Changes in Steady-state Conformational Equilibrium Resulting from Cytoplasmic Mutations of the Na,K-ATPase α-Subunit*

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Mutations comprising either deletion of 32 amino acids from the NH₂ terminus (αM32) or a Glu²³³ → Lys substitution in the first M2-M3 cytoplasmic loop (E233K) of the α-subunit of the Na,K-ATPase result in a shift in the steady-state E₁ ↔ E₂ conformational equilibrium toward E₂, form(s). In the present study, the functional consequences of both NH₂-terminal deletion and Glu²³³ substitution provide evidence for mutual interactions of these cytoplasmic regions. Following transfection and selection of HeLa cells expressing the ouabain-resistant αM32E233K double mutant, growth was markedly reduced unless the K⁺ concentration in the culture medium was increased to at least 10 mM. Marked changes effectized by this double mutation included 1) a 15-fold reduction in catalytic turnover (Vₘₐₓ/EPₘₐₓ), 2) a 70-fold increase in apparent affinity for ATP, 3) a marked decrease in vanadate sensitivity, and 4) marked (~10-fold) K⁺ activation of the Na-ATPase activity measured at micromolar ATP under which condition the E₀(Κ) → E₁ pathway is normally (α₁) rate-limiting and K⁺ is inhibitory. The decrease in catalytic turnover was associated with a 5-fold decrease in Vₘₐₓ and a compensatory ~3-fold increase in expressed αM32E233K protein. In contrast to the behavior of either αM32 or E233K, αM32E233K also showed alterations in apparent cation affinities. K⁺ₕₐₜ was decreased ~2-fold and K⁺ was increased ~2-fold. The importance of the charge at residue 233 is underscored by the consequences of single and double mutations comprising either a conservative change (E235D) or neutral substitution (E233Q). Thus, whereas mutation to a positively charged residue (E233K) causes a drastic change in enzymatic behavior, a conservative change causes only a minor change and the neutral substitution, an intermediate effect. Overall, the combined effects of the NH₂-terminal deletion and the Glu²³³ substitutions are synergetic rather than additive, consistent with an interaction between the NH₂-terminal region, the first cytoplasmic loop, and possibly the large M4-M5 cytoplasmic loop bearing the nucleotide binding and phosphorylation sites.

The Na,K-ATPase couples the hydrolysis of one ATP molecule to the translocation of 3 Na⁺ and 2 K⁺ ions against their electrochemical gradients, thus maintaining the normally high K⁺ and low Na⁺ concentrations inside animal cells. This enzyme complex comprises a large subunit, α (molecular mass, 112 kDa) and a small subunit, β (molecular mass, 35 kDa). α and β have been cloned and sequenced from a variety of tissues (see Ref. 1). The functional unit may be a heterodimer (αβ)₂, although a monomeric αβ unit can occlude both Na⁺ and (K⁺)₂Rb⁺, consistent with its being the minimal unit required for transport (2). α is the catalytic subunit, which spans the membrane probably 10 times and includes the cytoplasmic catalytic domain and the extracellular cardiac glycoside binding site(s) (3). Although this enzyme complex has eluded efforts to obtain ordered three-dimensional crystals of sufficient quality to allow precise description of key structural features such as topology and cation ligating structure(s), protein chemical and molecular biology techniques are providing important information about structure/function relationships (for review, see Ref. 4).

The reaction mechanism is probably consecutive (Cleland’s ping-pong mechanism), whereby Na⁺ is released before K⁺ binds (5, 6). Most reaction schemes are based on the original Albers-Post mechanism involving phosphorylated and dephosphorylated forms of the enzyme, both of which undergo conformational transitions (E₁P → E₂P and E₂ → E₁), which are coupled to ion translocation steps. The enzyme (and phosphoenzyme) include conformations with ion binding sites accessible to the cytosol, to the extracellular milieu and in a state in which the transported ion is occluded. ATP interacts not only with high affinity to catalyze phosphorylation of E₁ (ATP + E₁ → E₁P), but also with low affinity in a manner which effects release of occluded K⁺ from the form E₂(K), i.e. E₂(K) + ATP → ATP.E₂K → E₁ + K⁺.

Although considerable information has been obtained regarding transmembrane-located residues involved in cation binding and occlusion, the structural basis for conformational coupling of the scalar energy of ATP hydrolysis to the vectorial movement of Na⁺ and K⁺ remains a major unresolved issue. Previous site-specific alterations of cytoplasmic residues within several P-type ATPases, particularly those of the sarcoplasmic reticulum Ca-ATPase, have identified residues in both the M2-M3 and larger catalytic M4-M5 cytoplasmic loops, which support a model in which the E₁P → E₂P conformational transition is transmitted from the phosphorylation site to the cation binding pocket via the β-strand structure in M2-M3 and a “stalk” region connecting M4 to the catalytic loop (for review, see Ref. 7). It is pertinent that mutation of Leu³²² at the putative boundary of M4 and the stalk region connecting M4 to the M4-M5 loop affects conformation coupling as evidenced in the shift in equilibrium in favor of E₁P (8).

Another cytoplasmic region involved in conformational cou-
E233Q mutations were each prepared in the are the same as those described previously (11, 12). The E233D and a infected at 3% CO2 with 20 mM NaCl and varying KCl concentrations for calf serum, 100 units/ml penicillin G, 100 (E233D, E233Q, a1M32 deletion and substitution of Glu 233). We show that the magnitudes of the kinetic changes resulting from the double mutation give strong support to the existence of mutual interactions between these regions.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Transfection, and Cell Culture—**HeLa cell lines expressing pRo/CMV (Invitrogen) constructs of rat α1, α1M32, and E233K are the same as those described previously (11, 12). The E233D and E233Q mutations were each prepared in the Sau3A-BamHI (1780) cassette of rat α1 in M33mp18, using the site-specific, oligonucleotide-directed mutagenesis technique of Kunkel (13) as described previously (14). Mutant cDNAs were completely sequenced to verify the presence of the mutation and the absence of any unplanned substitutions. The mutated Sau3A-BamHI restriction fragment containing each of the substitutions for E233 were then excised, gel-purified, and ligated into either in M13mp18 or rat α1-m32 cDNA in a modified pIB30 shuttle vector, in place of the wild type Sau3A-BamHI cassette. After verifying the presence of mutant sequences and the sequences at the Sau3A and BamHI sites, the full-length mutant cDNAs were excised from the shuttle vector with HindIII and ligated into pRo/CMV (Invitrogen). Orientation of the α1 CDNAs were determined by restriction analysis.

Ten-cm plates of HeLa cells at approximately 15% confluence were transfected at 3% CO2 with 20 μg of the pRo/CMV-rat α1 mutant constructs (E233D, E233Q, α1M32E233D, α1M32E233Q, and α1M32E233K), using the Chene and Okayama (15) calcium phosphate technique. HeLa cells expressing the relatively ouabain-resistant rat α1 mutant enzymes were selected in 0.5 μM ouabain in Dulbecco's modified Eagle's medium plus 10% calf serum, 100 units/ml penicillin G, 100 mM streptomycin, 0.25 mM amphotericin B (Life Technologies, Inc.). For cells expressing the double mutant, rat α1M32E233K, and α1M32E233K, the culture medium was supplemented with an additional 15 mM KCl (20 mM total).

**Membrane Preparation—**NaI-treated microsomal membranes were prepared from the mutant cells as earlier described (14, 16), and the protein concentration was determined with a detergent-modified Lowry assay (17).

**Growth Curves—**The cells were grown in 24-well plates and at the times indicated in the text, the cells were trypsinized for 10 min at 37 °C, after which the cell counts were performed in a hemocytometer.

**Enzyme Assays—**Assays of Na,K-ATPase activity were carried out as described earlier by measuring the release of 32Pi from [γ-32P]ATP (18). Unless indicated otherwise, the membranes were preincubated for 10 min at 37 °C with all the reactants except ATP. The reaction was initiated by adding 0.2 volumes of ATP to 0.8 volumes of preincubated membranes. Final concentrations were 1 mM ATP, 3 mM MgSO4, 20 mM Tris-HCl (pH 7.4), 5 mM EGTA (pH 7.4), and 10 μM ouabain (Sigma). A final concentration of 10 mM ouabain was used to determine the baseline hydrolysis activity. The cation affinities were determined with 100 mM NaCl and varying KCl concentrations for K+ and 20 mM KCl and varying NaCl concentrations for Na+. Appropriate amounts of choline chloride were added to keep the ionic strength constant. To optimize the proportion of specific [γ-32P]ATP hydrolysis in the cation affinity assays, the concentration of ATP in the assay mix for the α1M32E233K mutant was lowered from 1 to 0.1 mM, which saturates the enzyme. K+ (Rb+) fluxes were performed as earlier described (12). Vanadate (orthovanadate from Fisher) was made up freshly before the experiment and was added at the concentrations indicated. The final Na+ and K+ concentrations were 100 and 10 mM, respectively. The Na,K-ATPase activities were measured with 1 mM MgSO4, 20 mM histidine (pH 7.4), 5 mM EGTA (pH 7.4), 20 mM NaCl, 1 μM ATP, 10 μM ouabain and KCl concentrations as indicated. For base-line activities, Na+ was omitted and 20 mM KCl included, with choline chloride added to maintain a constant (40 mM) chloride concentration.

**Phosphoenzyme Determination—**As in earlier studies with HeLa cells expressing ouabain-resistant rat α1 Na,K-ATPase, we used a culture medium containing 1 μM ouabain to select stable HeLa cell lines expressing the mutant α1 enzymes. In most cases, the functional alterations caused by the mutations did not preclude this selection procedure and the stable expression of the mutants enabled us to characterize their functional properties. An exception was the double mutant α1M32E233K, which failed to yield any ouabain-resistant HeLa cells in normal Dulbecco's modified Eagle's medium following numerous transfections. On the assumption that the combined effects of the NH2-terminal 1–32 residue deletion and the E233K substitution might be additive or even synergistic, and that the resultant secondary decrease in catalytic turnover might prevent survival of the α1M32E233K-transfected cells, we attempted to promote growth by raising extracellular K+ as done previously by Arguello and Lingrel in their studies with mutant S775A defective in cation ligation (19).

When HeLa cells transfected with α1M32E233K were selected with 1 μM ouabain in Dulbecco's modified Eagle's medium containing 20 mM KCl, a few ouabain-resistant colonies were obtained. In the experiment shown in Fig. 1, cells cultured in 20 mM K+ were either maintained in that medium or transferred into normal medium with 5.4 mM K+. Growth rates were followed by counting the cells for periods up to 10 days. As shown, the doubling time for the wild type α1-transfected cells was 16.4 ± 0.4 h, whereas for the α1M32E233K mutant growing in 5.4 mM K+ was 61.5 ± 7.5 h. Adjustment of the K+ concentration to 20 mM maintained the growth rate close to normal (23.5 ± 0.5 h). Another experiment (not shown) indicated that 10 mM K+ was sufficient to restore the growth rate to that of the cells grown in 20 mM K+. As described below and consistent with our original premise, elevated K+ concentration compensated for the markedly reduced pump turnover and the lower apparent affinity for K+ observed in kinetic analysis of α1- and α1M32E233K-transfected cells as described below.

**Ligand Interactions—**At a saturating ATP concentration, 10 mM NaCl sufficient to saturate the high-affinity phosphorylation site, the response of Na+-dependent ATP hydrolysis to varying concentrations of K+ is a convenient and sensitive indication of ligand-specific differences in the E2(K) → E1 pathway of the Na,K-ATPase reaction (10, 11). This part of the reaction becomes rate-limiting at low ATP concentration, and as shown,
first by Post et al. (20), K⁺ inhibits Na-ATPase activity of the α1 enzyme. In contrast, K⁺ activates Na-ATPase of α1M32 and E233K mutants, consistent with a rapid deocclusion via \( E_2(K) \) → \( E_1(K) \) → \( E_1 + K^+ \) in these mutant enzymes (10, 11).

In the experiments shown in Fig. 2, we compared the K⁺ activation profiles of the α1M32E233K mutant and the single mutants, α1M32 and E233K. The maximal stimulations by K⁺ are 100, 135, and 200%, respectively, suggesting synergism and hence mutual interactions of the NH₂-terminal and M2-M3 domains.

Fig. 2 also shows the K⁺ response profiles of mutants in which substitution of Glu₂³³ is either conservative (aspartate), neutral (glutamine), or positive (lysine), both in the case of single mutants (compare α1M32E233D, α1M32E233Q, and α1M32E233K in Fig. 2A) and double mutants (compare α1M32E233D, α1M32E233Q, and α1M32E233K in Fig. 2B). As shown, the magnitude of K⁺ activation is dependent on the charge of the residue, being minimal with α1M32E233D and moderate with α1M32E233Q compared with E233K and similarly, minimal with α1M32E233D and moderate with α1M32E233Q compared with α1M32E233K.

The notion that the distinct K⁺ activations signal differences in the \( E_1-E_2 \) conformational equilibrium is underscored by the alterations in ATP dependence of the Na,K-ATPase activity shown in Fig. 3. As in previous studies (10) a model describing a branched pathway of the \( E_2(K) \) deocclusion limb of the Albers-Post mechanism is relevant. In one branch of the pathway, low-affinity ATP binding to the \( K^- \)-occluded enzyme, \( E_2(K) \), is followed by rapid deocclusion (ATP-E₂(K) → ATP-E₁K → ATP-E₁ + K⁺), and in the other branch, slow release of K⁺ from E₂(X) via the sequence E₂(X) → E₁X → E₁ + K⁺ is followed by high-affinity ATP binding to E₁. A comparison of the results shown in Fig. 2 with those of Fig. 3 and Table I indicate that the magnitudes of K⁺ stimulation of the mutant enzymes at low ATP is directly related to their increased apparent affinities for ATP.

The experiments shown in Fig. 3 and Table I include previous results with α1, the α1M32 truncated mutant, and E233K. As reported previously, both the wild type α1 and truncated α1M32 enzymes have low (\( K'_{\text{ATP,L}} \)) and high (\( K'_{\text{ATP,H}} \)) apparent affinities for ATP (10). For α1 the values are 331 ± 44 \( \mu M \) and 5.44 ± 1.9 \( \mu M \), respectively, and for α1M32, the values are 130 ± 92 \( \mu M \) and 4.57 ± 2.3 \( \mu M \), respectively. With the E233K mutant, high- and low-affinity components were not readily distinguished. As shown previously, the data for E233K fit a single binding component, with an apparent \( K'_{\text{ATP}} \) of 56 ± 14 \( \mu M \). Presumably, the increase in low-affinity binding obscures high-affinity binding. With single mutants E233D and E233Q analyzed in the range 5–500 \( \mu M \) ATP, a single component for α1M32E233D, α1M32E233Q, and α1M32E233K was also obtained, with \( K'_{\text{ATP}} \) values of 41 ± 8 \( \mu M \), 17 ± 3 \( \mu M \), and 5 ± 1 \( \mu M \), respectively (Fig. 3B). Most
Cytoplasmic Mutations of Na,K-ATPase

Comparison of K⁺ effects on Na-ATPase with apparent affinities for ATP

| α-Subunit     | Maximal K⁺ stimulation, Na-ATPase (1 μM ATP) | K⁺, ATP(H) | K⁺, ATP(L) |
|---------------|---------------------------------------------|------------|------------|
| α1            | (Inhibition) 5.44 ± 1.9³ | 331 ± 44² | 130 ± 32²  |
| E233D         | (Inhibition) ND | 241 ± 10  | ND         |
| E233Q         | 138 ± 15     | ND         | 130 ± 16   |
| E233K         | 200 ± 5⁶ | 56 ± 14⁶   | 130 ± 32²  |
| α1M32         | 135 ± 3     | ND         | ND         |
| α1M32E233D    | 319 ± 40    | 41 ± 8    | ND         |
| α1M32E233Q    | 502 ± 127   | 17 ± 3    | ND         |
| α1M32E233K    | 985 ± 266   | 5 ± 1     | ND         |

³ Values taken from Daly et al. (10).
⁶ Values taken from Daly et al. (11).

ND, not determined (values shown were obtained with ATP varied from 10 to 500 μM). Assays were carried out as described in the legends to Figs. 2 and 3.

Glu²³³ with another region, most likely the M4-M5 catalytic domain as discussed below.

Based on the Albers-Post model and discussed in detail by Eisserer and Richards (21), a change in apparent affinity for ATP should alter the apparent affinity for K⁺ and vice versa. To determine whether the striking increase in ATP binding to α1M32E233K alters apparent cation affinities, K⁺ and K Na were determined from the plots shown in Fig. 4, A and B. With the data fitted to a noncooperative model, the apparent affinity of α1M32E233K for K⁺ was 2.4-fold lower than that of α1. The K⁺ for the double mutant was 1.2 ± 0.36 mM compared with 0.50 ± 0.11 mM for α1. A similar difference in affinity for extracellular K⁺ activation of (⁸⁶Rb)K⁺ influx was observed in transport experiments performed with intact α1- and α1M32E233K-transfected cells (not shown). A change in K Na (2.2-fold) was also observed, but in the opposite direction, with values of 1.00 ± 0.08 mM and 2.19 ± 0.20 mM for α1M32E233K and α1, respectively. This increase in apparent affinity for Na⁺ and decrease in apparent affinity for K⁺ support the idea that the conformational equilibrium of α1M32E233K favors the high-affinity Na⁺ binding conformation, E1.

Vanadate Sensitivity—To gain further insight into the effect of the α1M32E233K mutation on the steady-state E₁-E₂ conformational equilibrium, we used vanadate as a conformational probe. Vanadate has the ability to exist in a stable trigonal bipyramidal structure and may, as such, act as a transition state analog of inorganic orthophosphate (22). It competes with P₈ in binding to the E₂ conformation of P-type ATPases and is able to form a stable intermediate whereby the pump activity is inhibited (23). As shown in Fig. 5, the α1M32 and E233K mutants are at least 100-fold less sensitive to vanadate inhibition than α1. α1M32E233K is not inhibited by vanadate present up to at least 10 mM, at which concentration a paradoxical increase in activity was consistently observed. Although we have no explanation for this stimulation, it is plausible that it represents an effect due to a minor contaminant, possibly a form of vanadate other than orthovanadate, and that the stimulation is masked in other less resistant mutants by the inhibitory effects of orthovanadate.

Catalytic Turnover of Single and Double Mutations: Effect of Charge of Residue 233—We showed previously that the α1M32 and E233K mutants as well as the α₂ isoform have reduced catalytic turnovers, which probably reflects the poise in the steady-state E₁-E₂ equilibrium in favor of E₁. Catalytic turnover was determined as the ratio of Vₘₐₓ under optimal conditions of ATP, Na⁺, and K⁺, to the maximal level of phosphoenzyme formed in the presence of Na⁺, with oligomycin added to

Notable is the high ATP affinity of the α1M32E233K mutant, the value obtained being virtually the same as K Artem of the wild type α1 enzyme. To date, a similar remarkable change in apparent affinity for ATP has not been observed with other functional mutants. These data and those for the single mutants of Glu²³³ also provide insight into the nature of the interaction of the β-strand of M2-M3 encompassing the charged
trap the enzyme as Na$_2$E$_1$P (24, 25). Compared with α1, the catalytic turnovers of α2, α1M32, and E233K were reduced approximately 50%.

In the present study, we extended the estimates of turnover to double mutants as well as single mutants in which Glu$_{233}$ was replaced with aspartate or glutamine as well as lysine. As shown in Fig. 6, the turnover effected by the double mutation, α1M32E233K, is 5% that of the wild type α1 enzyme. This reduction in catalytic site activity is far greater than predicted by the combined effects of the single mutations, which should be no greater than the product of the fractional reductions effected by the single mutations, namely 0.45 × 0.55. The results also show that the extent of alteration (decrease) in turnover is dependent on the charge of residue 233.

Interestingly, comparison of phosphoenzyme formed in the absence and presence of oligomycin indicates an oligomycin-dependent increase (2-fold) in total phosphoenzyme of α1, which diminishes in the various mutants as a function of the charge at residue 233, deletion of the NH$_2$ terminus, and combinations of the two alterations (Fig. 6, inset). Thus, the

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**Fig. 4.** Effect of the mutation α1M32E233K on apparent cation affinities. ATP hydrolysis was assayed with varying cation concentrations as described under “Experimental Procedures.” The data are presented as Na$_2$K-ATPase activity as percent of $V_{max}$, apparent affinity for K$^+$. When fitted to a noncooperative model, the $V_{max}$ (nmol (mg × min)) values were estimated to be 124 and 45 for α1 and α1M32E233K, respectively. The $K_v$ (mM) values were 0.50 ± 0.11 for α1 and 1.22 ± 0.36 for α1M32E233K. α1; ○ α1M32, α1M32E233K, B, apparent affinity for Na$^+$. When fitted to a noncooperative model, the $V_{max}$ (nmol (mg × min)) values were estimated to be 83 and 25 for α1 and α1M32E233K, respectively. The $K_v$ (mM) values were 2.19 ± 0.20 for α1 and 1.00 ± 0.08 for α1M32E233K. Legends are the same as in A. Results shown are from representative experiments; values shown are the average of triplicate determinations.

**Fig. 5.** Vanadate sensitivity of mutant Na$_2$K-ATPases. ATP hydrolysis at varying vanadate concentrations was determined with 100 mM NaCl, 10 mM KCl, and 1 mM ATP as described under “Experimental Procedures.” Values are presented as percent of Na$_2$K-ATPase activity (control) measured in the absence of vanadate. Control activities in the presence of 100 mM NaCl, 10 mM KCl, 3 mM MgSO$_4$, and 5 mM EGTA (mmol/(mg × min)) were 153 ± 47, 107 ± 35, 70 ± 11, and 39 ± 5 for α1, E233K, α1M32, and α1M32E233K, respectively. α1; ● α1M32, ○ α1M32E233K. Values for E233K are taken from Daly et al. (11). Results shown are from a representative experiment, except for α1M32E233K in which case the average of five experiments is shown. Values shown are the average of triplicate determinations.

**Fig. 6.** Catalytic turnover of mutant enzymes. Maximal phosphoenzyme ($E_{P_{max}}$) was determined in the presence of 100 mM NaCl and oligomycin as described under “Experimental Procedures.” Values (pmol/mg protein) were 19 ± 7, 20 ± 12, 18 ± 1, 25 ± 2, 38 ± 5, 15 ± 9, 37 ± 5, and 69 ± 7 for α1, E233D, E233Q, E233K, α1M32, α1M32E233D, α1M32E233Q, and α1M32E233K, respectively. Data are presented as turnovers (min$^{-1}$). These were 81 ± 7, 54 ± 5, 46 ± 4, 34 ± 6, 17 ± 1, and 6 ± 0.7 for α1, E233D, E233Q, E233K, α1M32, α1M32E233D, α1M32E233Q, and α1M32E233K, respectively. The inset represents the ratios of phosphoenzyme in the presence of oligomycin over phosphoenzyme without oligomycin. These ratios were 1.91 ± 0.04, 1.70 ± 0.07, 1.51 ± 0.19, 1.40 ± 0.30, 1.27 ± 0.09, 1.46 ± 0.04, 1.27 ± 0.19, and 1.14 ± 0.01 for α1, E233D, E233Q, E233K, α1M32, α1M32E233D, α1M32E233Q, and α1M32E233K, respectively. Results shown are from an average of at least three experiments, each done in triplicate. Values for α1M32 and E233K are taken from Daly et al. (10, 11).
graded decrease in turnover and in oligomycin stimulation parallels the putative shifts in steady-state $E_1$-$E_2$ conformational equilibrium toward $E_2$ form(s).

**DISCUSSION**

We showed previously that changes caused by mutation of glutamate 233 to lysine in the catalytic $\alpha$ subunit of Na,K-ATPase alters the equilibrium between major conformational states of the dephospho- and phosphoenzyme forms during steady-state catalysis in favor of $E_1$ forms. A generally similar conformational shift was observed when residues 1–32 were removed from the cytoplasmic amino terminus of $\alpha$ (9, 26, 12). Evidence for the conformational shift was derived from kinetic studies showing that these mutations cause (i) an increase in the $E_2^a(K)$ $\leftrightarrow$ $E_1^a + K^+$ equilibrium in favor of $E_1$, accountable for by an increase in the rate of formation of $E_1$ from $E_2^a(K)$, (ii) an increase in apparent affinity for ATP at the step $E_2^a(K) + ATP \rightarrow ATP-E_1^a + K^+$ when the overall reaction is measured at $37^\circ$C (iii) less oligomycin-dependent increase in steady-state level of phosphoenzyme, which indicates a preponderance of $E_2^a$ versus $E_1^a$ form(s) in the mutants (cf. Fig. 6), and (iv) a decreased catalytic turnover. The decrease in sensitivity to vanadate is also diagnostic of a decrease in steady-state level of $E_2$ required for $P_i$ (vanadate) binding. Although mutation of amino acids (27) and tryptic cleavage (28, 29) in this $\beta$-strand region of the Ca-ATPase of the sarcoplasmic reticulum (reviewed by Möller et al. (4)) and tryptic cleavage of the Na,K-ATPase (30) block the $E_2^P$ to $E_1^P$ conversion (for review, see Ref. 31), mutations in this region of yeast proton pumps, like the Glu233 $\rightarrow$ Lys mutation in Na,K-ATPase, have resulted in active enzyme which can cycle through the entire reaction, but with reduced catalytic turnover and decreased sensitivity to vanadate (32–34). In the present study, the extent of the changes in kinetic behavior of the double mutant in which NH$_2$-terminal deletion and Glu233 $\rightarrow$ Lys were combined is remarkable. The magnitudes of the changes in the various kinetic parameters of $\alpha$M32E233K are far greater than predicted from “additive” effects of the two mutations. This synergy was apparent in the $K^+$ sensitivity profiles of Na,ATPase, the apparent affinities for ATP as well as the overall catalytic site turnover. As shown earlier, the response of Na,ATPase to $K^+$ at micromolar ATP reflects either (i) the relative rate of the formation of $E_1$ from $E_2^a(K)$ via the high-affinity branch $(E_2^a(K) \rightarrow E_1^a(K) \rightarrow E_1)$ versus the low-affinity branch $(E_2^a(K) + ATP \rightarrow ATP-E_1^a(K) \rightarrow E_1)$ of the reaction pathway and/or (ii) the relative ATP affinity for deocclusion via the low-affinity pathway.

Presumably, the extreme poise in conformational equilibrium of $\alpha$M32E233K in favor of $E_1$ precludes binding of ATP with low affinity during the catalytic cycle, to the extent that maximal activity is observed with ATP present at a concentration sufficient to saturate only the high-affinity site. This indicates that the enzyme can cycle through all the intermediates of the reaction cycle, without invoking low-affinity ATP binding, giving credence to the notion of a single physical site (35). Most likely, the $E_2^a$ to $E_1^a$ phase of the reaction cycle becomes rate-limiting to the extent that the overall catalytic turnover is reduced 15-fold. In contrast, turnover was decreased only $\sim$50% by each of the two mutations alone. In fact, the very slow turnover of $\alpha$M32E233K, though offset partly by a 3–4-fold increase in functional enzyme as measured by the maximal amount of phosphoenzyme, compromised the survival and growth of $\alpha$M32E233K-transfected cells in medium with regular K$^+$ concentration. It is also clear that this mutant is remarkably insensitive to vanadate.

The concept that conformational coupling in P-type ATPases occurs via interaction of the $\beta$-strand in the M2-M3 loop with the catalytic domain, as envisioned previously by Green and Stokes (36), was based largely on studies of the Ca-ATPase. Involvement of the M2-M3 loop of Na,K-ATPase in structural rearrangements associated with ligand binding and phosphorylation has also been apparent in distinctive conformational changes revealed by proteolytic cleavage patterns (37). Whereas evidence for M2-M3/M4-M5 loop interactions was weak using a yeast two-hybrid system (38), conclusive evidence was obtained in recent studies of specific iron-catalyzed cleavages of the $\alpha$-subunit (39). These interactions appear to involve the $\beta$-strand of M2-M3, and regions near the phosphorylation site and putative hinge region of the M4-M5 domain, but not the putative ATP binding site. Furthermore, the distinct cleavages in the presence of Na$^+$ versus K$^+$ indicate conformation-dependent interactions whereby the two loops interact when the enzyme is in the $E_2$ form and come apart in the $E_1$ conformation. The behavior of the $\alpha$M32E233K mutant indicates that the NH$_2$ terminus of the Na,K-ATPase impacts interactions between the M2-M3 and M3-M4 loops. Although the structural basis for this effect remains unknown, it may be implied that the NH$_2$ terminus favors the interaction of the two cytoplasmic loops, because truncation shifts the equilibrium toward $E_1$.

The kinetic alterations effected by the various mutations of residue 233 indicate that the shift is minimal with the conservative substitution of glutamate 233 by aspartate and moderate with substitution by glutamine compared with the nonconservative substitution by the positively charged lysine residue. This was evident with both the series of single mutants, E233D, E233Q, and E233K, as well as the double mutants, $\alpha$M32E233D, $\alpha$M32E233Q, and $\alpha$M32E233K. Although the sites of interaction between the NH$_2$ terminus and the M2-M3 and/or M4-M5 loops remain to be determined, it is likely that salt-bridge interactions are involved as suggested earlier (31, 40). Presumably such salt-bridge or ionic interactions occur close to the conformation-dependent specific iron-catalyzed cleavages. It may be relevant that an analysis of the behavior of a 1–27 deletion mutant of $\alpha$ indicates that the NH$_2$-terminal region critical in modulating the $E_1$-$E_2$ conformational equilibrium is the highly charged sequence $\alpha$MDELK$\alpha$K$^{32}$.

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