Cation Selectivity of Gastric H,K-ATPase and Na,K-ATPase Chimeras*

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Rhoda Blostein, Lisa Dunbar, Martin Mense, Rosemarie Scanzano, Ania Wilczynska, and Michael J. Caplan

From the Departments of Medicine and Biochemistry, McGill University, Montreal, Quebec H3G 1A4 Canada and the Department of Cell and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06510-8026

Chimeras of the catalytic subunits of the gastric H,K-ATPase and Na,K-ATPase were constructed and expressed in LLC-PK1 cells. The chimeras included the following: (i) a control, H519N (the first 85 residues comprising the cytoplasmic N terminus of Na,K-ATPase replaced by the analogous region of H,K-ATPase); (ii) H85N/H356–519N (the N-terminal half of the cytoplasmic M4–M5 loop also replaced); and (iii) H519N (the entire front half replaced). The latter two replacements confer a decrease in apparent affinity for extracellular K⁺. The 356–519 domain and, to a greater extent, the H519N replacement conferred increased apparent selectivity for protons relative to Na⁺ at cytoplasmic sites as shown by the persistence of K⁺ influx when the proton concentration is increased and the Na⁺ concentration decreased. The pH and K⁺ dependence of ouabain-inhibitable ATPase of membranes derived from the transfected cells indicate that the H519N and, to a lesser extent, the H356–519N substitution decrease the effectiveness of K⁺ to compete for protons at putative cytoplasmic H⁺ activation sites. Notable pH-independent behavior of H85N/H356–519N at low Na⁺ suggests that as pH is decreased, Na⁺/K⁺ exchange is replaced largely by (Na⁺ + H⁺)/K⁺ exchange. With H519N, the pH and Na⁺ dependence of pump and ATPase activities suggest relatively active H⁺/K⁺ exchange even at neutral pH. Overall, this study provides evidence for important roles in cation selectivity for both the N-terminal half of the M4–M5 loop and the adjacent transmembrane helix(es).

The gastric H,K-ATPase and ubiquitous Na,K-ATPase have the highest sequence similarity (62% amino acid sequence homology) of the phosphorylating class (P-type) of ion motive ATPases. The reaction sequence catalyzed by these enzymes involves ATP binding followed by cation-dependent phosphorylation and dephosphorylation of an aspartyl residue at the active site, as well as conformational and vectorial transitions of phospho- and dephosphoenzymes. These reactions transduce the chemical energy of ATP hydrolysis into cation binding at one side of the membrane followed by occlusion in an ion-binding pocket and then cation release at the opposite side of the membrane. Sequence similarity between these enzymes is greatest in the regions associated with ATP binding and phosphorylation (for review see Ref. 1). The basic structural and reaction sequence similarity notwithstanding, the catalytic subunit of each is associated with a distinct non-catalytic β subunit, and the two enzymes differ with respect to their cation selectivity and sensitivity to inhibitors. However, we showed earlier that the affinity or selectivity of these two enzymes for cations is not absolute. Although the selectivity and/or reactivity of the Na,K-ATPase for H⁺ (H₂O⁻) and the H,K-ATPase for Na⁺ are low, nevertheless the Na,K-ATPase can pump protons in place of Na⁺ ions at acidic pH (2), and the gastric H,K-ATPase, Na⁺ in place of protons at alkaline pH (3). This behavior underscores the similarities between these two enzymes and has the important implication that the cation binding domain probably involves a cluster of oxygen or possibly nitrogen atoms coordinating with either Na⁺ or hydrated protons (H₂O⁻) reminiscent of complexes formed with cyclic polyethers as discussed by Boyer (4).

To gain insight into the structural basis for the distinct cation selectivity of the two P-type pumps, we have initiated studies of the selectivity of Na⁺ versus protons for activation of activity using pumps comprising chimeric catalytic subunits of the rat α1 subunit of Na,K-ATPase and the gastric H,K-ATPase. Presumably, regions of dissimilarity in their α subunits include structures which confer the distinct ligand binding/reactivities of the two ion pumps.

In an earlier study (5), we showed that the replacement of the front-half of the Na,K-ATPase with that of the H,K-ATPase produced a functional chimera, H519N, which could be readily distinguished from the parent enzymes by virtue of its distinct sensitivity to ouabain. Unlike the parent H,K-ATPase, its activity is ouabain-sensitive, albeit with much lower sensitivity than that of the endogenous highly ouabain-sensitive pig kidney Na,K-ATPase of the LLC-PK₁ cells into which this chimera was transfected. In the present study, we have focused our attention on the cation selectivity changes affected by chimeric replacements within the front-half of the Na,K-ATPase, namely an N-terminal H85N chimera which served as a control and two others of which one (H85N/H356–519N) comprises an H356–519N as well as the H85N replacement, and the other, H519N, which comprises the entire front-half of the gastric H,K-ATPase appended to the back-half of the Na,K-ATPase.

EXPERIMENTAL PROCEDURES

Construction of Chimeras—The chimeras presented in this paper were generated between the rat Na,K-ATPase α1 subunit (cDNA provided by E. Benz, The Johns Hopkins University) and the rat gastric H,K-ATPase α subunit (cDNA provided by G. Shull, University of Cincinnati). Fragments of each cDNA were exchanged using the restriction sites ApoI, corresponding to H,K-ATPase amino acid number 85, Hpα1 at amino acid 356, and NarI at amino acid 519. The ApoI and Hpα1 sites were introduced into the cDNAs using the Kunkel method of site-directed mutagenesis. The Na,K-ATPase α subunit was mutated at
base pair (bp)4 465 Cys → Gly to form the ApoI site and at bp 1273 Cys → Thr and Cys → Ala at bp 1275 to form the HpaII site. The H,K-ATPase α subunit already contained an ApoI site but was mutated at bp 1268 Ala → Gly, bp 1269 Cys → Thr, and bp 1271 Gly → Ala to introduce the HpaII site. These mutations did not change the total surface charge of the H85N/356–519N was constructed using pSP72 (Promega, Madison, WI), which contains an ApoI site in the polylinker of pSP72 upstream of the CDNA insert, by subcloning the small ApoI fragment of the H,K-ATPase into the complementary portion of the Na,K-ATPase. Chimera H85N/H356–519N was created by inserting the HpaII/NarI fragment from the H,K-ATPase α subunit into chimera H85N. Both constructs were sequenced. To change the library into the mammalian expression vector pCB6 (kindly provided by M. Roth, University of Texas Southwestern) which carries resistance to the antibiotic G418 (Life Technologies, Inc.), transfected cells were selected in media containing 1.8 mg/ml G418. Clones were screened for expression of the chimera-mediated current using a cooperative model of K+ activation, with the Hill function n = 2; kinetic parameters for Na+ activation (K+Na and Vmax) were obtained using a 3-site non-cooperative model. Each value shown represents the mean ± S.E. of the difference between the means of triplicate determinations carried out in the presence of low (10−5 M) and high (5 × 10−3 M) ouabain concentrations.

Measurements of Pump Current in Transfected Oocytes—Electrophysiological measurements of the chimera-mediated current were similar to those described by Horisberger et al. (12). Initially, oocytes were loaded with Na+ by incubating them for 2 h in a solution free of K+ or Ca2+ ions (97 mM Na+, 0.82 mM Mg2+, 0.5 mM EGTA, 22.5 mM Cl−, 76 mM gluconate, 10 mM Hepes (pH 7.4)) The Na+-loading step was followed by a 15-min incubation in a K+-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 mM ouabain, and 10 mM Hepes (pH 7.4)), which contained 5 mM Ba2+ in order to block channel-mediated K+ currents. Finally, oocytes were transferred to the chamber of the voltage clamp set up that contained the same K+-free bath solution. The following protocol was followed to measure whole-cell currents at a holding potential of −50 mV; once the base line had stabilized, the K+-free bath solution was replaced with a K+-containing solution, and the K+-induced current was recorded for 5 min. The difference between the K+-induced and K+-free solution was that a fraction of Na+ ions had been replaced with K+ ions. The sum of the concentrations of Na+ and K+ was always 97 mM. Following recording of the current, the K+-containing solution was

1 The abbreviations used are: bp, base pair; MDCK, Madin-Darby canine kidney; MES, 4-morpholineethanesulfonic acid.

Results

In order to characterize domains of the catalytic α subunit which confer the distinct cation selectivities of the Na,K- and gastric H,K-ATPases, we have assessed the functional consequences of replacing regions of the one enzyme by those of the other. The focus of the present experiments has been on regions within the N-terminal half of these enzymes. These chimeras were expressed in both mammalian epithelial cell lines (MDCK and LLC-PK1) and, in the cases of H85N and H85N/H356–519N in Xenopus oocytes. All included replacement of the cytoplasmic N terminus of the Na,K-ATPase (residues 1–85) with the homologous region of the gastric H,K-ATPase. This strategy permits assessment of protein expression using antibodies raised against the N terminus of the H,K-ATPase. Accordingly, the chimera H85N served as a control for other chimeras.

Selection of Chimera-Transfected Cells in Ouabain—Following selection in G418 as described earlier (5), growth of the transfected cells in medium containing a low concentration (1 µM) of ouabain was used for selection of cells expressing functional rat H,K-/Na,K-ATPase chimeras. All three chimeras survived this selection, the H85N control and the chimeras involving...
ing the substitution of (i) the front-half of the cytoplasmic M4–M5 loop (H85N/H356–519N) and (ii) the entire front-half gastric H,K-ATPase/back-half Na,K-ATPase (H519N). Although as reported previously (5) the H519N-transfected cells did not grow initially in ouabain, they were gradually adapted to growth in ouabain by first selecting cells resistant to G418 and then replacing the medium containing G418 (cf. Ref. 5) with medium containing micromolar ouabain and elevated (5 mM) K+, a strategy first used by Arguello and Lingrel (15) for the S775A mutant. Relative to H85N and H85N/H356–519N, the doubling time of H519N-transfected cells was increased 2–3-fold.

**Pump-mediated Rb**<sup>+</sup> **Influx**—Preliminary experiments showed that the behavior of the control H85N in MDCK cells with respect to apparent affinities for activation of K<sup>+</sup> influx by extracellular K<sup>+</sup> (K<sub>act</sub>) or varying intracellular Na<sup>+</sup> (Na<sub>app</sub>) was similar to that of the endogenous ouabain-sensitive dog kidney enzyme (not shown), suggesting that this region does not have an important role in cation interactions. In subsequent studies, the behavior of transfected LLC-PK<sub>1</sub> rather than MDCK cells was studied for the following reasons. First, with membranes isolated from LLC-PK<sub>1</sub> cells, the fraction of total Na,K-ATPase activity insensitive to low (10<sup>−8</sup>–5 mM) but sensitive to high (2.5 × 10<sup>−3</sup> mM) ouabain concentrations was greater than that observed with membranes isolated from MDCK cells. Second, with control H85N-transfected LLC-PK<sub>1</sub>, but not MDCK cells, the pump-mediated K<sup>+</sup> influx into cells equilibrated and then assayed in Na<sup>+</sup>-free medium was essentially zero. This is probably due to the absence, or a substantially lower amount of nonspecific binding of Na<sup>+</sup> or, more likely, trapping of cations in interstitial spaces of LLC-PK<sub>1</sub> cells compared with MDCK cells.

To determine whether the H519N and H356–519N substitutions alter cation interactions, the transport behaviors of these chimeras were compared with that of the H85N control under conditions of varying pH, Na<sup>+</sup>, and K<sup>+</sup> concentrations, with monensin added to maintain a constant concentration of extracellular K<sup>+</sup> with respect to apparent affinities for activation of K<sup>+</sup> influx into transfected LLC-PK<sub>1</sub> cells equilibrated with 20 mM Na<sup>+</sup> at pH 6.0 (Fig. 1A) and pH 7.4 (Fig. 1B). Higher Na<sup>+</sup> concentrations were avoided in order to minimize changes in intracellular pH due to putative Na<sup>+</sup>/H<sup>+</sup> exchange. The data are plotted as percentages of V<sub>max</sub> values given in the legend. The results show that the substitution of the N-terminal half (residues 356–519) of the M4–M5 loop of the gastric pump into the homologous regions of the Na,K-ATPase decreases the apparent affinity for K<sup>+</sup> and that the fold change relative to H85N is similar for both H85N/H356–519N and H519N (~6–7-fold higher K<sub>0.5(K</sub>) at pH 6.0 and 4.4-fold higher K<sub>0.5(K</sub>) at pH 7.4; see values in legend). Accordingly, a significant difference between H85N/H356–519N and H519N could not be detected with cells assayed in 20 mM Na<sup>+</sup>. With cells equilibrated at pH 7.4 and assayed at lower Na<sup>+</sup> concentration (10 mM) the fold difference from the control H85N was reduced similarly for both H85N/H356–519N and H519N chimeras at pH 7.4 (2-fold higher K<sub>0.5(K</sub>), experiment not shown). However, with both the sodium concentration reduced and the proton concentration increased (Fig. 1C; cells assayed at 10 mM Na<sup>+</sup> and pH 6.0), K<sub>0.5(K</sub>), values 4- and 8-fold higher than that of H85N were observed with H85N/H356–519N and H519N, respectively (see values in legend). In subsequent K<sup>+</sup> influx assays, the medium K<sup>+</sup> concentration was 4 mM in order to ensure close to saturation of all chimeric pumps. V<sub>max</sub> values of all chimeras were reduced over 50% as the pH was decreased from pH 7.4 to pH 6.0 (see legend) due, presumably, to a decrease in overall catalytic activity as pH is decreased.

![Figure 1. Effect of varying K⁺ concentration on ouabain-sensitive ([³⁸Rb]⁺ K⁺ influx into transfected LLC-PK₁ cells at pH 6.0 and pH 7.4. Assays were carried out as described under “Experimental Procedures” with cells equilibrated with 10 mM Na⁺ and the indicated concentrations of KCl in the medium. Cells were equilibrated and assayed in medium containing 20 mM Na⁺ at either pH 6.0 (A) or pH 7.4 (B) or 10 mM Na⁺ at pH 6.0 (C). Values for K<sub>0.5(K</sub>), were obtained from a competitive 2-site model described by the following form of the Hill equation: \( v = \frac{V_{\text{max}}[K]^{n}K_{\text{app}}^{5}/[K]^{5}}{K_{\text{app}}^{5}+[K]^{5}} \) where K<sub>app</sub> = K<sub>1/2</sub>. For H85N, H85N/H356–519N, and H519N, respectively, values of K<sub>app</sub> (mm) were 0.19, 1.10, and 1.36 at pH 6.0 and 20 mM Na⁺ (A), 0.31, 1.35 and 1.35 at pH 7.4 and 20 mM Na⁺ (B), and 0.19, 0.75, and 1.56 at pH 6.0 and 10 mM Na⁺ (C), respectively. Values of V<sub>max</sub> (nmol/mg/min) for the three chimeras were, respectively, 2.81, 1.41, and 0.47 (A), 6.55, 4.72, and 2.5 (B), and 1.11, 2.42, and 0.78 (C).]
brated with either 0 or 20 mM Na$^+$ are shown in Fig. 3. For all three chimeras, activity in the presence of Na$^+$, presumably Na$^+$/K$^+$ exchange, decreases as a function of decreasing pH. In the absence of Na$^+$, pump activity of the control H85N is barely detectable but persists in H85N/H356–519N and H519N chimeras, particularly the latter.

Although the experiments presented in Figs. 2 and 3 suggest that under Na$^+$-free conditions the H85N/H356–519N and H519N chimeras catalyze an exchange of protons for K$^+$, it is unlikely that intracellular Na$^+$ is completely removed, even in cells equilibrated and assayed in Na$^+$-free medium in the presence of monensin (see Fig. 5 of Ref. 8). Several explanations are plausible. Either the apparent affinity for Na$^+$ of the H519N chimera and, to a lesser extent, the H85N/H356–519N chimera are markedly increased compared with the control H85N and/or one or both of these chimeras can use protons in place of Na$^+$ (H$^+$/K$^+$ exchange) or together with Na$^+$ ((H$^+$/Na$^+$)/K$^+$ exchange).

To gain explicit information about proton versus Na$^+$ selectivity affected by the H356–519N and H519N substitutions, it was necessary to assay activity using Na$^+$-free preparations. Well washed porous membranes, although lacking sidedness, are nominally Na$^+$-free and thus satisfy this criterion. In the experiments described below, we tested the effects of pH and varying Na$^+$ and K$^+$ concentrations on this component of ATPase activity of LLC-PK$_1$ membranes, henceforth referred to as “ouabain-sensitive ATPase.” With membranes, it was feasible to assay activity at concentrations of protons (pH 5.5) greater than feasible with cells (pH ≥ 6.0).

Cation Dependence of ATPase Activity—We first characterized the three chimeras with respect to apparent affinity for ATP. This allowed for subsequent assays to be carried out at optimal but not excessive concentrations of ATP, thereby maximizing the sensitivity of the assays. As shown in Fig. 4, the chimeric replacements altered markedly the apparent affinity of the enzyme for ATP. This holds true particularly for H519N. Thus, $K'_\text{ATP}$ values determined at pH 7.4 with optimal Na$^+$ (100 mM) and K$^+$ (10 mM) concentrations were 124, 45, and 1.92 μM ATP for H85N, H85N/H356–519N, and H519N, respectively. $K'_\text{ATP}$ was reduced approximately 4-fold further when the pH was reduced to pH 5.5 (experiments not shown). Based on these results, the effects of cations were tested under the conditions of saturating concentrations of ATP. Thus (and unless indicated otherwise), the ATP concentration was 50 μM for experiments carried out at low pH (pH 5.5) and 250 μM for experiments carried out at pH 7.4 with the H85N and H85N/H356–519N chimeras. Assays of the H519N chimera were carried out at 10 μM ATP at both pH values. Despite the low Na,K-ATPase activity of the H519N preparations at pH 7.4 (see legend to Fig. 4), for all three chimeras, these concentrations of ATP allowed for maximal percentages (at least 25% of the total ATP hydrolyzed) of pump-associated ATP hydrolysis.

Evidence for Ouabain-sensitive K$^+$-ATPase Catalyzed by H85N/H356–519N and H519N—The Na$^+$ dependence of ouabain-sensitive ATPase activity of membranes derived from mutant-transfected LLC-PK$_1$ cell membranes with constant K$^+$ concentration (10 mM) at pH 5.5 is shown in Fig. 5. The results indicate that in the absence of Na$^+$, an ouabain-sensitive ATPase activity is observed with the H85N/H356–519N and H519N chimeras at pH 5.5, whereas little, if any, activity is observed with the control H85N chimera. In fact, the activity of H519N at pH 5.5 was similar in the absence or presence of Na$^+$.

The pH sensitivity profiles of K$^+$ influx into cells equilibrated with either 0 or 20 mM Na$^+$ are shown in Fig. 3. For all three chimeras, activity in the presence of Na$^+$, presumably Na$^+$/K$^+$ exchange, decreases as a function of decreasing pH. In the absence of Na$^+$, pump activity of the control H85N is barely detectable but persists in H85N/H356–519N and H519N chimeras, particularly the latter.
presence of K⁺. For these chimeras, the data were fitted to a 3-site non-cooperative model. Their Na⁺ activation profiles indicate similar values of K<sup>⁺</sup>Na of which the significance vis a vis experiments carried out at higher K⁺ concentration is discussed below.

Experiments aimed to assess the K⁺ dependence of the putative H⁺/K⁺-ATPase activity effected by the chimeric substitutions are described in Fig. 6. The results indicate that at acidic pH and absence of Na⁺, the activities of all three chimeras increase with addition of 0.5 to 2 mM K⁺. With higher K⁺ concentration (≥ 10 mM), the activities of the H519N and, to a lesser extent, the H85N/H356–519N chimeras remain relatively high, whereas that of the control becomes markedly inhibited. These results suggest that the H356–519N and H519N replacements alter K⁺/H⁺ antagonism at cytoplasmic H⁺ activation site(s). Support for this notion was obtained by the following observations (experiments with H85N and H85N/H356–519N, not shown). (i) When K⁺ inhibition was determined as a function of pH, K⁺ inhibition increased (comparison of activity at 10 mM to that at 0.5 mM K⁺) as the pH was decreased from pH 6.0 to pH 5.0 in the case of the H85N control but not the H85N/H356–519N chimera. (ii) Analogous differences in K⁺/Na⁺ antagonism at cytoplasmic Na⁺ activation sites were observed (experiments not shown). Thus, the apparent affinity for Na⁺ at pH 7.4 decreased at least 2-fold as the K⁺ concentration was increased from 10 to 50 mM in the case of the H85N control chimera, whereas little change was observed with the H85N/H356–519N chimera, i.e. K<sub>Na⁺</sub> values for H85N were 1.8 and 4.2 mM at 10 and 50 mM K⁺, respectively; the corresponding values for the H85N/H356–519N chimera were 2.2 and 2.5 mM (experiment at 50 mM K⁺, not shown). Similarly, in experiments carried out at pH 7.4 (not shown), evidence of Na⁺/K⁺ antagonism was obtained with the K⁺ concentration varied at low (5 mM) Na⁺ concentration; maximal activity was observed at 2–5 mM K⁺, and increasing the K⁺ concentration from 5 to 50 mM effected marked inhibition of the Na⁺,K⁺-ATPase of the control H85N but not H85N/H356–519N chimera. These differences in effects of cytoplasmic K⁺ are probably the basis for the notable differences in levels of pump-mediated K⁺ influx into intact cells relative to Na⁺,K⁺-ATPase activity of porous membranes among the different chimeras (compare V<sub>max</sub> values in the legends of Figs. 1B and 4). It is plausible that the high K⁺:Na⁺ concentration ratio of the intact cells results in underestimation of the maximal pump activity in H85N and to a lesser extent H85N/H356–519N.

**pH Dependence of Activity Measured in the Presence of Low Na⁺ Concentration**—To gain insight into the nature of cation exchanges associated with the altered sodium versus proton selectivity caused by the H356–519N and H519N replacements, the pH sensitivities of the three chimeras were determined in the presence of 2 mM K⁺ and relatively low Na⁺ concentration (5 mM). The results shown in Fig. 7 indicate a pH-dependent decrease in activity of the H85N control and H519N chimera typical of wild type Na⁺,K⁺- and H⁺,K⁺-ATPases. The important observation was that the H356–519N replacement results in little if any decrease in activity. This result taken together with (i) the decrease in activity at low pH in the absence of Na⁺ (Fig. 5A) suggests that at low pH the H85N/H356–519N chimera catalyzes an electrogenic or electroneutral exchange of cytoplasmic Na⁺ plus H⁺ for extracellular K⁺ ions in the presence of Na⁺ or H⁺/K⁺ exchange in the absence of Na⁺.

**Vanadate Sensitivity**—The question of whether the alterations in apparent cation selectivity are secondary to major conformational changes was tested by probing the steady-state conformational equilibrium between E<sub>2</sub> and E<sub>3</sub> forms of the enzyme. The strategy was to use inorganic orthovanadate which acts as a transition state analog of inorganic orthophosphate thus binding to the E<sub>2</sub> conformation of P-type ATPases to form a relatively stable intermediate (16, 17). A comparison of the vanadate sensitivity of H85N and H85N/H356–519N assayed in the presence of 100 mM Na⁺ and 10 mM K⁺ at pH 7.4
indicated that for both, the $I_50$ value for vanadate inhibition was $3 \times 10^{-5}$ M, and a difference between the two could not be detected. This behavior contrasts with that of $E_1/E_2$ conformational mutants of Na,K-ATPase (18) and of other P-type pumps (19, 20). In contrast to H85N/H356–519N, the H519N substitution results in a decrease in vanadate sensitivity. Even at the highest concentration tested ($10^{-2}$ M), the activity was only reduced $< 50\%$. This behavior taken together with the very high apparent affinity of H519N for ATP indicate that the steady-state conformational equilibrium of this chimera is shifted toward $E_1$.

**DISCUSSION**

The notion that specific residues in transmembrane segments of the catalytic subunit of P-type pumps bind transported cations directly is well supported by experiments involving site-specific alterations of the sarcoplasmic reticulum Ca$^{2+}$-ATPase (reviewed in Refs. 1 and 21) and, more recently, the Na,K-ATPase (for review see Ref. 22). In the cases of the two most closely related P-type pumps, the Na,K-ATPase and gastric H,K-ATPase, it was also shown that Na$^+$ ions and protons interact with sites of overlapping cation specificity (2, 3). Since cation binding, occlusion, and translocation are intimately coupled to ATP hydrolysis, it is not unexpected that alterations in the large M4–M5 cytoplasmic loop bearing the ATP binding and phosphorylation sites impact the cation selectivity of putative cation ligating residues in adjacent transmembrane helices. The point of evidence is the increase in the apparent affinity for protons relative to Na$^+$ observed with the chimeric enzyme comprising residues 356–519 of M4–M5 of the Na,K-ATPase replaced by the analogous region of the H,K-ATPase. Thus, compared with the H85N control, the chimeric H85N/H356–519N enzyme functions as an H,K-ATPase in the absence of Na$^+$ and at elevated proton concentration (Figs. 2A and 5A).

The experiments in Fig. 6 showing distinct effects of K$^+$ on the ATPase activity of the H85N/H356–519N chimera provide insight into the basis for the alteration in usage of protons versus Na$^+$ caused by the H356–519N replacement. The relevant observations are as follows. Similarly to the wild type Na,K-ATPase under conditions of low pH and absence of Na$^+$ (2, 23), ATPase activity of the H85N and H85N/H356–519N chimeras was carried out at pH 5.5 at 250, 50, and 10 $\mu$M ATP, respectively. Maximal activities of the three chimeras were 5.34, 4.66, and 1.25, respectively.

![Figure 5](image1.png)

**FIG. 5.** Na$^+$ activation kinetics at pH 5.5 and 7.4. A, activity at pH 5.5 in the presence of 10 mM KCl. Values shown are percentages of the maximal activities observed. B, activity at pH 7.4 in the presence of 10 mM KCl. Values shown for H85N and H85N/H356–519N are percentages of the $V_{max}$ values calculated as described in Fig. 2. For H85N and H85N/H356–519N, values of $K_{Na}$ (mM) are 1.8 and 2.2, respectively.

![Figure 6](image2.png)

**FIG. 6.** Effect of K$^+$ concentration on ouabain-sensitive ATPase activity measured at acidic pH in the absence of Na$^+$. Assays of the H85N, H85N/H356–519N, and H519N chimeras were carried out at pH 5.5 at 250, 50, and 10 $\mu$M ATP, respectively. Maximal activities of the three chimeras were 5.34, 4.66, and 1.25, respectively.

![Figure 7](image3.png)

**FIG. 7.** pH sensitivity profile of Na,K-ATPase activity measured at low Na$^+$ concentration. Assays were carried out as described in Fig. 4 except with 5 mM NaCl and 2 mM KCl and the same ATP concentrations as those used for Fig. 6. Values of activity (nmol/mg/min) representing 100% activity are shown in parentheses.
substitution and probably accounts for the substantial pump-mediated K⁺ influx into the H85N/H356–519N, but not H85N-transfected cells equilibrated and assayed in Na⁺-free medium at acidic pH (Fig. 2A). Presumably, the concentration of intracellular K⁺ is high in these metabolically active cells. Even with Na⁺ present at concentrations up to ~5 mM, the ATPase activity of the H85N/H356–519N chimera is notably greater than that of the control at acidic pH. The observation that at higher pH K⁺/Na⁺ antagonism is also diminished in the H85N/H356–519N chimera indicates that the affinity for K⁺ at intracellular cation activation sites is diminished irrespective of whether the activating cation is H₂O⁺ or Na⁺. It is interesting that at both low and high pH, the H356–519N substitution also lowers the apparent affinity for extracellular K⁺ at activating site(s) (Fig. 1, A and B).

When the chimeric replacement comprises, instead, not only the N-terminal half of the M4–M5 loop but rather the entire N-terminal half of the catalytic subunit, the selectivity of protons over Na⁺ is increased to the extent that the enzyme catalyzes ouabain-sensitive H⁺/K⁺ exchange at a rate 60% that of Na⁺/K⁺ exchange at pH 6.0. Furthermore, at even lower pH (pH 5.5), the K⁺-ATPase activity catalyzed by H519N in the absence of Na⁺ is similar to that observed in the presence of Na⁺. Other consequences of the H519N replacement are the further marked reduction in cytoplasmic K⁺/H⁺ antagonism (Fig. 6) as well as reduction in apparent affinity for extracellular K⁺ under the condition of suboptimal Na⁺ concentration and elevated proton concentration (pH 6.0; Fig. 1C). Overall, these results imply differences in the chimeras, in residue(s) within the cation binding pocket, which comprise the shared ligands for K⁺, Na⁺, and H₂O⁺ and thus affect selectivity for cation binding at both intra- and extracellular sites.

Although the ATPase experiments carried out with porous membranes support the conclusion that the H85N/H356–519N pump catalyzes H⁺/K⁺ exchange, it is likely that at acidic pH and in the presence of Na⁺ at low concentration, this chimera catalyzes H⁺ plus Na⁺/K⁺ exchange either concurrently or even exclusively, with greater activity than deduced for the wild type Na,K-ATPase (3) or even H519N chimera under similar conditions of reduced pH. It is pertinent to recall that alterations not only in Na⁺ versus proton usage, but also in Na⁺:K⁺ stoichiometry, were reported earlier for the wild type Na,K-ATPase under conditions of lower pH and/or reduced Na⁺ concentration (3, 24). As already discussed, the relatively high ouabain-sensitive K⁺-ATPase and K⁺ influx into the H519N-transfected cells in the absence of Na⁺ indicate that this chimera resembles more closely the gastric H,K-ATPase than H85N/H356–519N. A noteworthy distinction between this chimera and the H85N/H356–519N chimera is their distinct response to acidification at low Na⁺ concentration. It is plausible that the decrease in ATPase activity of the H519N but not the H85N/H356–519N chimera reflects mainly the replacement of 3Na⁺/2K⁺ exchange by (lower activity) 2H⁺/2K⁺ exchange as the pH is decreased to pH 5.5 (Fig. 7).

Although there may be differences between the wild type Na,K-ATPase and gastric H,K-ATPase in step(s) involving certain conformational transition(s), the H356–519N substitution, per se, has only a modest 3-fold effect on apparent affinity for ATP and little, if any, on the steady-state Eₜ/Eᵦ equilibrium as deduced from experiments using inorganic orthovanadate as a probe of the Eᵦ conformation. Therefore, we conclude that the functional consequence of this substitution on H⁺ versus Na⁺ translocation reflects a more subtle change in secondary structure of the M4–M5 loop. A likely cation-ligating region affected by such a change is the juxtapositioned M4 helix. Consistent with this notion are experiments indicating the converse. Thus, in studies of the proton pump of Saccharomyces, notable conformational changes have resulted from mutation within M4. In that case, changes in the Eᵦ/Eᵦ' conformational equilibrium as evidenced in altered vanadate sensitivity were observed (25). In the present study, extension of the H356–519N chimeric replacement to include M4 (as well as the rest of the N terminus) also reduced dramatically the vanadate sensitivity. In another relevant study, mutation of Glu-327 in M4 (sheep) of the Na,K-ATPase (Glu-343 of rat gastric H,K-ATPase) indicated that this residue is important in stabilizing a K⁺-induced conformation (26).

The functional alteration caused by the H356–519N substitution is somewhat surprising in view of the high degree of homology among P-type ATPases in the cytoplasmic regions adjacent to M4 (1). In the region preceding the catalytic phosphorylation site, S362R would be expected to alter electrostatic and Van der Waals interactions, and L366V, helix stability. Interestingly the Ser-362 residue is close to the border region of the putative stalk segment and cytoplasmic loop. Therefore, it may be in a critical position to effect a conformational change transmitted via the stalk to M4.

In view of the likely ligation of cations by oxygen-containing residues in M4, M5, and M6 and possibly residues in other transmembrane helices in the C-terminal half of the Na,K-ATPase, it is likely that residues in M4 which are different in H85N/H356–519N and H519N confer their differences in cation selectivity. Although the H519N and, to lesser extent, H85N/H356–519N substitutions confer increased proton selectivity to the Na,K-ATPase, the apparent affinity of H519N for protons relative to sodium falls short of that of the “parent” gastric H,K-ATPase which can only use sodium when the pH is raised to ≥pH 8.0. On the other hand, resemblance of these chimeras, particularly the latter, to the so-called colonic H,K-ATPase which appears to function as an (H,Na)-ATPase (27) is intriguing. An obvious inference from the results of the present study is that, in addition to those in M4, differences in ligating residues in the C-terminal M5–M10 half of the catalytic subunit confer the distinct cation selectivity of the Na,K-ATPase and gastric H,K-ATPase.

Failure to confer ouabain resistance to ouabain-sensitive cells has been used recently to identify regions of Na,K-ATPase/H,K-ATPase chimeras important for conferring cation selectivity (28). Such a strategy does not account for the likelihood that each of a number of distinct regions participate in cation binding and ligation and, second, that the two enzymes differ in other critical functions, for example catalytic site activity, such that the cells are metabolically compromised. In the case of the H519N chimera, the transfected LLC-PK cells can be compelled to adapt to the low activity chimeric pumps by raising the medium K⁺ concentration. The change in their cation selectivity is remarkable. Although their relatively low activity has thus far precluded certain functional assessments, critical transport and ATPase assays have provided insight into the major alterations affected by the H519N substitution.

The present study underscores the complexity of the structural basis for cation selectivity. Part of the selectivity is conferred by extramembranous cytoplasmic region(s). Our results show that the H356–519N replacement increases the apparent affinity for protons, specifically H₂O⁺, compared with alkali cations at cytoplasmic sites. The kinetics of transport and ATP hydrolysis catalyzed by the H85N/H356–519N chimera is consistent with the conclusion that this alteration enhances protons acting at cytoplasmic Na⁺ sites such that as the concentration is increased to the micromolar range, they support K⁺ influx and counteract K⁺ inhibition at these sites. When the replacement is extended to the entire N-terminal half of the
enzyme, the apparent affinity for protons is increased at least an order of magnitude as evidenced in the considerable K\(^+\) influx observed at pH 7.4 in the absence of added Na\(^+\) ions. Protons, more specifically H\(_3\)O\(^+\), bind also at extracellular sites, causing a marked decrease in apparent affinity for extracellular K\(^+\), an effect that is also altered by the chimeric replacements. This behavior is consistent with a ping-pong model of cation transport involving a cation-ligating structure that alternatively binds cations at one side and then the other side. At cytoplasmic sites of the H519N chimeric pump, hydronium ions can bind and be transported like Na\(^+\); at extracellular sites, they can also bind and, like Na\(^+\), act as competitive inhibitors of K\(^+\) binding.

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