Structural Basis for α1 Versus α2 Isoform-distinct Behavior of the Na,K-ATPase*

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The Na,K-ATPase or sodium pump is an integral membrane protein complex found in the plasma membrane of virtually all animal cells. It catalyzes the exchange of three intracellular Na+ ions for two extracellular K+ ions using the energy of hydrolysis of one molecule of ATP. Consequently, the sodium pump plays an essential role in the maintenance of the electrochemical alkali cation gradients, providing the driving force for the transport of various nutrients into the cell. The Na,K-ATPase is a member of the family of P-type ATPases, which during the course of their catalytic cycle undergo phosphorylation and dephosphorylation of a conserved aspartate residue located in the large catalytic loop between transmembrane segments 4 and 5 of the catalytic α subunit. During the catalytic cycle both dephospho- and phosphoenzymes undergo conformational transitions commonly referred to as E1 ↔ E2 and E2 ↔ E1M, respectively. In addition to the large catalytic α subunit, the Na,K-ATPase comprises a smaller, highly glycosylated β subunit that is important for the proper folding of α and its insertion into the plasma membrane. At present, four isoforms of α and three isoforms of β have been described, and these are distributed in a tissue- and developmentally dependent manner.

The α2 isoform is located primarily in skeletal muscle and in brain, predominantly in glial cells. Our earlier studies indicated that it differs from the ubiquitous α1 subunit primarily in the steady-state E1/E2 equilibrium. Thus, compared with α1, the E1/E2 poise of α2 is shifted toward E1. This shift is reminiscent of the changes in α1 effected by deleting 32 residues from its N terminus (mutant α1M32) which corresponds to the E1-shifted trypsinized kidney enzyme first described by Jorgensen (1). Except for a modest (∼1.5-fold) increase in K⁺Na⁺ (12), α2 resembles α1 with respect to apparent affinity for extracellular K⁺ when ouabain-sensitive K⁺ influx is assayed under physiological conditions of ATP concentration. However, marked differences between α2 and α1 become apparent when, at micromolar ATP, the E2K⁺ (E2K⁺ ) → E1 conversion is rate-limiting. Under these conditions, α2 resembles closely the α1M32 mutant. We showed previously (2, 3) that, compared with α1, both α2 and α1M32 have faster rates of K⁺ declusion as seen in K⁺ stimulation rather than α1-like inhibition of Na,K-ATPase activity at 1 μM ATP, with a concomitantly lower K⁺ATP for low affinity ATP binding and decreased (50%) catalytic turnover.

One region of marked primary sequence diversity among the otherwise homologous α1, α2*, and α3* isoforms is the N terminus. In previous studies we compared the kinetics of α1/α2 chimeras in which the first 32 residues were interchanged. The results showed that although removal of 32 residues from the N terminus of α1 yields an enzyme with α2*-like kinetics as mentioned above, substitution of the first 32 residues of α1 with those of α2* is without effect. Similarly, substituting the analogous N-terminal sequence of α1 into that of α2 does not dramatically alter the kinetics of α2. It was therefore suggested that either removal of the N terminus, as in the case of α1M32, or alteration of its primary or secondary structure, as in the case of α2*, likely results in a weakening of intramolecular interactions between the N terminus and some other regions of the α protein (2).

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The experiments described in the present study were designed to extend the chimera approach to additional domains of isoform diversity to pinpoint regions that confer the α1/α2 distinct behavior. As a prelude to this analysis, we first show that cytoplasmic interactions that underlie the $E_1^*E_2^*$ conformational transitions seen with α1 (3, 4) are notably similar in α2. The subsequent analysis of α1/α2 chimeras shows that the N-terminal segment encompassing the cytoplasmic N terminus and the first (M2-M3) cytoplasmic loop of α2, the so-called “Actuator” or A domain (5) and, to a lesser extent, the most divergent portion of the nucleotide binding or N domain within the large M4-M5 loop, are responsible for the $E_1^*$ shift of α2.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Transfection, and Cell Culture—**All of the mutant and chimeric αs used in this study were derived from the ouabain-insensitive rat α1 and α2 cDNAs previously described by Jewell and Lingrel (6). The E231K mutation was introduced into a rat α2 cDNA that had been excised from pRcCMV with HindIII(875, 2282), using PCR amplification with synthetic nucleotides and the TaqPlus polymerase reaction kit (Stratagene). The reaction mixtures were eluted from QIAquick spin columns (Qiagen) and digested with DpnI, EcoRI, and SalI. The EcoRI(7065, 1202) α2 fragment was gel-purified and ligated into a modified pBluescript shuttle vector in place of the wild type EcoRI-SalI fragment. Clones containing the E231K mutation (G800 → A) were identified by the presence of adenine nucleotide 800, and the complete sequence of the substituted fragment was verified before excising the full-length α2E231K cDNA from the shuttle vector and ligating it into pRcCMV (Invitrogen). Orientation of the α2E231K cDNA in pRcCMV was determined by restriction enzyme analysis.

The α2M30 mutant was constructed by introducing a 30-residue deletion into the 5′ HindIII(906, 2282) restriction fragment cassette of the rat α2 cDNA as described previously (2). The mutant cassette was then ligated into the rat α2 cDNA in place of the wild type HindIII-EcoRI cassette. The full-length mutant cDNA was released from the shuttle vector by digestion with HindIII(875, 2282) and ligated into the expression plasmid pCDNA3.1 (Invitrogen), and orientation of the cDNA was determined by restriction analysis.

Chimeras α2-(1–209)/α1 and α1-(1–311)/α2 were prepared by excising 5′ HindIII-BbsIII fragments of the two cDNAs. The HindIII site of each cDNA is in the 5′-untranslated region, and the BbsIII site splits the codons for Ala-347 in α1 and the corresponding Ala-345 in α2. It should be noted that the two isoforms have identical sequences in the region between residues 311 and 346 of α1. The full-length cDNAs for the chimeric proteins were then excised from the shuttle vectors and cloned into pRcCMV as above.

The N-terminal chimera, α2-(1–63)/α1, denoted as α2/α1, was prepared using the oligonucleotide-directed technique of Kunkel (7). Starting with the 5′ ScaI(2305, 3875) cassette of α1-(1–322)/α2 (3), nucleotide mutations encoding the α2 amino acid sequence from residue 33 through 63 were introduced. This mutant 5′ cassette was sequenced and ligated into an α1 shuttle vector in place of the wild type cassette, and the full-length cDNA chimeras was transfected into pRcCMV. Because residues 64–94 of rat α2 are naturally identical to residues 66–96 of α1, the entire N-terminal cytoplasmic tail of the α2/α1 protein has the α2 sequence.

The chimera in which the isoform divergent portion within the M4-M5 loop, α2-(427–562)/α1, denoted as α2/α1, was prepared using the Kunkel (7) technique to introduce the α2 sequence into five short segments of the H5-H6 cytoplasmic loop of rat α1. Three of the substituted segments are encoded by nucleotides in the Sall(135),BamHI(1776) cassette: I, 429–442 α1 replaced by 427–440 α2; II, 457–473 α1 replaced by 455–471 α2; and III, 489–499 α1 replaced by 487–498 α2. The other two segments are in the BamHI,SalI, and EcoRI cassettes: IV, 515–530 α1 replaced by 512–527 α2; and V, 552–565 α1 replaced by 549–562 α2. After sequence verification, the mutated cassettes were ligated into an α1 shuttle vector in place of the wild type cassettes, and the α2/α1 cDNA was then transfected into pRcCMV. The mutated 5′ ScaI-SalI cassette used above to prepare α2/α1 was also substituted into the α2/α1 cDNA in place of the wild type cassette to produce α2/α1.

HeLa cells were transfected with the pRcCMV-α and pCDNA3 constructs using either the calcium phosphate method (8) or the LipofectAMINE technique (LipofectAMINE, Invitrogen), and cells expressing the relatively ouabain-resistant rat α enzymes were selected as previously described (6, 9). HeLa cells expressing the mutant α enzymes were amplified in Dulbecco’s modified Eagle’s medium plus 10% newborn calf serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 1 μM ouabain as described previously (2).

**Membrane Preparation—**Nat-treated microsomal membranes were prepared from the mutant cells as described earlier (6, 9). Protein content was determined with a detergent-modified Lowry assay (9).

**Tryptic Cleavage—**Digestion was carried out at a trypsin/enzyme ratio of 200 units trypsin/100 μg α2 cDNA for 30 min in 100 mM NaCl, 0.2 mM MgSO4, and 20 mM Tris glycylglycine (pH 7.4) in a buffer comprising 100 mM NaCl, 0.2 mM MgSO4, and 20 mM Tris glycylglycine (pH 7.4) at a final enzyme concentration of 35 μg/ml. Digestion was terminated by adding 10-fold excess (units) soybean trypsin inhibitor (Type I-S, Sigma) in a buffer containing 50 mM choline chloride, 0.2 mM MgCl2, and 20 mM Tris glycylglycine (pH 7.4).

**Enzyme Assays—**Na,K-ATPase activity was measured as the release of 32P from [γ-32P]ATP as previously described (11). Briefly and unless indicated otherwise, the membranes were preincubated for 10 min at 37 °C with all reagents added except [γ-32P]ATP. The reaction was initiated by the addition of [γ-32P]ATP. Final concentrations for Na,K-ATPase activity measurements were 100 mM NaCl, 10 mM KCl, 3 mM MgSO4, 20 mM histidine (pH 7.4), 5 mM EGTA (pH 7.4), and 5 μM ouabain (Sigma). 5 μM ouabain was used to determine base-line hydrolysis activity. As in earlier studies and unless indicated otherwise, assays of Na,K-ATPase activity were carried out using 1 mM ATP to maintain close to saturating ATP concentration and maximize sensitivity of the assay of the relatively low activity cultured cells (cf. Refs. 3, 9, and 12). Na-ATPase activity was measured at 37 °C, preequilibrated previously (2), with varying amounts of added KCl and choline chloride to maintain constant chloride (40 mM) concentration. Base-line activity was determined with 40 mM KCl replacing NaCl. For studies of vana-date sensitivity, inorganic orthovanadate (Fisher) solutions were prepared before the experiment and added with the [γ-32P]ATP solution to initiate the reaction. Na,K-ATPase activities obtained at various vanadate concentrations and expressed as the percentage of that obtained in the absence of vanadate were analyzed by fitting the data to a one-compartment model using a nonlinear least-square analysis of a general logistic function, as described elsewhere (13). Curve fitting was carried out using the Kaleidagraph computer program (Synergy). Each experiment was carried out at least three times, with one or more chimeras analyzed concurrently with the α2 and α1 isoforms.

**Rate of $E_F^* → E_P^*$—**After formation of $E_F$, in the presence of high chloride concentration (14), the rate of $E_F^* → E_P^*$ was determined by measuring the rate of disappearance of total phosphoenzyme after rapid dilution of the salt (to allow “normal” relaxation of $E_F^*$, $E_P^*$) plus the addition of KCl to catalyze rapid hydrolysis of $E_P^*$ (15). Accordingly, the enzyme was first phosphorylated in medium containing 600 mM NaCl to stabilize $E_F^*$, 1 mM MgCl2, 1 mM EGTA, and 20 mM Tris-HCl (pH 7.4) with 1 μM [γ-32P]ATP for 30 s at 0 °C to obtain maximum phosphoenzyme. Dephosphorylation was then initiated by 6-fold dilution with a “chase” medium containing final concentrations of 100 mM NaCl, 10 μM unlabelled ATP, 10 mM EGTA, and 10 mM Tris-HCl (pH 7.4), which simultaneously lowered the NaCl concentration to 100 mM. Samples were taken for measurement of [32P]IE for periods up to 30 s. Background phosphoenzyme levels were obtained by allowing the chase to continue for 60 s. The data were fitted to a first-order decay model using the Kaleidagraph nonlinear fitting program (Synergy). At least two different membrane preparations obtained from at least two different clones were assayed. The data presented are representative of at least three independent experiments. Each value shown is the mean ± S.D. of triplicate determinations.

**RESULTS**

**Cytoplasmic Interactions of α2**—Our earlier studies showed that the steady-state $E_1^*/E_2^*$ conformational equilibrium of α2, compared with that of the ubiquitous α1 isomer, is poised toward $E_1$. In fact, the kinetic behavior of α2 is generally similar to that of mutants of α1 in which the poise is shifted toward $E_1$. These mutations include a 32-residue deletion of the cytoplasmic N terminus (mutant α1M32) and a Glu-233 → Lys replacement (mutant α1E233K) in the first cytoplasmic loop. Furthermore, the combination of these two mutations of the so-called Actuator domain (mutant α1M32E233K) in α1 results in a remarkably synergistic shift in poise toward $E_1$ state(s). The findings were interpreted to indicate that interactions between these regions of the Actuator domain (5) and the
catalytic loop are critical for conformational coupling of the Na,K-ATPase (4).

Experiments carried out in the present study to address the question of whether cytoplasmic interactions of a2α are analogous to those of α1 are summarized in Fig. 1 and Table I. Expression of a2M30 and a2E231K in HeLa cells yielded functional enzymes capable of supporting cell growth in 1 μM ouabain. Cells transfected with the double mutant α2M30E231K, analogous to a1M32E233K (4), failed to grow even in elevated K+ (cf. Ref. 16). Therefore, a comparable “mutation” was prepared by enzymatically cleaving the N terminus of a2E231K in the E1(Na+) conformation with trypsin (cf. Ref. 1). The single mutants and the cleaved a2E231K (a2E231K-Tryp) were then assessed for shifts in the E1/E2 equilibrium using the following criteria: (i) the effect of K+ on Na-ATPase activity as a measure of E2(K+) → E1 (see Ref. 2), (ii) KATP for low affinity binding to E2(K+), KATPLP, and (iii) sensitivity of Na,K-ATPase activity to inhibition by inorganic orthovanadate.

Thus, at micromolar ATP concentrations sufficient to saturate only the high affinity phosphorylation site the response of Na-ATPase to K+ is a sensitive means to characterize mutant-specific differences in the K+-deocclusion pathway of the reaction cycle [E2(K+) → E1 + K+] that becomes rate-limiting under these conditions (2, 17). As shown previously and summarized in Table I (upper panel), a low concentration of K+ (1 mM KCl) inhibits the Na-ATPase activity of α1 but stimulates that of α1M32 and α1E233K with a further and notably synergistic stimulation of the double mutant α1M32E233K. The

**TABLE I**

| α Subunit      | Maximal K+ effect on Na-ATPase activity (%) | Na,K-ATPase activity (μM ATP) | IC50 vanadate normalized |
|----------------|---------------------------------------------|-------------------------------|--------------------------|
| α1             | Inhibition                                  | 405 ± 66 (4)                  | 1                        |
| α1M32α         | 135 ± 3                                     | 145 ± 31 (3)                  | 500                      |
| α1E233K        | 260 ± 5                                     | 56.3 ± 14 (3)                 | 250                      |
| α1M32E233Kβ    | 985 ± 286                                   | 5 ± 1                         | Stimulation              |
| α2α            | 170 ± 32                                    | 209 ± 56 (17)                 | 20                       |
| α2M30          | 384 ± 96                                    | 117 ± 42 (14)                 | ≥10000                   |
| α2E231K        | 922 ± 250                                   | 61.2 ± 13 (6)                 | 2740                     |
| α2E231K-Tryp   | ≥2000                                       | ≤16                           | ND                       |

a Na-ATPase activity measured at 20 mM NaCl and 1 μM ATP in the presence or absence of 1 mM KCl.

b From Daly et al. (2, 3).

c From Daly et al. (30).

d From Boxenbaum et al. (4).
pattern for \(\alpha^2\) cytoplasmic mutants is remarkably similar (see Fig. 1 and the lower panel of Table I). With respect to the \(\alpha^2\) mutants, K\(^+\) stimulates the Na-ATPase activity of \(\alpha^2M30\) and \(\alpha^2E231K\), more than 2000%, and that of \(\alpha^2E231K\)-Tryp, more than 2000%. In fact, the apparent stimulation may be an underestimation of the true stimulation since trypsinolysis of \(\alpha^2E231K\) undoubtedly results in a heterogeneous pool of enzyme species that includes untrypsinized enzyme having inherently lower sensitivity to K\(^+\) activation. It should be mentioned that trypsinization of \(\alpha^2\) in the presence of Na\(^+\) increased K\(^+\) activation such that \(\alpha^2\)-Tryp resembled \(\alpha^2M30\) (data not shown; cf: tryptic cleavage of \(\alpha^2\) described in Ref. 18), which is not surprising since the region encompassing the tryptic cleavage site (T2; see Ref. 1) is conserved between \(\alpha^2\) and \(\alpha^2\). These results suggest that \(\alpha^2M30\), \(\alpha^2E231K\), and \(\alpha^2E231K\)-Tryp all shift the \(E_1/E_2\) equilibrium of \(\alpha^2\) progressively further toward \(E_1\).

As shown in Table I, \(\alpha^2M30\), \(\alpha^2E231K\), and \(\alpha^2E231K\)-Tryp also exhibit increased affinities for low affinity ATP binding relative to \(\alpha^2\), with a synergistic effect of the N-terminal deletion and the E231K mutation in the M2-M3 loop. It should be noted that, as with the K\(^+\) stimulation of Na-ATPase activity shown above, the K\(^+\) ATP(L) of \(\alpha^2E231K\)-Tryp is an overestimation of the actual value of the double mutant due to the presence of untrypsinized enzyme.

To gain further insight into the effects of these mutations on conformational equilibrium, we investigated the sensitivity of the Na,K-ATPase activity of the mutants to inhibition by vanadate. Inorganic orthovanadate is a transition state analog of inorganic phosphate that binds to P-type ATPases in the \(E_2\) conformation during steady-state catalysis. Consequently, sensitivity of an enzyme to inhibition by vanadate is a measure of the proportion of enzyme in the \(E_2\) state (19). As shown by the representative experiment in Fig. 2 and summarized in Table I,
α2M30 and α2E231K are both less sensitive to vanadate inhibition than α2*, suggesting that these mutations further shift the $E_1/E_2$ equilibrium of α2* toward $E_1$. We were unable to determine accurately the IC$_{50}$ for vanadate inhibition of α2E231K-Tryp because the residual Na,K-ATPase activity was too low relative to the background (15 nmol/mg/min).

**Chimeras of the α1 and α2* Isoforms**—The above analysis of cytoplasmic mutations of the α2* isofrom of the Na,K-ATPase support the notion that interactions between the cytoplasmic domains of α2* that modulate conformational shifts are fundamentally similar to those of α1. The analysis also suggests that it is the α2-specific regions of the A domain and/or the catalytic domain with which A interacts that underlie the predilection of $E_2$ domains of the A domain and/or the catalytic domain with which A interacts that underlie the predilection of $E_2$ (see Fig. 3A). Only the $E_2/K^+$ state of $E_2$ has been shown to be the predominant form of the ATPase with a preponderance of the $E_1$ state(s). Accordingly, an α1/α2 chimera approach was used to address this issue.

From a comparison of the primary structure of the cytoplasmic regions of α1 and α2* (Fig. 3B), it is important to note that the β strand region in the M2-M5 loop of α1 encompassing Glu-233 is highly homologous to that of α2*. In contrast, the two isoforms differ significantly in the primary sequence of their N termini and in the sequence encompassing the ATP binding site in the M4-M5 cytoplasmic loop. Therefore, chimeras were constructed in which the entire N terminus (residues 1–65) and the divergent portion of the nucleotide binding (N) domain in the large M4-M5 loop (residues 429–565) of α1 were substituted with the analogous residues of α2*. It should be noted that our original intent was to investigate 5 individual divergent regions contained within residues 429–565 (see “Experimental Procedures,” cassettes I–V) on the $E_1/E_2$ equilibrium of α1. Because the individual domains showed no effect on the K$^+$ inhibition of Na-ATPase of α1 relevant to the $K^+$ deactivation pathway, we constructed an α1/α2 chimera where all 5 cassettes of α2* were substituted into α1. Two additional chimeras were constructed. In these chimeras, the first 311 residues of α1, which encompass the entire A domain, are interchanged between α1 and α2* because this region comprises the portion of the cytoplasmic domain that undergoes large rotational displacements during $E_1/E_2$ transitions (5, 20). A schematic representation and the designation of the chimeras are illustrated in Fig. 3A.

To assess the effect of these different regions on the $E_1/E_2$ poise of α1, we first investigated the effect of K$^+$ on Na-ATPase activity measured at micromolar ATP for each of the chimeras as described above. As shown in Fig. 4, the Na-ATPase activities of wild type α1 and chimeras α2$_{α1}$/α1,$^3$ α2$_{α2}$/α1, and α2$_{α1+1}$/α1 are all inhibited by K$^+$. In contrast, wild type α2* and α2-(1–309)/α1 are stimulated by K$^+$ at least up to 1 mm, consistent with a faster $E_2(K^+)$ transition and, hence, with a preponderance of the $E_1$ conformation. It is noteworthy that although the α2$_{α1}$/α1 construct encompasses the most divergent portion of the primary sequence, it is only upon replacement of the homologous N-terminal segment (residues 1–311) of α1 with that of α2*, chimera α2-(1–309)/α1, that the shift toward $E_1$ is observed.

Fig. 5 shows the determination of $K_{ATP}^{α1}$ using the Lineeweaver-Burk transformation of the simple Michaelis-Menten analysis of Na,K-ATPase activity as a function of ATP concentration, with values presented in the inset. Only the α2-(1–309)/α1 chimera, like wild type α2*, showed an increased affinity for ATP. Thus, the $K^+$ stimulation of α2-(1–309)/α1 described in Fig. 4 correlates with its increased apparent affinity for ATP, consistent with a shift in the $E_1/E_2$ poise in favor of $E_1$. Chimeras α2$_{α1}$/α1 and α2$_{α1+1}$/α1 show no difference in $K_{ATP}^{α1}$ relative to α1.

To further examine the contribution of the various domains

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$^3$Chimera abbreviations: α2$_{α1}$/α1, α2-(1–63)/α1; α2$_{α2}$/α1, α2-(427–562)/α1; α2$_{α1+1}$/α1, α2-(1–63, 427–562)/α1.

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L. Segall, L. K. Lane, and R. Blostein, unpublished information.
Vanadate sensitivity of Na,K-ATPase activity of α1, α2*, and chimeras. Assays were carried out as in Fig. 2. One series of experiments was carried out with chimeras α1(1–311)/α2 and α2(1–309)/α1 assayed concurrently with α1 and α2*, and a representative experiment is shown in panel A. In a second series, chimera α2*/α1 was assayed concurrently with α1 and α2*, and a representative experiment is shown in panel B. Symbols are as in Fig. 4.

Fig. 6. Vanadate sensitivity of Na,K-ATPase activity of α1, α2*, and chimeras. Assays were carried out as in Fig. 2. One series of experiments was carried out with chimeras α1(1–311)/α2 and α2(1–309)/α1 assayed concurrently with α1 and α2*, and a representative experiment is shown in panel A. In a second series, chimera α2*/α1 was assayed concurrently with α1 and α2*, and a representative experiment is shown in panel B. Symbols are as in Fig. 4.

to the poise of $E_1/E_2$, we used vanadate as a conformational probe for $E_2$ forms as described above. In one set of experiments, chimeras α1(1–311)/α2 and α2(1–309)/α1 were analyzed concurrently with α1 and α2* (Fig. 6A), and in another, the α2*/α1 chimera was compared with α1 and α2* (Fig. 6B). As shown previously (21), the Na,K-ATPase activity of α2* is ~25-fold less sensitive to vanadate than α1 (Fig. 6A). Interestingly, the α1(1–311)/α2 enzyme is 3.7-fold less sensitive to vanadate than α1 and resembles α1 with respect to $K^+$ inhibition of Na-ATPase and $K'_\text{ATP}$, whereas α2(1–309)/α1 is 8.5-fold less vanadate-sensitive than α1 and resembles α2* with respect to $K^+$ activation of Na-ATPase and $K'_\text{ATP}$. A modest shift in conformational poise is also indicated by the behavior of the α2*/α1 chimera. This replacement of the divergent region of the $N$ domain of α1 by that of α2 reduces the vanadate sensitivity of α1 by a factor of 2.3. In other experiments (not shown) a similar ~2-fold shift was effected by including the loop insertion of α2* with the α2* $N$ terminus, i.e. a 2-fold higher IC$_{50}$ for vanadate seen for α2*/α1 compared with α1, which was further doubled, resulting in a 4-fold higher IC$_{50}$ compared with α1 for α2*/α1. Taken together these findings imply a major contribution of the N-terminal segment encompassing residues 1–309 and a smaller contribution of the L region of α2* in effecting shifts away from $E_2$.

It has been observed that the α2* isoform and the 32-residue deletion mutant of α1, α1M32, are both enzymes with their $E_1/E_2$ equilibrium shifted toward $E_1$ forms. Both enzymes show similar $K^+$ sensitivities of Na-ATPase activity at low ATP, $K'_\text{ATP}$ values, catalytic turnovers, and $K^+$ deocclusion rates. One major difference between the two enzymes is their sensitivity to inhibition by vanadate. Thus, α2* has a 25-fold lower IC$_{50}$ than α1M32. Because vanadate sensitivity reflects the steady-state levels of $E_2$, we investigated differences not only in the $E_2/K^+$ to $E_1$ transition rate but also the $E_1P$ to $E_2P$ transition rate. As shown previously (22) and represented in Fig. 7, α1M32 has a 5-fold slower conversion of $E_1P$ to $E_2P$ than α1, consistent with a preference for the $E_1P$ form. This does not hold true for α2*; its $E_1P$ to $E_2P$ transition rate is only slightly slower than that of α1, providing an explanation for its higher sensitivity to vanadate compared with α1M32. The implication of vanadate sensitivity as a measure of the $E_1/E_2$ poise during steady-state catalysis is discussed further below.
Structural Basis for α1 Versus α2 Kinetic Differences

| α subunit | Na+/Na,K-ATPase (1 μM ATP) | K<sub>ATP,L</sub> | Vanadate IC<sub>50</sub> | Catalytic turnover<sup>c</sup> | Rate of E<sub>d</sub>K<sup>1</sup> → E<sub>d</sub>P<sup>a</sup> | Rate of E<sub>d</sub>P → E<sub>d</sub>P<sup>d</sup> |
|-----------|-----------------|-----------------|-----------------|----------------|----------------|----------------|
| α1        | Inhibition       | 1               | 1               | 1              | 0.02           | 1              |
| α1M32     | 135 ± 3         | 0.4             | 500             | 0.5            | 0.08           | 5              |
| α2<sup>a</sup> | 170 ± 32       | 0.5             | 20              | 0.6            | 0.07           | 1              |

<sup>a</sup> Data taken from Table I.
<sup>b</sup> Data taken from Table I and normalized to values obtained for α1.
<sup>c</sup> From Daly et al. (3) and Segall et al. (21, 22) and normalized to the value obtained for α1.

<sup>d</sup> From Daly et al. (3).

<sup>e</sup> From Fig. 7 and normalized to the rate for α1.

DISCUSSION

Our earlier studies showed a notable similarity between the α2<sup>a</sup> isoform of the Na,K-ATPase and the “E<sub>i</sub>-shifted” mutants of α1, particularly the deletion mutant α1M32. Nevertheless, the behavior of α1/α2 chimeras in which the first 32 residues of the N termini were interchanged showed that the kinetic difference between α1 and α2<sup>a</sup> could not be explained by their distinct N-terminal 1–32 residue per se. More likely, those findings indicate that the difference is due to the interaction of the N terminus with another, isoform-distinct region(s) of the enzyme.

As a preliminary step toward defining the structural basis for α1/α2 differences, we have used a mutagenesis approach to obtain evidence for cytoplasmic interactions between the N terminus, the M2-M3 loop, and large M4-M5 loop of α2<sup>a</sup>, as noted previously for α1 (3, 4). As shown in Table I (lower panel), a kinetic analysis of cytoplasmic mutants of α2<sup>a</sup>, namely α2M30 and α2E231K, show that both mutations effect shifts in the E<sub>d</sub>/E<sub>i</sub> poise of α2<sup>a</sup> analogous to those seen for α1, although even further toward E<sub>i</sub> HeLa cells transfected with the double mutant, α2M30E231K, analogous to α1M32E233K, failed to grow. Noting that the catalytic turnover of α1M32E233K is ≤500 min<sup>−1</sup> (4), it is likely that the catalytic turnover of α2M30E231K is much too low to support HeLa cell growth in 1 μM ouabain. Consequently, the N terminus of α2E231K was enzymatically cleaved by trypsinolysis of membranes isolated from the α2E231K-transfected cells as originally described by Jorgensen (1). Like α1M32E233K, α2E231K-Tryp showed a strong, synergistic effect on E<sub>i</sub> ↔ E<sub>d</sub>, shifting it even further in favor of E<sub>i</sub> forms. Therefore, we conclude that the interaction of the cytoplasmic domains of α2<sup>a</sup> that modulate conformational shifts are fundamentally similar to those of α1.

As already mentioned, the primary structure of the β-strand region in the M2-M3 loop encompassing Glu-231 of α2<sup>a</sup> is highly homologous to that of α1 containing Glu-233. In contrast, the primary structures of the two isoforms differ significantly in their N termini (domain nt, 56% identity) and a region within the N domain of the M4-M5 loop and referred to here as the L region (61% identity). It is noteworthy that for the remainder, the sequence identity is very high, namely 86% between the end of the nucleotide and the beginning of the L region (residues 429–565; see Fig. 3B) and 92% from the C-terminal end of the L region to the C terminal of the protein. Therefore, α1/α2 chimeras were constructed in which domains of divergent primary sequence were interchanged. Thus, the N terminus (nucleotide residues 1–65) and L region (residues 429–565) of α1 were substituted with the analogous regions of α2<sup>a</sup>. This was done either individually (α2<sub>nt</sub>/α1 and α2<sub>nt</sub>/α1, respectively) or in combination (α2<sub>nt</sub>/α1) (see Fig. 3A for a schematic representation). In addition to the above, chimeras encompassing the entire Actuator domain, i.e. α1-(1–311)/α2 and α2-(1–309)/α1, were analyzed. As shown in recent structural studies, this domain undergoes large rotational motions (estimated at 110° in the sarcoplasmic reticulum Ca-ATPase) in the course of the conformational transitions (20). The present results show that although a switch of the entire N terminus is without effect, inclusion of the entire isoform-divergent N-terminal segment up to residue 309 of α2<sup>a</sup> is capable of conferring α2<sup>a</sup>-like kinetics to α1. This is apparent from the K<sup>c</sup> activation of Na,K-ATPase activity at low ATP when E<sub>d</sub>(K<sup>c</sup>)E<sub>i</sub> is rate-limiting as well as a decrease in K<sub>ATP,L</sub>. However, this N-terminal segment of α2<sup>a</sup> only partially decreases the vanadate sensitivity of α1 (~8.5-fold compared with 20–25-fold for α2<sup>a</sup> relative to α1). In addition, however, the L region of α2<sup>a</sup> confers a 2.3-fold decrease in vanadate sensitivity to α1, such that synergistic effects of the two domains, residues 1–309 in the N-terminal segment and residues 427–562 in the N domain, can account for the α2<sup>a</sup> versus α1 differences.

Although it is attractive to hypothesize that the E<sub>i</sub> shift of α2-(1–309)/α1 is due to the A domain, one cannot exclude the possible contribution of amino acid replacements in other regions, namely M1, M2, and M3 and the extracellular M1-M2 and M3-M4 loops. Of these regions, it is noteworthy that Coppi et al. (31) showed that an α2-(1–129)/α1 chimera is not E<sub>i</sub>-shifted because, like α1, its Na-ATPase activity is inhibited by K<sup>c</sup> at low ATP concentration (31). This result provides a basis for eliminating M1 and the M1-M2 loop as candidates for effecting the E<sub>i</sub> shift. For the rest, there are four replacements, namely Ser-132 → A in M2, His-288 → Gln in M3, and Glu-309 → Gly and Thr-311 → Ser in the M3-M4 loop. Experiments are currently under way to determine whether and/or which ones of these replacements are important or whether it is the A domain per se that confers the α2-like E<sub>i</sub> shift seen with the α2-(1–309)/α1 mutant.

It is instructive to consider likely α1/α2 differences in the interactions of the A domain with the N and P domains in the E<sub>i</sub> and E<sub>d</sub> conformations as well as interactions within regions of the A domain. With the Na,K-ATPase, metal-catalyzed oxidative cleavage studies of domain interactions reveal that in the E<sub>i</sub> conformation, the N domain docks onto the phosphorylation (P) domain, and A moves apart; in E<sub>d</sub>, A docks onto P, and N is displaced (32), consistent with putative domain interactions deduced from crystal structures of sarcoplasmic reticulum calcium ATPase in E<sub>i</sub> and E<sub>d</sub> states (5, 20, 33). Converged effects of α1 versus α2 distinct residues within the N-terminal segment encompassing domain A and the L region within domain N can be explained by the recent model for regulation of the aforementioned domain interactions regulated by the N terminus (22). Thus, assuming that the secondary structure of the α2<sup>a</sup> N terminus, like that of α1, has the propensity to form three short helices (H1, H2, and H3), intramolecular interactions between helices 1 and 2 of the N
terminus allow the M2-M3 loop in A and the N domain to come together in the E₂ conformation. In the E₁ conformation, H₂ interacts with the M2-M3 loop to keep it apart from N. Thus, isoform diversity in the N terminus impacts intramolecular interactions of H2 with M2-M3 within the A domain. The minor E₁ shift effects swapping of the isoform divergent portion of the N domain (L swap) probably indicates interaction between N and P domains in the Ca ATPase M2-M3 loop does not interact with either the N domain in E₁ but could interact with both N and P in E₂. With only N in E₂, there are no nearby sites of interaction between the N terminus and either the N or P domains, consistent with an intermediary role of the M2-M3 loop of the A domain in E₁ ⇔ E₂ conformational changes.22).

The present kinetic analysis involves evaluation of the overall reaction cycle under different conditions of rate limitation or ligand perturbations as well as certain partial reactions relevant to conformational transitions. The usefulness of this approach is indicated by the comparison of α2β and α1M32 as summarized in Table II. Compared with α1, α1M32 but not α2β, exhibits a substantial slowing of the E₁P → E₂P conversion, providing an explanation for its much larger decrease in sensitivity to vanadate compared with α2β, i.e. IC₅₀ values of α1M32 and α2β are decreased about 500- to 20-fold, respectively, compared with α1. Accordingly, the E₁ shift in E₁P/E₂P poise of α2β is due to a shift in dephosphoenzyme [E₂P(K*)] → E₁P but not phosphoenzyme [E₂P] → E₁P. This result indicates the “non-equivalence” of the two conformational transitions and also highlights a limitation of the sole use of vanadate as a probe of conformation since the steady-state proportion of enzyme in the E₂ state reflects the rates of transition between both dephospho- and phosphoenzyme states.

This report deals only with the structure/function analysis of isoform-specific kinetic behavior. Isoform-specific interactions of the pump with other proteins such as cytosolic second messengers may be important for adapting sodium pump function to cell-specific requirements as suggested by Blanco et al. (23). Thus, targets for protein kinase C phosphorylation present in the N terminus of α1 are absent in α2. The significance of this difference is underscored by the observation that in transfected opossum kidney cells (24) protein kinase C-mediated phosphorylation of α1 at these residues promotes its translocation to the plasma membrane. Similarly, in α1 but not α2, the presence of Tyr-5 within a consensus sequence for phosphorylation by tyrosine kinases of the Src family has been implicated in the insulin stimulation of pump activity in the proximal convoluted tubules of the rat kidney (25). Furthermore, although insulin acts via tyrosine kinases in rat proximal convoluted tubules to stimulate pump activity by increasing the apparent Na⁺ affinity (26) in skeletal muscle, it promotes translocation of α2 to the plasma membrane (27) via the action of protein kinase C (28). There is also direct evidence for a role of the isoform-specific primary sequence within the M4-M5 loop domain, in regulation of the pump by second messengers. In agreement with our findings, Pierre et al. (29) failed to detect an effect of the introduction of an α2β distinct region of the large catalytic loop (residues 489–499, contained within the L region) on the E₁E₂ conformational equilibrium of α1. However, a role for this domain in the isoform-specific response of transfected opossum kidney cells to protein kinase C activation was observed in the differential response of the isoforms to hormones via the action of second messengers. Taken together, the foregoing studies suggest that the primary sequence diversity may in part be relevant to isoform-specific pump regulation, separate from a role in isoform-distinct pump kinetics.

In conclusion, we have shown that the E₁ shift in the conformational equilibrium of α2β can be largely accounted for by the N-terminal third of the α subunit that comprises mainly the A domain, with a small contribution of the isoform-specific sequence of the N domain within the M4-M5 loop. In addition, despite the kinetic similarities of the α2 isoform with cytoplasmic mutants of α1, such as α1M32, the E₁ shift of α2 results primarily from differences in the dephosphoenzyme conformational transition, i.e. E₂P(K*) → E₁P, with its E₁P → E₃P transition rate similar to that of α1.

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Structural Basis for α1 Versus α2 Isoform-distinct Behavior of the Na,K-ATPase
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