sodium) indicates that for construct
the light of the large shift in K reaction cycle of this pump.

A facilitate the substitution of protons for sodium ions in the tially lower K and 0.73 and 1.4 mM for the K\textsubscript{1/2}(K\textsuperscript{+}) values were 1.2 and 2.75 mM, respectively. The corre-

Potassium Dependence of Ouabain-sensitive ATPase—In light of the large shift in K\textsubscript{v} for the potassium activation of pump-mediated current observed in the electrophysiological experiments (Fig. 3), it was important to investigate the potassium dependence of the ATPase activity (Fig. 6). At pH 7.5 the K\textsubscript{1/2}(K\textsuperscript{+}) values for ecto + A were 4.65 mM, whereas for H85N and ecto K\textsubscript{1/2}(K\textsuperscript{+}) values were 1.2 and 2.75 mM, respectively. The corre-

Vanadate (VO\textsubscript{4}\textsuperscript{3-}) Inhibition of Ouabain-sensitive ATPase— It is possible that the observed shifts in apparent affinity for potassium are due to a change in the steady-state E\textsubscript{1}/E\textsubscript{2} conformational equilibrium in favor of E\textsubscript{1}. To gain insight into this issue, we examined the inhibition of enzymatic activity by inorganic orthovanadate. Like inorganic phosphate, orthovanadate acts as a transition state analog, preferentially binding to the E\textsubscript{2} conformation of P-type ATPases to form a relatively stable intermediate (18–20). Thus, if the ecto + A construct preferentially accumulates in the E\textsubscript{2} conformations, we would expect to observe a larger IC\textsubscript{50} value for vanadate inhibition. Measurements of the vanadate sensitivities of the two control chimeras, H85N and ecto, and the ecto + A mutant were carried out under V\textsubscript{max} conditions at pH 7.5 and 6.0. IC\textsubscript{50} values for the vanadate-sensitive component of ouabain-sensi-

FIG. 5. pH dependence of sodium-independent ouabain-sensitive ATP hydrolysis. The pH dependence of the ATPase activity was determined with 60 and 0 mM Na\textsuperscript{+} in the incubation solution. The solutions also contained 3 mM ATP, 3 mM Mg\textsuperscript{2+}, 20 mM K\textsuperscript{+}, and 10 \mu M ouabain to abolish the endogenous sodium pump activity (details are given under “Experimental Procedures”). At 60 mM Na\textsuperscript{+} the pH dependence of the ATPase activity for all three constructs is almost identical to what has been observed for wild type Na,K-ATPase (data not shown). The plot represents the fraction of sodium-independent ATPase activity at different pH values. A very strong pH dependence can be seen for construct ecto + A (○), while the control constructs ecto (○) and H85N (●) show little or no pH dependence. Assays were done in triplicate, and the data represent averages over at least three assays.

FIG. 6. Potassium dependence of ouabain-sensitive ATPase ac-

The pH dependence of the ATPase activity was measured at pH 7.5 (○) and pH 6.0 (●). The incubation solution con-
tained 3 mM ATP, 3 mM Mg\textsuperscript{2+}, and 100 mM Na\textsuperscript{+}. To inhibit the endogenous sodium pump all solutions contained 10 \mu M ouabain (for details refer to “Experimental Procedures”). Each assay was performed in triplicate, and for each condition the data shown are the averages of at least three assays. The data were fitted to a Hill function. At pH 7.5 the following K\textsubscript{v}(K\textsuperscript{+}) were computed: for H85N (A) 1.2 mM, for ecto (B) 2.8 mM, and for ecto + A (C) 4.6 mM. At pH 6.0 K\textsubscript{v}(K\textsuperscript{+}) was determined to be 0.74 mM for H85N (A), 1.4 mM for ecto (B), and 2.5 mM for ecto + A (C).

Cation Selectivity of Na,K- and H,K-ATPases

In ouabain to abolish the endogenous sodium pump activity (details are shown). It is interesting that the relationship between the IC\textsubscript{50} value of 15 IC\textsubscript{50} for H85N was 42, and 151 \mu M for H85N, ecto, and ecto + A, respectively. At pH 6.0, IC\textsubscript{50} values were 26, 61, and 203 \mu M for H85N, ecto, and ecto + A, respectively (data not shown). It is interesting that the relationship between the IC\textsubscript{50} (VO\textsubscript{4}\textsuperscript{3-}) values obtained for the three constructs is similar to that observed for the apparent K\textsuperscript{+} affinities. This finding is consistent with the interpretation that the conformational equilibrium of the ecto + A construct is shifted to favor of E\textsubscript{2} forms. Finally, it should also be noted that the IC\textsubscript{50} of 15 \mu M for H85N corresponds well to observations of the wild type Na,K-ATPase conducted by Friedrich et al. (21) and Smith et al. (22).

To rule out the possibility that the changes in vanadate sensitivity are secondary to changes in K\textsuperscript{+} affinity, vanadate titrations were also carried out in the absence of K\textsuperscript{+}. These titrations were carried out at a low (micromolar) ATP concen-
Cation Selectivity of Na,K- and H,K-ATPases

**DISCUSSION**

The data presented here demonstrate that residues of the TM4 segments of the Na,K-ATPase and H,K-ATPase α-subunits are important for determining the distinct cation selectivities of these enzymes. A helical wheel analysis led us to propose that three TM4 residues might be especially relevant to cation selectivity. A construct ecto + A was generated in which the corresponding residues of H,K-ATPase α-subunit TM4 were substituted into the sequence of Na,K-ATPase α-subunit (L319F, N326Y, and T340S). When the activity of ecto + A was assayed at pH 6.0, it exhibited sodium-independent ATP hydrolysis, which was more than 50% of its V_max activity. This activity was not observed for the wild type-like H85N construct, and the ecto control construct had only slightly elevated sodium-independent ATPase activity. The sodium-independent ATPase activity was strongly pH-dependent, and at pH 7.5 it was not detectable. The likeliest explanation for the sodium-independent ATPase activity of ecto + A is that protons can substitute for sodium ions in this catalytic cycle of the construct. This conclusion is strongly supported by the pH dependence of the sodium-independent ATP hydrolysis, which is shown in Fig. 5. An increase in pH from pH 6.0 to pH 6.4 (corresponding to a 2.5-fold decrease in proton concentration) reduced the sodium-independent ATPase activity almost 2-fold.

In addition to the sodium-independent ATPase activity observed for ecto + A, we found that the K_1/2(Na^+) for this construct decreased from 8.2 mM at pH 7.5 to 1.5 mM at pH 6.0 (see Table I). This is kinetically consistent with a loss, at low pH, of the normal requirement of Na,K-ATPase for cooperative binding of three sodium ions to drive ATP hydrolysis and pump turnover. Replacement of one or two Na^+ ions by protons in the transport cycle would explain the observed behavior. If at pH 6.0, for example, two internal cation binding sites were occupied with protons or hydronium ions, the sodium stimulation as shown in Fig. 4 could reflect the binding of only one sodium ion to the third, highly specific sodium binding site. Indeed, the K_1/2(Na^+) of 1.5 mM is in reasonably good agreement with K_1/2 values reported by Schneeberger and Apell (23) for the binding of the third sodium ion.

In considering the structural basis for the change in the sodium versus proton selectivity observed with the ecto + A construct, it is tempting to focus on the exchanges L319F and N326Y, which introduce two bulkier aromatic residues into the presumpted cation translocation pore. The larger side chains of these residues might be expected to present a greater physical barrier for the binding and transport of the larger sodium ion compared with a proton. The validity of this argument is, of course, predicated on the extent to which the ions are hydrated.

In addition to the altered selectivity for sodium versus protons, a large (5–10-fold) decrease in apparent affinity for potassium was observed with construct ecto + A in ATPase assays and in current measurements (Table I). This observation led us to propose that in addition to altering the cation selectivity, the substitution of the TM4 residues also leads to a shift in the E_m/E_0 conformational equilibrium. Further experimental evidence supports this interpretation. Thus, the IC_50 shifts that were observed for vanadate inhibition of the ecto + A construct resemble the shifts observed for potassium K_1/2. It should be noted, however, that although these findings are consistent with a shift in conformational equilibrium, they do not constitute rigorous proof of such an alteration.

We cannot, for example, exclude the possibility that the intrinsic affinities of the K^+ and vanadate binding sites were altered. Thus, it is possible that the mutation E314R in the ectodomain between TM3 and TM4 reduces the intrinsic K^+ affinity because the replacement of a negatively charged glutamate with a positively charged arginine residue might cause a stronger electrostatic repulsion near the access to the extracellular K^+ binding sites. This could explain the small K_1/2(K^+) shift observed for the ecto construct, but the vanadate IC_50 shift and the large K_1/2(K^+) shift found for the ecto + A construct are unlikely to be due to a change in the intrinsic affinities for K^+ and vanadate. The shift in vanadate IC_50 persisted in the absence of potassium (Fig. 7), so we can rule out the possibility that it is only secondary to a change in the intrinsic potassium affinity (24). The possibility that the vanadate binding site itself has been altered also seems very unlikely, because none of the mutated residues are part of the presumed vanadate binding site or are even a close neighbor in the amino acid sequence. The proposed vanadate binding pocket is very highly conserved for all P-type ATPases and is thought to correspond

---

**Table I**

Summary of all K_1/2 and IC_50 values determined in this study

| Construct          | pH 7.5 | pH 6.0 | pH 6.0 |
|--------------------|--------|--------|--------|
| K_1/2(Na^+)        | 8.4 ± 0.7 | 9.2 ± 0.3 | 8.2 ± 1.0 |
| K_1/2(K^+)         | 0.56 ± 0.03 | 1.16 ± 0.05 | 5.6 ± 0.4 |
| K_1/2(K^+)_max     | 1.2 ± 0.1 | 2.8 ± 0.15 | 4.6 ± 0.5 |
| IC_50(VO_4^3-)     | 0.74 ± 0.1 | 1.4 ± 0.2 | 2.5 ± 0.4 |
| IC_50(VO_4^3-)_max | 14.9 ± 2.4 | 41.6 ± 9.9 | 151.0 ± 41.3 |
| IC_50(VO_4^3-)_max for H85N | 26.5 ± 5.4 | 61.0 ± 4.5 | 203.5 ± 49.5 |

---

**Fig. 7.** Vanadate inhibition titration of ouabain-sensitive ATPase activity in the absence of potassium. The assay was carried out in the absence of potassium and in the presence of 1 mM [γ-32P]ATP and 2 mM NaCl. The solution also contained 5 μM ouabain to suppress contributions from the endogenous sodium pump (for details see “Experimental Procedures”). The data illustrate that the large shift in vanadate IC_50 for ecto + A (○) compared with ecto (▲). Vanadate inhibition of construct H85N was virtually identical to the inhibition observed for ecto (not shown). The data shown are a representative single experiment carried out in triplicate.
to the region between amino acids 369 to 378 of the Na,K-ATPase (25, 26).

Additional support for a change in the E1/E2 conformational equilibrium comes from the observation that the ouabain sensitivity of the ecto + A construct is reduced compared with that of H85N. In the electrophysiological experiments 5 mU ouabain inhibited the current produced by H85N virtually completely, whereas for ecto + A a current corresponding to 15–20% of the total potassium-induced current remained in the presence of this drug. None of the residues altered in our study are thought to play a role in ouabain binding. Ouabain predominantly binds this drug. None of the residues altered in our study are thought to play a critical role in distinguishing between Na+

In a recent study of gastric H,K-ATPase and Na,K-ATPase chimeras (4), it was shown that the N-terminal half of the cytoplasmic loop between TM4 and TM5 (chimera H519N) confer marked increases in selectivity for protons relative to Na+ ions, even at pH 7.4, when compared with the H85N control. The data presented here demonstrate that residues in TM4 contribute to the dramatic shift observed with H519N. It seems likely that specificity is determined through a concerted interaction between the identified residues in TM4 and sequences within the loop between TM4 and TM5. Construction of further chimeras will be required to test this proposition directly.

The results of the present study indicate that transmembrane residues can also influence conformational equilibria. Similar conclusions can be drawn from the previous Na,K-/H,K-ATPase chimera studies. Thus, the H519N but not the H85N/H356–519N displayed reduced vanadate sensitivity (4). Similarly, Vilsen (5) has shown that the mutation of Leu-332 to alanine in TM4 of the Na,K-ATPase resulted in a conformational equilibrium shift toward E2. Perhaps not surprisingly, therefore, it appears that residues in the transmembrane domains contribute to the determination of conformational equilibria as well as participating in the formation of the ion binding and conduction pathway. Conversely, analysis of the H85N/H356–519N chimera demonstrates that changes in cation affinities can be produced through mutations or substitutions outside of predicted transmembrane domains (4). The small but significant sodium-independent ATPase activity observed with ecto at low pH is also consistent with the conclusion that sequences flanking membrane domains can influence cation selectivity. The mechanism through which these separate domains of the pump molecules interact to modulate specificity and conformation remain to be established.

In conclusion, this study elucidates three specific residues of TM4 that are important for the distinct cation selectivity properties of Na,K- and H,K-ATPases. These residues also impact upon transitions among the conformational states of these enzymes. Together with our recent work (4) it becomes evident that cation selectivity of P-type ATPases is determined not only by transmembrane domains but by an interplay of transmembrane domains and their flanking regions. These findings underscore the complex and cooperative nature of cation translocation by P-type ATPases.

Acknowledgments—We are grateful to Vanathy Rajendran and Ania Wilczynska for excellent technical support. We are also indebted to Biff Forbush, Alexander Grishin, Joe Hoffman, and Clifford Slayman for valuable discussions and advice. Special thanks to Tiffany Runyan Garrison for reading and critiquing the manuscript.

REFERENCES

1. Moller, J. V., Juul, B., and le Maire, M. (1996) Biochim. Biophys. Acta 1286, 1–51
2. Courtois-Coutry, N., Roush, D., Rajendran, V., McCarthy, J. B., Geibel, J., Kashgarian, M., and Caplan, M. J. (1997) Cell 90, 501–510
3. Doucet, A. (1997) Exp. Nephrol. 5, 271–276
4. Blaschko, B. D., Dunbar, L. A., Mense, M., Scanlan, R., Wilczynska, A., and Caplan, M. J. (1999) J. Biol. Chem. 274, 18574–18581
5. Vilsen, B. (1997) Biochemistry 36, 13312–13324
6. Andersen, J. P., and Vilsen, B. (1995) FEBS Lett. 359, 101–106
7. Krieg, P. A., and Melton, D. A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2533–2535
8. Fyfe, G. K., and Canessa, C. M. (1998) J. Gen. Physiol. 112, 423–432
9. Horisberger, J. D., Jaunin, P., Good, P. J., Rossier, B. C., and Geering, K. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8397–8400
10. Vilsen, B. (1992) FEBS Lett. 314, 301–307
11. Jorgensen, P. L., and Petersen, J. S. (1982) Biochim. Biophys. Acta 705, 38–47
12. Ketelaar, P. M., and Oudshoorn, R. G. M. (1995) Biochemistry 34, 9435–9440
13. Gordon, J. A. (1991) Methods Enzymol. 201, 477–482
14. Forbesch, B. D. (1983) Anal. Biochem. 128, 159–163
15. Galy, S. E., Lane, L. K., and Blaschko, R. (1984) J. Biol. Chem. 269, 23944–23948
16. MacLennan, D. H., Toyofuku, T., and Lytton, J. (1992) Ann. N. Y. Acad. Sci. 671, 1–10
17. Kuntzweiler, T. A., Wallick, E. T., Johnson, C. L., and Lingrel, J. B. (1995) J. Biol. Chem. 270, 2999–3006
18. Harris, S. L., Perlin, D. S., Seto-Young, D., and Haber, J. E. (1991) J. Biol. Chem. 266, 24439–24445
19. Bozhenkova, N. D., Daly, S. E., Javadi, Z. Z., Lane, L. K., and Blaschko, R. (1998) J. Biol. Chem. 273, 23086–23092
20. Ambesi, A., Pan, R. L., and Slayman, C. W. (1996) J. Biol. Chem. 271, 22999–23005
21. Friedrich, T., Bamberg, E., and Engel, A. (1996) Biochim. Biophys. J. 71, 2348–2350
22. Smith, R. L., Zinn, K., and Cantley, L. C. (1980) J. Biol. Chem. 255, 9852–9859
23. Schneeberger, A., and Apell, H. J. (1995) J. Membr. Biol. 168, 221–228
24. Sachs, J. R. (1987) J. Gen. Physiol. 89, 291–320
25. Rao, R., and Slayman, C. W. (1993) J. Biol. Chem. 268, 6708–6713
26. Serrano, R., and Portillo, F. (1990) Biochim. Biophys. Acta 1018, 195–199
27. Anker, B. M., Moosmayer, M., and Imesch, E. (1994) Mol. Membr. Biol. 11, 237–245
28. Vilsen, B. (1995) FEBS Lett. 363, 179–183
29. Grishin, A. V., Sverdlov, V. E., Kostina, M. B., and Modyanov, N. N. (1994) FEBS Lett. 349, 144–150