Mechanistic Basis for Kinetic Differences between the Rat α1, α2, and α3 Isoforms of the Na,K-ATPase*

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Previous studies showed that the α1, α2, and α3 isoforms of the catalytic subunits of the Na,K-ATPase differ in their apparent affinities for the ligands ATP, Na⁺, and K⁺. For the rat isoforms transfected into HeLa cells, K⁺/ATP for ATP binding at its low affinity site is lower for α2 and α3 compared with α1; relative to α1 and α2, α3 has a higher Kₐₙa and lower Kₐₚ (Jewell, E. A., and Lingrel, J. B (1991) J. Biol. Chem. 266, 16925–16930; Munzer, J. S., Daly, S. E., Jewell-Motz, E. A., Lingrel, J. B, and Blostein, R. (1994) J. Biol. Chem. 269, 16668–16676). The experiments described in the present study provide insight into the mechanistic basis for these differences. The results show that α2 differs from α1 primarily by a shift in the E₁ = E₂ equilibrium in favor of E₁ form(s) as evidenced by (i) a 20-fold increase in IC₅₀ for vanadate, (ii) decreased catalytic turnover, and (iii) notable stability of Na,K-ATPase activity at acidic pH. In contrast, despite its lower K⁺/ATP compared with α1, the E₁ = E₂ poise of α3 is not shifted toward E₁. Distinct intrinsic interactions with Na⁺ ions are underscored by the marked selectivity for Na⁺ over Li⁺ of α3 compared with either α1 or α2 and higher Kₐₙa for cytoplasmic Na⁺, which persists over a 100-fold range in proton concentration, independent of the presence of K⁺. The kinetic analysis also suggests α3-specific differences in relative rates of partial reactions, which impact this isoform’s distinct apparent affinities for both Na⁺ and K⁺.

The Na,K-ATPase or sodium pump is an integral membrane protein complex that couples the exchange of three intracellular Na⁺ ions for two extracellular K⁺ ions to the hydrolysis of one molecule of ATP. The sodium pump is essential to the maintenance of the electrochemical gradients of Na⁺ and K⁺ across the plasma membrane of virtually all animal cells and consequently provides the driving force for the transport of nutrients such as glucose and amino acids into the cell. This enzyme is a P-type ATPase transporter and, as such, is directly phosphorylated on a conserved aspartate residue within its cytoplasmic domain. Both the phosphorylated and dephosphorylated forms of the enzyme exist in at least two states that undergo conformational transitions (E₁P → E₂P and E₂ → E₁) that are coupled to the ion-translocating steps.

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The Na,K-ATPase comprises two essential subunits: a large catalytic α subunit (~110 kDa), containing the phosphorylation site as well as the binding sites for Na⁺, K⁺, ATP and for the cardiac glycoside ouabain, and a smaller, highly glycosylated β subunit (~35–55 kDa) that ensures the proper folding and delivery of the α subunit to the plasma membrane, possibly also modulating cation affinity (1, 2). A third subunit, γ (~7 kDa), was found in the kidney (3), where it functions as a regulator of the pump (for a review, see Ref. 4). Several isoforms of α and β have been described. In the case of α, there are four known isoforms that are expressed in a tissue- and development-specific manner. In the rat, they are distributed as follows. α1 is the ubiquitous, “housekeeping” isoform; α2 is expressed in muscle, nerves, and adult heart; α3 is present in nerves, brain, and fetal heart; and recently the protein for α3 was found in rat testis, where it may participate in spermatogenesis and spermatotyli (5, 6). For a comprehensive review on Na,K-ATPase isozyme structure and diversity in function, see Ref. 7.

Both our laboratory and others have compared the functional properties of the α1, α2*, and α3* isoforms of the rat Na,K-ATPase within the same mammalian membrane environment following transfection of their cDNAs into HeLa cells. (α2* and α3* are the rat α2 and α3 isoforms rendered relatively resistant to ouabain, to permit their distinction from endogenous ouabain sensitive enzyme (8).) The results showed that the isozymes exhibit differences in their apparent ligand affinities (see Table I). Both α2* and α3* have a 3-fold higher apparent affinity for ATP binding (low affinity site) compared with the α1 isoform (K⁺/ATP values of 120 and 130 μM, respectively, compared with 331 μM) (9). In addition, while α1 and α2* show similar apparent affinities for Na⁺ and K⁺, measurements both in broken membranes and in intact cells revealed a lower apparent affinity of α3* for intracellular Na⁺ and a higher apparent affinity for extracellular K⁺ (9, 10).

The present study investigates whether the aforementioned differences in apparent affinities for ligands are the consequence of primary differences in ligand interactions or whether and to what extent they reflect differences in the E₁ = E₂ conformational equilibrium. The results of the present study, together with our earlier work (11), show that the kinetic differences of the α2* isoform are a consequence of a shift in the poise of the conformational equilibrium toward the E₁ form of the enzyme. In the case of α3*, the mechanistic basis for the observed differences in ligand binding is less straightforward. The notable differences in apparent cation affinities are not explained by a shift in the poise of the E₁ = E₂ balance. Nevertheless, they do reflect, at least partly, a change in the rates of limiting steps of its reaction cycle.

EXPERIMENTAL PROCEDURES

Cell Culture and Membrane Preparation—HeLa cells expressing the rat α1, α2*, and α3* enzymes (a generous gift from Dr. J. B Lingrel) were grown in Dulbecco’s modified Eagle’s medium supplemented with...
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Values for apparent kinetic constants were determined using a noncooperative model of ligand binding as in papers cited below. Values shown are the mean ± S.D. of the number of experiments shown in parenthesis.

| Parameter measured | α1 | α2 | α3 | Na,K-ATPaseα | Na,K-ATPaseβ | Na,K-ATPaseγ |
|-------------------|----|----|----|--------------|--------------|--------------|
| K\(_{\text{ATP}}\) (mM) | 0.36 ± 0.07 (7) | 0.12 ± 0.02 (4) | 0.13 ± 0.04 (4) | Na,K-ATPaseα | Na,K-ATPaseβ | Na,K-ATPaseγ |
| K\(_{\text{Na,}}\) (mM) | 8.96 ± 1.28 (4) | 12.48 ± 1.60 (3) | 78.72 ± 17.60 (6) | Na,K-ATPaseα | Na,K-ATPaseβ | Na,K-ATPaseγ |
| K\(_{\text{K}}\) (mM) | 1.15 ± 0.13 (3) | 1.05 ± 0.11 (3) | 3.08 ± 0.06 (3) | Na,K-ATPaseα | Na,K-ATPaseβ | Na,K-ATPaseγ |

\(a\) From Daly et al. (9).
\(b\) From Munzer et al. (10).
\(c\) From Jewell and Lingrel (8).

Results

In the experiments described below, the properties of α2* and α3* are described in terms of their similarities or differences relative to the ubiquitous α1 isofrom.

Assessment of Differences in the E₁/E₂
Conformational Equilibrium

Vanadate Sensitivity—Inorganic orthovanadate is a transition state analog of inorganic phosphate that binds to P-type ATPases in the form of the enzyme from which phosphate is released in the E₂ conformation (18). Accordingly, sensitivity of these enzymes to vanadate inhibition is a measure of the proportion of enzyme in the E₂ conformation during steady-state catalysis. Thus, for mutants of various P-type pumps, including plant and yeast proton pumps and the Na,K-ATPase, this criterion has provided insight into shifts in the E₁/E₂ distribution (for recent examples, see Refs. 19–21). From the present comparative analysis of the vanadate sensitivity of the highly homologous α1, α2*, and α3* Na,K-ATPase isoforms, it is evident that the Na,K-ATPase activity of α2* is ~20-fold less sensitive to vanadate inhibition than α1 (Fig. 1A), suggesting a shift in the E₁/E₂ distribution toward E₁. In contrast, no significant difference in vanadate sensitivity of α3* compared with α1 could be detected. Although the representative experiment shown in Fig. 1A was carried out at 1 mM ATP and 3 mM MgCl₂, similar results (not shown) were obtained at 3 mM ATP, with 5 mM MgCl₂ added to maintain a 2 mM excess of Mg²⁺ (IC₅₀ values were 0.50 ± 0.15, 12.1 ± 4.3, and 1.2 ± 0.4 mM for α1, α2*, and α3*, respectively). Fig. 1B shows that similar results were also obtained when pump turnover was measured in the absence of K⁺ (Na⁺-ATPase activity), precluding effects of vanadate secondary to differences in interactions with K⁺.

Catalytic Turnover—Catalytic turnover of the Na,K-ATPase is estimated as the ratio of V\(_{\text{max}}\) to E₉\(_{\text{max}}\). The latter was measured at 0°C in the presence of 100 mM NaN₃, with K⁺ omitted and oligomycin added to trap the enzyme in the (Na,K)-ATPase mode (see “Experimental Procedures”). As shown in Table II, in the presence of K⁺ (Na,K-ATPase mode), the catalytic turnover of α2* is ~40% that of α1 as observed previously (9). The turnover of α3* is also lower (~50% of α1). In contrast, when the turnover of Na,K-ATPase is estimated as the product of Na⁺-ATPase activity (V\(_{\text{max}}\)) and Na,K-ATPase α3* turnover of α3* is also similar (20 mM Na⁺; Table II) or even higher (100 mM Na⁺; not shown) than that of α1, the former probably reflecting the lower K\(_{\text{cat}}\) for α3*. Turnover of α2* remains reduced in both the presence and absence of K⁺.

pH Sensitivity—The pH sensitivity profile of Na,K-ATPase and its relevance to the issue of rate-limiting reaction(s) was addressed in earlier studies of Forbush and Klodos (22). Their experiments showed that the pathway involving deocclusion of

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The abbreviations used are: MES, 4-morpholineethanesulfonic acid; E₉, the phosphoenzyme form of the Na,K-ATPase; Na⁺, intracellular Na⁺; K⁺, extracellular K⁺. The values shown are the mean ± S.D. of at least three independent experiments. Each value shown is the mean ± S.D. of triplicate determinations.

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10% newborn calf serum, 100 units of penicillin G, 100 μg/ml streptomycin, and 1 μg ouabain as described previously (9).

NaI-treated microsomal membranes were prepared from the transfected HeLa cells as described elsewhere (8, 12). Protein concentrations of the membrane preparations were determined using the Lowry assay as modified by Markwell et al. (13).


denote irreversible (for recent examples, see Refs. 19–21).

\(\text{IC}_{50}\) values were 0.80 ± 0.15, 12.1 ± 4.3, and 1.2 ± 0.4 mM for α1, α2*, and α3*, respectively. Fig. 1B shows that similar results were also obtained when pump turnover was measured in the absence of K⁺ (Na⁺-ATPase activity), precluding effects of vanadate secondary to differences in interactions with K⁺.

Catalytic Turnover—Catalytic turnover of the Na,K-ATPase is estimated as the ratio of V\(_{\text{max}}\) to E₉\(_{\text{max}}\). The latter was measured at 0°C in the presence of 100 mM NaN₃, with K⁺ omitted and oligomycin added to trap the enzyme in the (Na,K)-ATPase mode (see “Experimental Procedures”). As shown in Table II, in the presence of K⁺ (Na,K-ATPase mode), the catalytic turnover of α2* is ~40% that of α1 as observed previously (9). The turnover of α3* is also lower (~50% of α1). In contrast, when the turnover of Na,K-ATPase is estimated as the product of Na⁺-ATPase activity (V\(_{\text{max}}\)) and Na,K-ATPase α3* turnover of α3* is also similar (20 mM Na⁺; Table II) or even higher (100 mM Na⁺; not shown) than that of α1, the former probably reflecting the lower K\(_{\text{cat}}\) for α3*. Turnover of α2* remains reduced in both the presence and absence of K⁺.

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FIG. 1. Vanadate sensitivity of Na,K-ATPase and Na-ATPase activity. ATP hydrolysis at varying vanadate concentrations was determined as described under “Experimental Procedures.” Representative experiments are shown. A, effect of vanadate on Na,K-ATPase activity. Data are presented as percentage of Na,K-ATPase activity (control) measured in the absence of vanadate. Control activities were as follows: 194.62 ± 3.29, 143.53 ± 2.22, and 49.40 ± 3.14 nmol/(mg × min) for α1, α2*, and α3*, respectively. IC50 values for vanadate inhibition were as follows: α1, 3.25 ± 0.65 μM; α2*, 114 ± 2.0 μM (p < 0.005 relative to α1); and α3*, 3.30 ± 0.61 μM. B, effect of vanadate on Na-ATPase activity. IC50 values for vanadate inhibition were as follows: α1, 0.24 ± 0.01 μM; α2*, 6.93 ± 2.10 μM (p < 0.005 relative to α1); and α3*, 0.11 ± 0.01 μM. ○, α1; ▽, α2*; ◻, α3*.

### Table II

| Parameter measured | α1                  | α2*                 | α3*                 |
|--------------------|---------------------|---------------------|---------------------|
| Na,K-ATPase turnover (min⁻¹) | 8290 ± 853          | 4971 ± 845*         | 4148 ± 1194*        |
| Na-ATPase/Na,K-ATPase (a × b) | 0.021 ± 0.003       | 0.015 ± 0.004       | 0.042 ± 0.003       |
| Na-ATPase turnover (min⁻¹) | 170                 | 76                  | 173                 |

a Values of turnover normalized to Vmax using values of K+ shown in Table I are 11274 ± 1160, 5568 ± 946, and 4687 ± 1349 min⁻¹ for α1, α2*, and α3*, respectively.

b p < 0.005 relative to α1.

Identification of Na+ in the transport cycle is partially rate-limiting at acidic pH, whereas the E1 → E1P and E1P → E2P processes are more rate-limiting at alkaline and neutral pH, respectively. In the present study, we compared the pH profiles of the three isoforms over the pH range of 6.0–8.5. As shown in Fig. 2, the activity of all three isoforms declines as pH is decreased below or increased above the optimal. Compared with α1, the activity of α2* is diminished to a lesser extent at acidic pH and more at alkaline pH, consistent with relatively faster E3(K) → E1 and slower E1 → E2P transitions and hence with a preponderance of enzyme in E1 state(s). Although the pH profile of α3* resembles that of α1 at acidic pH, it decreases more significantly than α1 on the alkaline side of the optimum, the significance of which is discussed below.

**Distinct Interactions with Ligands: Comparison of α3* vs α1 and α2***

Since the differences in ligand binding affinities of α3* and α1 hold true despite their similar sensitivities to vanadate, it may be inferred that α3* differs from α1 primarily in its interaction with alkali cation ligands. Nevertheless, certain aspects of α3* kinetic behavior have remained enigmatic and a further comparative analysis of ligand interactions was carried out to explore the mechanistic basis for α3*-specific properties.

**Cytoplasmic Na+ Effects and the Role of Li+ as a Na+ Congener**—To determine whether α3* has an intrinsically lower affinity for Na+ than α1, ATP hydrolysis was measured in the absence of K+ and with the Na+ concentration varied up to 10 mM to favor interactions primarily with cytoplasmic Na+ activation sites. Comparisons of α3* and α1 were carried out at pH values of 6.2, 7.4, and 8.0. The results shown in Fig. 3 indicate that large differences in KNa persist at all pH values tested, consistent with an intrinsic difference in apparent Na+ affinity between α1 and α3*. For α3* compared with α1, respectively, KNa values were 0.125 ± 0.036 and 0.552 ± 0.018 mM at pH 6.2, 0.046 ± 0.005 and 0.335 ± 0.046 mM at pH 7.4, and 0.066 ± 0.030 and 0.753 ± 0.372 mM at pH 8.0 (p < 0.05 at all pH values).

In the case of α1, Li+ can act as a congener of cytoplasmic Na+ as well as extracellular K+ (24, 25). The experiment shown in Fig. 4 was carried out to test whether this holds true for α2* and α3*. The results show that Li+ can replace Na+ and stim-
ulate ATP hydrolysis for both the α1 and α2* enzymes but not in the case of α3*. This finding underscores the large difference in cation selectivity of α3* compared with α1 or α2*.

**Interactions with Extracellular K**⁺ and Li**⁺**—In an earlier study, we showed that K⁺ stimulates Na-ATPase activity of α2* at micromolar ATP concentration but inhibits both α1 and α3*. The explanation that $E_{g}(K) \rightarrow E_{1}$ of α2* is faster relative to other reactions of the cycle was substantiated by measurements of the $E_{2}(K) \rightarrow E_{1}$ rate of α1 and α2* (9). With Li⁺ replacing K⁺, Na-ATPase activity of all three isoforms was stimulated in the order $α2^* ≈ α3^* ≫ α1$, consistent with Li⁺ acting as a congener of K⁺ and with a known faster deocclusion compared with K⁺ (26). Nevertheless, the observation that K⁺ inhibits α1 and α3* to similar extents whereas Li⁺ stimulates $α2^*$ and $α3^*$ but not α1 suggests distinct properties of α3* with respect to interaction with extracellular alkali cations and/or the rate of another reaction step. Therefore, to gain insight into the basis for these isoform-specific differences of Li⁺ and K⁺, we compared $α3^*$ and α1 with respect to rates of K⁺-occlusion and deocclusion as described below.

**$K_{0.5}$ for K⁺ Oclusion**—The K⁺ dependence for formation of the K⁺-occluded enzyme, $E_{1} + K⁺ \leftrightarrow E_{2}(K)$, was measured indirectly as the decrease in phosphoenzyme ($E_{32p}P$ formed by phosphorylation with [γ-32P]ATP) following equilibration of the enzyme in the absence or presence of varying concentrations of K⁺ as described previously (9, 27). The decrease in $E_{32p}P$ resulting from reprimucination with K⁺ is a measure of the amount of $E_{2}(K)$. Fig. 5 shows a representative K⁺-occlusion profile of α3* and α1. Using the simple model $B_{\text{max}}[S]/(K_{0.5} + [S])$ to describe K⁺ occlusion, where $B_{\text{max}}$ is the maximally bound enzyme (23), the values of the equilibrium constant for K⁺ occlusion, $K_{0.5}$, were 0.031 ± 0.007 and 0.046 ± 0.008 mM for α1 and α3*, respectively, and, therefore, not significantly different ($p > 0.5$). (This contrasts with the >3-fold increase in $K_{0.5}$ for α2* compared with α1 (11).) Maximum formation of $E_{2}(K)$ with both α1 and α3* was observed at 1 mM KCl as shown previously for α1 (11).

**$E_{2}(K) \rightarrow E_{1}$**—The rate of the $E_{2}(K) \rightarrow E_{1}$ deocclusion process was estimated indirectly by measuring the rate of $E_{1}$ formation from $E_{2}(K)$ as described previously (11, 27). The results of the representative experiment shown in Fig. 6 indicate that the K⁺ deocclusion rate for α3*, similar to that determined earlier for α2* (11), is faster than that of α1. The fold increase obtained in three similar independent experiments was 3.4 ± 0.5. Since $K_{0.5}$ for K⁺ occlusion is similar for α1 and α3*, the rate of $E_{1} + K⁺ \rightarrow E_{2}(K)$ must also be 3-fold faster for α3*. The implication of the faster deocclusion vis-à-vis the effects of K⁺ on Na-ATPase at micromolar ATP concentration is considered below.

**DISCUSSION**

In the present study, we have extended earlier comparisons of the rat α1, α2*, and α3* isoforms expressed in the same (HeLa) cell environment (8, 10) to gain insight into the mechanistic basis for their distinct behavior. The following discus-

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Fig. 2. PH dependence of Na,K-ATPase activity. Na,K-ATPase activity was measured in the presence of 100 mM NaCl, 10 mM KCl, 3 mM MgSO₄, 1 mM EGTA with either 30 mM MES (pH 6.5) or 30 mM Tris/HCl (pH ≥7.0) as described under "Experimental Procedures." The Na,K-ATPase activities corresponding to 100% were 260.70 ± 0.93, 170.29 ± 3.68, and 192.21 ± 1.87 nmol/min/mg for α1, α2*, and α3*, respectively. pH values indicated were measured at room temperature. Symbols are as described in legend to Fig. 1.

Fig. 3. Na⁺ activation kinetics of α1 and α3* at varying pH. Na-ATPase activity was measured in the presence of 1 μM ATP with varying concentrations of NaCl as indicated. Data are presented as percentage of maximal Na-ATPase activity (control) measured at 10 mM NaCl. pH values shown were measured at room temperature. The data were fit to a noncooperative three-site model as described by Garay and Garrahan (23); i.e. $v = V_{\max}/(1 + K_{\text{Na}}/[Na⁺])^{3}$, where $K_{\text{Na}}$ is the apparent affinity for intracellular Na⁺. $K_{\text{Na}}$ values were 0.125 ± 0.036 and 0.052 ± 0.018 mM at pH 6.2, 0.46 ± 0.005 and 0.353 ± 0.046 mM at pH 7.4, and 0.066 ± 0.030 and 0.753 ± 0.372 mM at pH 8.0 for α3* compared with α1 ($p < 0.05$ at all pH values). Symbols are as described in the legend to Fig. 1.
The maximal Li+-stimulated ATP hydrolysis was determined at 1 μM ATP as described under “Experimental Procedures.” The data are expressed as percentage of maximal Li+-ATP hydrolysis at varying LiCl concentrations. Symbols are as described in the legend to Fig. 1.

The α2 Isoform—From a comparison with the ubiquitous α1 isoform, it is now clear that the main distinguishing property of the α2 isoform is its poise in conformational equilibrium in favor of E1 forms. There are several observations in support of this conclusion. Thus, earlier studies showed that the E3(K) → E1 transition associated with K+-deocclusion is faster for α2* than for α1 and that the apparent affinity for low affinity ATP binding is 2–3 times higher for α2* compared with α1 (8–10). Further supportive evidence is the present finding of a ~20-fold increase in IC50 for vanadate inhibition of α2* compared with either α1 or α3*, indicating a shift in equilibrium away from the E2 state(s). Another point of evidence is the α2*-distinct pH profile. As already discussed, the smaller decrease in activity of α2* at acidic pH and greater decrease at alkaline pH also implicate shifts of rates of partial reactions in favor of E1 form(s).

It is noteworthy that the distinctive behavior of α2* vis-à-vis α1 is remarkably similar to the behavior of α1 mutants obtained by deletion of the first 32 residues from the amino terminus and by a Glu233Lys substitution in the cytoplasmic loop between M2 and M3. Generally similar differences from α1 were obtained in the aforementioned properties for these mutants, including faster E3(K) → E1, slower catalytic turnover, lower K+ATP for low affinity ATP binding, and decreased sensitivity of Na,K-ATPase activity to vanadate inhibition, all leading to the conclusion that these are mutants with the E1 → E2 equilibrium shifted toward E1 (21, 29). Current studies aim to identify distinct cytoplasmic regions of α2* that underlie its altered conformational transitions are under way.

The α3 Isoform—A mechanistic explanation for the distinct kinetic properties of α3* is more complex. As shown previously, differences in K+/Na+ antagonism at cytoplasmic Na+ activation sites underlie much of the variability in K+Na+ noted in studies with intact cells comprising high intracellular K+ versus studies with membrane fragments analyzed at relatively low K+ concentration (see Ref. 30 and Table I). When the complexity of competing effects of Na+ and K+ is eliminated by analyzing the reaction cycle in the absence of K+, and under conditions of enzyme turnover with Na+ varied up to 10 mM to limit its interaction to primarily cytoplasm-facing sites, a markedly lower apparent affinity of α3* for Na+ is observed.
(Fig. 3). This behavior supports the notion of α3-distinct cation ligation. It is noteworthy that an earlier analysis of a series of α1/α3* chimeras expressed in HeLa cells failed to identify a region clearly responsible for the differences in apparent Na\(^+\) affinity between the two isoforms (31). This is not surprising in view of the present experiments, which indicate that a difference in cation binding or selectivity is not the sole determinant of α3- versus α1-distinct apparent affinities for Na\(^+\) and K\(^+\) activation as discussed further below. The remarkable ability of α3* to utilize Na\(^+\) over Li\(^+\) focuses on its distinctive cation interactions with cytoplasmic Na\(^+\) and should be an invaluable criterion for identification of specific residues comprising either the selectivity filter, gate, or Na\(^+\) binding pocket of the catalytic subunit.

Despite the evidence of an intrinsic difference in \(K_{Na}\) of α3* compared with α1 and α2*, several observations suggest that the apparent Na\(^+\) and K\(^+\) affinities are affected, in an isoform-specific manner, by the rates of reactions associated with Na\(^+\) and K\(^+\) translocation. Thus, if one considers the sequence \(E_1 + Na^+ + ATP \rightarrow Na^+E_1P \rightleftharpoons E_2P + Na^+\), a decrease in the rate of \(E_2P \rightarrow E_3P\) reaction from \(E_1\) or a higher rate of the subsequent \(E_3P \rightarrow E_4P\) reaction should effectively increase \(K_{Na}\). The greater decline in activity of α3* at alkaline pH suggests greater rate limitation of the former reaction in the case of α3* (cf. Ref. 22). Furthermore, the conclusion that the phosphorylation reaction also contributes to rate limitation under \(V_{max}\) conditions at physiological pH is supported by the lower turnover of α3* compared with α1 under near \(V_{max}\) conditions in the presence of both Na\(^+\) and K\(^+\) (Na,K-ATPase activity).

On the other hand, compared with α1, the Na\(^+\)E\(_2\)P → E\(_3\)P transition is probably intrinsically faster for α3* for the following reason. Inhibition of Na-ATPase by low concentrations of K\(^+\) is observed at micromolar ATP concentration with both the α1 and α3*, but not the α2*, isoform (9). For α1, this behavior is thought to be a consequence of the rate-limiting \(E_2(K) \rightarrow E_1\) process, which at low ATP is slower than \(E_2 \rightarrow E_1\) in the absence of K\(^+\). This holds true despite the evidence for an intrinsically faster rate of formation of \(E_1\) from \(E_2(K)\) noted in this study.

The most straightforward explanation for the foregoing paradox is that reaction steps comprising the conformational transition of phosphoenzyme that lead to release of three Na\(^+\) ions to the extracellular milieu (Na\(_2\)E\(_2\)P → E\(_3\)P + 3Na\(^+\)) as well as the K\(^+\) deocclusion process (E\(_3\)P(K) → E\(_1\) + K\(^+\)) are proportionately faster for α3*, with the result that the K\(^+\) deocclusion process is similarly rate-limiting at low ATP concentration, for α3* as for α1. Relevant to the presumably faster Na\(_2\)E\(_2\)P = E\(_3\)P + 3Na\(^+\) for α3* is the observation that the human α3 enzyme, expressed in Xenopus oocytes, shows almost no voltage dependence of pump current over the range of -150 to +50 mV in the presence of extracellular Na\(^+\) and K\(^+\) (32). (The greater voltage dependence of α2 observed in that study is consistent with more rate limitation of this step as well as with the preference of this isoform for the E\(_1\) conformation.) This led these authors to suggest that there is an isoform-specific difference in the backward rate constant for the Na\(^+\) release step. Accordingly, less rate limitation of the Na\(_2\)E\(_2\)P → E\(_3\)P + 3Na\(^+\) process would be consistent with the loss of voltage dependence of the α3-catalyzed reaction in the presence of extracellular Na\(^+\). Decreased voltage dependence may, in turn, reflect either the decreased affinity of α3 for allosteric Na\(^+\) inhibition of pump flux (33) or a decrease in Na\(^+\) antagonism of K\(^+\)\(_{\text{ext}}\) binding (see Ref. 34). Thus, in either case, an increase in the relative rate of the forward E\(_1\)P ↔ E\(_2\)P process of the Na\(^+\) and K\(^+\) reaction cycle is observed. Studies are currently under way to compare the voltage dependence of pump current in rat isoform-transfected HeLa cells.

With the human enzyme, little, if any difference in \(K_{K}\) of α3 was observed except under conditions of extreme hyperpolarization in presence of Na\(^+\)\(_{\text{ext}}\) (32); in SF9 cells, the apparent affinity for K\(^+\) was somewhat lower for α3 compared with α1 (6). It is unlikely that the difference in \(K_{K}\) seen with the rat α3* enzyme is due to the mutation to ouabain resistance, since the difference persists when ouabain-resistant rat kidney α1 and rat axolemma (~70% α3*) enzymes are compared (30). More likely, the discordant findings suggest that the isoforms do not differ in their intrinsic K\(^+\) binding/occlusion but rather in the relative rates of reaction(s) associated with K\(^+\)-activated dephosphorylation of E\(_3\)P. Accordingly, an increase in the rate of the proceeding K\(^+\) deocclusion process [E\(_3\)P(K) → E\(_1\)] or preceding E\(_1\)P → E\(_2\)P transition would effectively decrease and increase, respectively, the apparent affinity for K\(^+\). Although the present study indicates that these two processes are faster for α3, the relative magnitudes of the increases probably differ when the enzyme is analyzed in both a different membrane environment and at a different temperature (Xenopus membranes assayed at ambient temperature (32) versus HeLa membranes assayed at 37 °C).

In conclusion, the main distinguishing feature of α2* is its shift in conformational equilibrium toward \(E_1\). On the other hand, the α3* isoform resembles α1 in its conformational equilibrium. Its lower apparent affinity for Na\(^+\)\(_{\text{ext}}\) is due, in large part, to intrinsic differences in cation binding and, to some extent, to differences in rates of limiting forward and reverse steps in the catalytic cycle. The higher apparent affinity of α3 for K\(^+\)\(_{\text{ext}}\) is probably secondary to differences in the rates of reactions preceding and subsequent to K\(^+\)-activated dephosphorylation.

Although the relationship between the kinetics of the individual isoforms and their distinct physiological roles in various tissues remains elusive, a few clues are notable. In neuronal tissue, the lower affinity of α3 for Na\(^+\)\(_{\text{ext}}\) suggests that α3 may only be activated when the Na\(^+\)\(_{\text{ext}}\) concentration reaches its maximum following repeated firing, possibly acting as a “spare pump” to restore membrane potential (8, 10). It is also plausible that the higher ATP affinity of α3 compared with the ubiquitous α1 allows it to function at the low ATP concentrations found near the membrane (7). This α3-distinct property (α2 as well) is reminiscent of the shift in ATP affinity of α1 conferred by interaction with the γ subunit in the renal outer medulla, which may allow the pump to function more efficiently under the near anoxic conditions found in this portion of the kidney (35). It is also plausible that the higher apparent ATP affinity of α2 and the maintenance of activity under acidic and metabolically depleted conditions are advantageous for skeletal muscle tissue during exercise. In fact, it has been shown that in both the rat (36) and human (37) exercise induces the translocation of α2β1 pumps from an intracellular pool to the plasma membrane of skeletal muscle. That the presence of α3 in neonatal rat cardiac myocytes, with its low Na\(^+\) affinity, would be particularly effective in raising cytosolic Ca\(^{2+}\) is supported by the coincidence of the switch from α3 to α2 to the shortening of the action potential duration in rat heart during ontogeny (38–40). Although studies with cardiac myocytes in which α1 is down-regulated implicate α1 as having a major role in regulation of cardiac Ca\(^{2+}\) (41), a specific role of α2 in Ca\(^{2+}\) signaling during heart contraction was clearly evidenced in studies of genetically reduced levels of α2 in the heart (42). The information provided by the present study taken together with earlier studies of isoform-specific kinetics repre-
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