Functional Consequences of a Posttransfection Mutation in the H2-H3 Cytoplasmic Loop of the α Subunit of Na,K-ATPase*

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During kinetic studies of mutant rat Na,K-ATPases, we identified a spontaneous mutation in the first cytoplasmic loop between transmembrane helices 2 and 3 (H2-H3 loop) which results in a functional enzyme with distinct Na,K-ATPase kinetics. The mutant cDNA contained a single G850 to A substitution, which resulted in the replacement of glutamate at 233 with a lysine (E233K). E233K and α cDNAs were transfected into HeLa cells and their kinetic behavior was compared. Transport studies carried out under physiological conditions with intact cells indicate that the E233K mutant and α have similar apparent affinities for cytoplasmic Na⁺ and extracellular K⁺. In contrast, distinct kinetic properties are observed when ATPase activity is assayed under conditions (low ATP concentration) in which the K⁻ deocclusion pathway of the reaction is rate-limiting. At 1 μM ATP K⁺ inhibits Na⁺-ATPase of α, but activates Na⁺-ATPase of E233K. This distinctive behavior of E233K is due to its faster rate of formation of dephosphoenzyme (E₁) from K⁺-occluded enzyme (E₂(K)), as well as 6-fold higher affinity for ATP at the low affinity ATP binding site. A lower ratio of V₉⁻max to maximal level of phosphoenzyme indicates that E233K has a lower catalytic turnover than α. These distinct kinetics of E233K suggest a shift in its E₉/E₆ conformational equilibrium toward E₁. Furthermore, the importance of the H2-H3 loop in coupling conformational changes to ATP hydrolysis is underscored by a marked (2 orders of magnitude) reduction in vanadate sensitivity affected by this Glu→Lys mutation.

The Na,K-ATPase is an integral membrane protein complex that catalyzes the exchange of three cytoplasmic sodium ions for two extracellular potassium ions coupled to the hydrolysis of one molecule of ATP. It is a heterodimeric protein comprising a catalytic α subunit and a smaller heavily glycosylated β subunit (for reviews, see Refs. 1 and 2). Although an additional protein, γ, has been found associated with the α and β subunits (3), its function is unknown. The Na,K-ATPase is a member of the family of P-type ATPases, which are characteristically phosphorylated by ATP, and the phosphoenzyme intermediate undergoes rapid turnover during the reaction cycle. The phospho- as well as dephosphoenzymes exist in at least two distinct conformational states.

During transport both sodium and potassium ions are occluded within the Na,K-ATPase (for review, see Ref. 4). The nature of the cation occlusion site(s) and the structures involved in gating of these sites to both the cytoplasmic and extracellular milieu are unknown. Approaches using chemical labeling (5, 6) and site-directed mutagenesis (7–12) have identified a number of functionally important carboxyl-containing amino acid residues in transmembrane regions H4, H5, H6, H8, and H9. Point mutations of putative cation binding amino acid residues that resulted in the expression of functional enzymes were characterized by an altered affinity for ATP, K⁺, and/or Na⁺.

The prediction that the highly charged amino terminus had a role as a cation gate (13) now seems unlikely. Instead, interaction of this region with other region(s) of the α protein affects the K⁺ deocclusion pathway of the reaction, probably via alteration in E₂(K)/E₁ conformational equilibrium. Our recent study (14) demonstrated that the highly charged sequence comprising residues 24–32 of the amino terminus of the α subunit is important in modulating the K⁺ deocclusion pathway of the reaction cycle. Furthermore, this modulation is not due to the 24–32 nanopeptide per se, but rather to its interaction(s) with other isoform-specific region(s).

This paper describes the characterization of a spontaneous, posttransfection mutation in the H2-H3 cytoplasmic loop of the α isoform of the Na,K-ATPase. It differs from the wild type enzyme by having a lysine substituted for glutamic acid in position 233, and is designated E233K. The mutant enzyme is functional and its apparent affinities for sodium and potassium are unaltered. However, it exhibits a decrease in catalytic turnover, an increase in apparent affinity for ATP, and an increase in the rate of conversion of E₂(K) to E₁.

EXPERIMENTAL PROCEDURES

Recovery and Analysis of Mutant E233K from HeLa Genomic DNA—Two sets of synthetic oligonucleotide primers were prepared for polymerase chain reaction amplification of the 5' and 3' halves of a putative spontaneous mutant in the NH₂-terminal chimeric mutant of rat α cDNA (α11–14ε2 cDNA) that had been incorporated into the HeLa genomic DNA in a pRc/CMV (Invivogen) construct. The 5' primer set included a 32-mer complementary to the sequence of the sense strand of the T7 promoter of pRcCMV and a 33-mer complementary to the antisense sequence between bases 1828–1860 of the rat α cDNA. The 3' primer set included a 28-mer complementary to the sense strand of rat α between bases 1684 and 1711 and a 32-mer complementary to the antisense sequence in the SP6 promoter of pRcCMV at the 3’ end of the rat cDNA. The 5’ and 3’ halves of the α11–14ε2 cDNA were amplified from 1 μg of HeLa genomic DNA utilizing these primer sets (10 pmol of each primer) and the TaqPlus polymerase reaction kit (Stratagene). Aliquots of the amplified DNA, with or without digestion with HindIII and BamHI, were analyzed on 0.8% agarose gels and compared to products obtained by amplification of 1 ng of purified pRcCMV-rat α plasmid DNA. An aliquot of each reaction mixture was then utilized as...
Mutation in the H2-H3 Cytoplasmic Loop of Na,K-ATPase

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FIG. 1. K⁺ sensitivity of Na-ATPase. ATP hydrolysis was assayed in the presence of 1 μM ATP, 20 mM NaCl, and various concentrations of KCl as described under "Experimental Procedures." Data are presented as percent of Na-ATPase activity (control) measured in the absence of added KCl. Control activities in the presence of 20 mM NaCl, 1 mM MgSO₄, 20 mM choline chloride, and 5 μM ouabain to inhibit endogenous activity (μmol/mg × h), were 118 ± 41 and 205 ± 48, for α₁ and E233K, respectively. ○, α₁; ●, E233K. Values shown are the means ± S.D. of 11 and 4 experiments for α₁ and E233K, respectively.

template for additional amplification with the same primer sets. Amplified DNA from the 5’ and 3’ halves of rat α₁(1–14α2) was digested with HindIII and BamHI, gel-purified, and ligated into M13mp18/19 that had been digested with HindIII and BamHI. Competent DH5αF’ Escherichia coli were transformed with each ligation mixture and plated, and the resultant plaques were picked and amplified in liquid Escherichia coli 1m M MgSO₄, 20 mM choline chloride, and 5 mM ouabain to inhibit endogenous activity (μmol/mg × h), were 118 ± 41 and 205 ± 48, for α₁ and E233K, respectively. ○, α₁; ●, E233K. Values shown are the means ± S.D. of 11 and 4 experiments for α₁ and E233K, respectively.

After detection of a single base substitution at base 950 in the pooled M13mp18–5’α and M13mp19–3’α DNA. The 5’ and 3’ halves of the rat α₁(1–14α2) DNA from the pooled clones were completely sequenced using the Sanger dideoxy method (15), Sequenase version 2.0 (Amersham), and synthetic oligonucleotides as primers.

Membrane Preparations and Enzyme Assays—Membranes were isolated, and assays of Na,K-ATPase, Na-ATPase, and ouabain-sensitive K⁺(Rb⁺) influx were carried out as described previously (18). Assays of K⁺-occluded enzyme and the formation of E₂ from K⁺-occluded enzyme are also described elsewhere (14).

RESULTS

During the course of studies with a rat α₁/α₂ NH₂-terminal chimeric mutant cDNAs transfected into HeLa cells, we observed that the Na,K-ATPase of one clone had functional properties distinct from rat α₁. The observation that the enzymic properties of several other clones selected from the same chimeric cDNA (rat α₁(1–14α2)) transfection were indistinguishable from those of α₁ suggested that a spontaneous, postransfection mutation had occurred within the coding region of the rat α₁ cDNA. As described under “Experimental Procedures,” the alteration was identified as the substitution of lysine for glutamate at position 233 in the cytoplasmic region between transmembrane helices H2 and H3. The functional difference between this mutant (E233K) and the rat α₁ enzyme was apparent when pump-mediated ATP hydrolysis was measured at micromolar ATP, under which condition ouabain-sensitive Na⁺–dependent ATPase activity of the α₁ isoform is inhibited by K⁺ (19). Accordingly, although K⁺ stimulates the dephosphorylation step of the reaction (E₂P + K → E₂(K) + P), and becomes occluded within the pump protein, its deocclusion is extremely slow. Stimulation of deocclusion is effected by ATP binding with low affinity (E₂(K) + ATP → ATP-E₁ + K⁺; Ref. 19). As a consequence, K⁺ is an inhibitor of the overall reaction at low ATP, and an activator at high ATP concentration. In fact, as shown previously (14, 18, 20) and described below, at micromolar ATP the response of Na-ATPase to K⁺ is a sensitive means of characterizing isoform- or mutant-specific differences in the K⁺ deocclusion pathway of the reaction.

K⁺ Sensitivity of E233K—As shown in Fig. 1, the Na-ATPase activity of membranes isolated from rat α₁-transfected cells is inhibited by the addition of 0.1–5 mM KCl, whereas the activity of E233K is markedly stimulated. At 1 mM KCl, the Na-ATPase activities of the mutant and α₁ enzymes are 200% and 50%, respectively, of their control activities measured in the absence of KCl.

The change in K⁺ sensitivity at low ATP concentration caused by the mutation is suggestive of a change in rate of a reaction step following dephosphorylation of the K⁺-sensitive form of phosphoenzyme commonly referred to as E₂P in the Albers-Post model. According to a branched pathway of K⁺ deocclusion (Scheme I), it is assumed that ATP can bind with either (i) low affinity to the K⁺-occluded form of the enzyme, E₂(K), at a step preceding the release of potassium and the formation of ATP-E₁ (pathway a), or (ii) with high affinity at a step following the slow release of potassium from E₂(K) (pathway b). Accordingly, potassium inhibition is dependent upon the apparent affinity of the enzyme for ATP at its low affinity site and/or the rate of release of K⁺ from E₂(K). Analysis of kinetic parameters of pathways a and b is described below.

Kinetic Analysis of the Reaction Modeled According to ATP Binding to Low and High Affinity Sites—The kinetic parameters Kᵢ, the apparent affinity for ATP, and Vₘₐₓ, for pathways a and b were obtained from measurements of Na,K-ATPase activity versus ATP concentration varied from 0.1 to 500 μM. Reciprocal plots of the data are shown in Fig. 2, and the kinetic parameters are given in Table I. In the case of the α₁ enzyme, the data points can be fitted to a two-component sys-
tem, each linear in the range of 1–8 μM and 25–500 μM ATP. The constants for the apparent affinities for ATP at low and high affinity sites, designated $K_L$ and $K_H$, respectively, are $331 \pm 44$ μM and $5.44 \pm 1.9$ μM for α1; $V_{\text{max}}$ of the high affinity component, $V_H$, is $0.684 \pm 0.20$ μmol/(mg × h) and represents 4.2% of the activity of the $V_{\text{max}}$ of the low affinity component, $V_L$ ($16.2 \pm 3.0$ μmol/(mg × h)). These values for α1 are similar to those reported previously (14). In contrast, the reciprocal plot of the E233K mutant is a straight line within the entire range of 1–500 μM, resulting in an apparent affinity of $56.3 \pm 14$ μM and a $V_{\text{max}}$ of $9.90 \pm 1.1$ μmol/(mg × h). Although these values are taken from representative experiments and there is some variability in $V_{\text{max}}$ values, it was observed that the activity of membranes prepared from E233K-transfected cells is generally lower than that of the rat α1-transfected cells.

An additional component of activity was observed in the range of 0.1–0.5 μM ATP (data not shown), which represented ~1–2% of $V_L$. The constant for its apparent affinity for ATP, $K_{VH}$, was similar for both enzymes (0.19 ± 0.09 μM for α1 and 0.21 ± 0.01 μM for E233K). As suggested previously (14), this very high affinity component may represent an alternate minor pathway of ATP hydrolysis, involving $K^+$ release directly from $E_2(K)$, i.e. $E_2(K) \rightarrow E_2 + K^+$ followed by the conversion of $E_2$ to $E_1$. The nature of this minor component was not investigated further.

**K+ Oclusion/Deoclusion**—The marked difference in effect of $K^+$ on Na-ATPase of E233K and α1 as depicted in Fig. 1 can be attributed to the higher affinity of E233K for ATP (lower $K^+$) and/or higher rate of $K^+$ deoclusion from $E_1(K)$ via pathway b. Since the low specific activity of the Na,K-ATPase expressed in HeLa cell membranes prevents the direct measurement of $E_2(K)$ and, therefore, the $K^+$ deoclusion pathway of the reaction, an indirect method was used as described recently (14). In this assay, formation of the $K^+$-occluded enzyme is reflected by the decrease in phosphoenzyme ($E^2(P)$) formed by phosphorylation with [γ-$^32P$]ATP following equilibration of the enzyme at room temperature (i) without and (ii) with $K^+$. The reduction in $E^2(P)$ resulting from preincubation with $K^+$ ($\Delta E^2(P)$) is a measure of the amount of $E_2(K)$.

As shown in Fig. 3, and in agreement with our previous studies (14), the maximum formation of $E_2(K)$ with the α1 isofrom is achieved after preincubation of the enzyme with 1 mM KCl, whereas with the α1E233K mutant maximum $E_2(K)$ requires at least 4 mM KCl. Using a simple model, $B_{\text{max}}(S)/K_{0.5} + [S])$, to describe the binding as in earlier studies of $K^+$ occlusion in the kidney enzyme (21), the values of $K_{0.5}$ obtained are $1.0 \pm 0.5$ and $0.12 \pm 0.03$ mM for the E233K and α1 enzymes, respectively (mean ± S.D. of three experiments). Maximum levels of $E_2(K)$, expressed as a percentage of phosphoenzyme formed without $K^+$ preincubation (enzyme preincubated without KCl minus the KCl baseline) were 93% ± 10% and 97% ± 5% for E233K and α1, respectively.

The shift in the equilibrium $E_1 + K^+ \leftrightarrow E_2(K)$ toward $E_1$ can be accounted for by either a slower rate of occlusion or a faster rate of deoclusion. Although the rate of formation of $E_1$ from $E_2(K)$ is not a direct measure of $K^+$ release from $E_2(K)$, it is characterized by single exponential kinetics (14) and, as a first approximation, is an estimate of the rate of deoclusion through pathway b.

In the representative experiments shown in Fig. 4, the rate of $E_1$ formation from $E_2(K)$ was determined as follows: α1 and E233K enzymes were first equilibrated with 8 mM KCl to form $E_2(K)$. $\Delta E^2(P)$, the difference ($E^2(P)$ formed following preincubation in the absence of $K^+$ minus ($E^2(P)$ formed following preincubation with 8 mM $K^+$)) was taken to represent 100% $K^+$-occluded enzyme (Fig. 3). Deoclusion was measured by following the rate of increase in $\Delta E^2(P)$ at 10°C, a temperature at which deoclusion is sufficiently slow to permit manual assays. We showed previously that (i) maximal $E^2(P)$ formed from $E_1$ was similar at 10°C and 0°C, and remained constant over the period used to follow deoclusion at 10°C, and that $\Delta E^2(P)$ remained constant for up to 30 s at 0°C. Therefore, the time course of increase in $\Delta E^2(P)$ at 10°C reflects the rate of the rate-limiting step in the sequence $E_2(K) \rightarrow E_1K \rightarrow E_1$ at that temperature.

As can be seen in Fig. 4, the rate of formation of $E_1P$ from $E_2(K)$ is significantly faster for the E233K mutant compared to the α1 enzyme; rate constants are (s$^{-1}$) $0.09 \pm 0.005$ and $0.02 \pm 0.004$ for E233K and α1, respectively. In other control experiments carried out with α1 and E233K, deoclusion was allowed to proceed for 10 s at 10°C in ATP-free Na$^+$ medium, after which the $E_1$ formed was measured by rapid dilution and conversion to $E_1P$ at 0°C (5-s phosphorylation at 0°C). The rate constants estimated from the 10-s decrease in $E_2(K)$ were similar to those obtained with 1 μM [γ-$^32P$]ATP present during deoclusion. This result indicates that the presence of 1 μM ATP during deoclusion did not significantly affect the rate of deoclusion under these conditions.

**Phosphoenzyme Turnover and the Effect of Oligomycin**—Since oligomycin stabilizes Na$^+$ occlusion in $E_1$ (22, 23) and

![Figure 2](image)

**Figure 2.** Reciprocal plots of Na,K-ATPase activity as a function of ATP concentration. ATP hydrolysis at various concentrations of ATP was determined as described under "Experimental Procedures." Data are presented as 1/($V_{\text{max}}$). Values of $V_{\text{max}}$ obtained in the range of 25–500 μM ATP for α1 and 1–500 μM ATP for E233K (μmol/(mg × h)) were 12.8 and 11.2, for α1 and E233K, respectively. Symbols are as described in legend to Fig. 1. Results shown are from a representative experiment; values shown are the average of triplicate determinations.

| α subunit | $V_{\theta}/V_L$ | $K_L$ | $K_H$ | $K_{VH}$ |
|----------|----------------|-------|-------|---------|
| α1       | 0.042 ± 0.009 (6) | $331 \pm 44$ (6) | $5.44 \pm 1.9$ (6) | $0.19 \pm 0.09$ (3) |
| E233K    | $V_H$ not detected | 56.3 $\pm$ 14 (3) | Not apparent | $0.21 \pm 0.01$ (2) |
traps the enzyme in the E1P state, the extent to which EP increases in the presence of oligomycin is a measure of the steady-state distribution of the dephospho- and phosphoenzyme during steady-state catalysis. As shown in Table II (see legend), phosphoenzyme measured at 0°C in the presence of oligomycin is increased 2.4-fold in the presence of 8 mM K+ at 0°C minus (E2P formed in the presence of 8 mM K+ at 0°C)). Symbols are as described in the legend to Fig. 1. Results shown are from a representative experiment; values shown are the average of triplicate determinations.

**Table II**

| Phosphoenzyme | Oligomycin | Oligomycin |
|---------------|------------|------------|
| α1            | 7598 ± 1331 (11) | 8.69 ± 0.24 | 19.9 ± 4.1 |
| E233K         | 4481 ± 196 (3) | 19.3 ± 0.68 | 21.4 ± 1.0 |

*Values of turnover were obtained from the ratio of V_{max} of Na,K-ATPase activity measured at 37 °C in the presence of 100 mM NaCl, 10 mM KCl, and 1 mM ATP, to the maximal amount of EP measured at 0 °C in the presence of 100 mM NaCl, 1 mM ATP, and 20 μg/ml oligomycin. Results shown are from a representative experiment; values shown are the average ± S.D. of triplicate determinations. Mean values ± S.D. of the ratio of EP_{a,oligomycin}/EP_{b,oligomycin} of at least three independent experiments were 2.4 ± 0.082 and 1.4 ± 0.26, for α1 and E233K, respectively.*

**Fig. 5. 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 ATP, 1 mM EGTA, 30 mM MES (4-morpholineethanesulfonic acid)/Tris (pH 6.5) (or 30 mM Tris/HCl (pH 7.0) or 50 mM Tris/HCl (pH 7.5–8.9)), and 5 μM ouabain (to inhibit endogenous Na,K-ATPase activity) or 10 mM ouabain (base-line activity). The Na,K-ATPase activities corresponding to 100% were (μmol/mg × h) 12.2 ± 0.27 and 9.03 ± 0.030 at pH 7.5, for α1 and E233K, respectively. pH values shown were measured at room temperature. Values of activity shown are the means of two representative experiments. Symbols are as described in legend to Fig. 1.

for α1 (50% decrease). In contrast, the activity profile on the alkaline side of the optimum was similar for both enzyme forms.

**Apparent Cation Affinities**—To determine if the substitution of glutamate 233 with a lysine residue alters the apparent affinity of the mutant enzyme for cations, we compared the transport behavior and Na,K-ATPase kinetics of HeLa cells transfected with the E233K and α1 enzymes as described previously (18). Briefly, Rb\textsuperscript{in} influx sensitive to high (10 mM) ouabain was measured in medium containing either various concentrations of sodium in the presence of monensin to control and maintain intracellular sodium concentration and saturating extracellular KCl concentration, or various concentrations of extracellular KCl, in the presence of monensin and 10 mM NaCl as described previously (25). We found that the apparent affinities for cytoplasmic Na\textsuperscript{+} (Na\textsubscript{cyt}) of the E233K mutant and α1 enzymes are indistinguishable. In five separate experiments with the data fitted to a three-site cooperative model, the apparent affinities for Na\textsubscript{cyt} (K_{Na}) are (μM) 20.9 ± 2.0 for E233K and 20.1 ± 2.7 for α1. Since intracellular Na\textsuperscript{+} could not be decreased below ~15 mM with monensin present (25), we also compared the apparent affinities for Na\textsuperscript{+} in assays of Na,K-ATPase at 1 mM ATP and constant (10 mM) K\textsuperscript{+}. No
Another possibility is that binding of phosphate is altered. The former is less likely according to the argument that changes in \( K_\text{p} \) effected by mutations in cytoplasmic domains are probably secondary to changes in \( K_{\text{ATP}} \) as discussed below.

**DISCUSSION**

The experiments described in this report provide evidence for a functional role of the H2-H3 cytoplasmic loop in conformation coupling in the Na,K-ATPase. The changes effected by mutation of glutamate 233 to lysine are consistent with the conclusion that this substitution alters the equilibrium between the major conformational states of the dephospho- and phosphoforms during steady-state catalysis. An increase in the steady-state distribution of \( E_1 \) and \( E_2 \) in favor of \( E_1 \) effected by the Glu233 → Lys mutation is apparent as (i) an ~4-fold increase in the rate constant for \( E_1 \) formation from \( E_2^\beta(K) \), accounting for the higher \( K_m \) for \( K^+ \) occlusion of E233K compared to \( E_1 \), (ii) a 6-fold increase in the apparent affinity for ATP at the step \( E_2^\beta(K) + ATP \rightarrow ATP-E_1 + K^+ \) when the overall Na,K-ATPase reaction is studied at 37°C, and (iii) a lesser decrease in activity as pH is lowered to pH 6.8 under which condition deocclusion of \( K^+ \) from \( E_2^\beta(K) \) becomes the main rate-limiting reaction in the wild type enzyme (24). The results are also consistent with a shift in the \( E_1P \leftrightarrow E_2P \) equilibrium toward \( E_1P \) caused by the Glu233 → Lys mutation. The evidence is two-fold: the smaller increase in \( E_2^{32P} \) effected by oligomycin and lower turnover of E233K compared to \( E_1 \), at least at physiological pH under which condition the \( E_1 \rightarrow E_2P \) transition is partly rate-limiting (24).

Glu233 is in a region previously identified as having a role in the \( E_1P \leftrightarrow E_2P \) conformational transition (for review, see Ref. 30). When Na,K-ATPase in the \( E_1 \) conformation is exposed to cleavage at either Leu266 or Arg262 in the H2-H3 cytoplasmic loop, as described by Jörgensen and co-workers (reviewed in Ref. 31), the \( E_1P \rightarrow E_2P \) conformational transition is blocked. In the sarcoplasmic reticulum Ca-ATPase, tryptic cleavage at Arg262 as well as site-specific mutation of residues in the predicted \( \beta \)-strand domain of the H2-H3 loop also blocked the \( E_1P \rightarrow E_2P \) conformational transition (reviewed in Ref. 32).

As pointed out by Green and Stokes (33), the H2-H3 loop is well conserved among P-type ATPases. In their model of P-type ion pumps, based largely on studies of the sarcoplasmic reticulum Ca-ATPase (34), their suggestion that the anti-parallel \( \beta \)-strand is positioned close to the phosphorylation site is supported by vanadate protection of Ca-ATPase from proteolytic degradation (35). In the yeast \( H^+ \)-ATPase, Perlin and co-workers (27, 28) have shown that perturbations of residues in this region not only alters the distribution of conformational intermediates during steady-state catalysis, but also decreases the sensitivity of this P-type pump to vanadate inhibition, consistent also with the conclusion that this region interacts with the catalytic phosphorylation domain.

In studies of Na,K-ATPase, involvement of the H2-H3 loop in structural rearrangements associated with ligand binding and phosphorylation has been apparent in distinctive conformational changes revealed by proteolytic cleavage patterns (36). The importance of these interactions in conformation coupling is emphasized by the remarkable vanadate insensitivity caused by the Glu233 → Lys mutation. This vanadate insensitivity suggests interaction of Glu233 located in the putative \( \beta \)-strand region of the H2-H3 loop with the phosphate binding domain within the catalytic H4-H5 loop. It is also possible that the vanadate insensitivity of E233K is the consequence of a decrease in steady-state level of \( E_2 \) required for \( P_i \) (vanadate) binding (cf. the vanadate-insensitive mutants of PMA1; Ref. 28).

When deocclusion of \( K^+ \) from the \( K^+ \)-occluded state, \( E_2^\beta(K) \), is
analyzed as a branched pathway reaction (Scheme I; cf. Refs. 14 and 37), values of Na,K-ATPase activity as a function of varying ATP concentration can be fitted to a biphasic reciprocal plot in the case of the α1 enzyme. The ratio of V_{max} values for the high affinity to low affinity components, V_{H}/V_{L}, is 0.043 for α1. In contrast, there is little, if any, evidence of a high affinity component (V_{H}) pathway b with the E233K enzyme. This is not unexpected, since, according to the simple Michaelis-Menten relationship shown in Equation 1, activity attributed to pathway a, compared to activity attributed to pathway b, is much greater in the E233K enzyme than it is in the α1 enzyme when ATP is reduced to micromolar concentrations.

\[ v = V_a[S] + V_b[S] \]  

(Eq. 1)

With the E233K mutant, the 6-fold decrease in K_{a} results in a similar -fold increase in the rate of K+ deocclusion through pathway a, thus largely masking the activity through pathway b. Nevertheless, an increase in the rate of E_{1}(K) \rightarrow E_{2}(K) is also observed with E233K under conditions in which E_{2}(K) is first formed by equilibrating the enzyme with a saturating concentration of K^{+}. The increased rate of K^{+} deocclusion during subsequent incubation at 10 °C also accounts for the higher K_{a} for K^{+} under equilibrium binding conditions. It should be noted that the contribution of pathway b to overall activity may increase as temperature is increased. Under the conditions of the deocclusion assays, the relative rate of deocclusion through pathway a compared to that through b at 10 °C is one-half that at 37 °C.

It is of interest to compare this mutation with those in other cytoplasmic regions of the α1 subunit. In the H4-H5 loop, mutation of aspartate at the phosphorylation site of the Torpedo californica, pig, or sheep kidney enzymes results in complete loss of Na,K-ATPase activity (38–40), and the latter two groups reported an increase in ATP affinity. Mutation of Lys^{507} of the T. californica enzyme at the putative ATP binding site decreases both activity and ouabain binding capacity (38), and substitution of Cy^{513} in the rat α1 enzyme decreases ATP affinity (41). In contrast to these mutations, a deletion mutant in which residues 1–32 have been removed from the cytoplasmic amino terminus of α1 results in a fully active enzyme with kinetic changes that are similar to those observed with the E233K mutation (14). This mutation results in a 2.5-fold increase in affinity for ATP at its low affinity binding site and an increase in rate through pathway b (14). With this mutant (α1M32), as with E233K, the rate of conversion of E_{2}(K) to E_{1} and the apparent affinity for K^{+} occlusion were increased, and turnover was substantially reduced. Similarly, a NH2-terminal truncated mutant of the Bufo marinus enzyme was recently shown to have a reduced E_{1} to E_{2} conformational change (42). As shown elsewhere, the α2 isofrom behaves similarly to α1M32 and may be regarded as a conformational variant of α1.

These cytoplasmic mutants contrast with those in which substitutions in transmembrane helices also result in active, functionally altered enzymes, most of which are characterized by changes in affinities for Na^{+} and K^{+}. Of these, one mutation localized to H5 (Glu^{779} \rightarrow Glu; Ref. 11) alters (decreases) only Na^{+} affinity. An Asn^{826} \rightarrow Leu mutation in H4 decreased the apparent affinity for Na^{+} but slightly increased it for K^{+} (8). Another group of transmembrane substitutions resulted in decreases in affinities for both Na^{+} and K^{+}. The substituted residues are, in H4: Glu^{229} \rightarrow Gln (43) and Glu^{327} \rightarrow Leu (9); in H5: Gln^{871} \rightarrow Ala (7, 12), with quantitatively smaller changes noted in Glu^{761} \rightarrow Asp (12); and in H6: Thr^{809} \rightarrow Ala (7). In contrast to these mutations, substitution of Ser^{775} in H5 with either alanine or cysteine decreases apparent K^{+} affinity dramatically, 31-fold in the case of Ser^{775} \rightarrow Ala and 13-fold in the case of Ser^{775} \rightarrow Cys, with no evidence of a change in Na^{+} affinity (10).

Substitutions in transmembrane helices effecting an increase in K_{a} are also associated with an increase in apparent affinity for ATP. In the analysis of Eisner and Richards (26) mentioned earlier, pump-mediated K^{+} influx was empirically described by a relationship which showed that decreasing K_{ext} concentration (presumably equivalent to increasing K_{a}) increases the apparent affinity for ATP. Similarly, increasing ATP concentration (presumably equivalent to decreasing K_{ATP}) decreases the apparent affinity for external K^{+} (and also increases V_{max}). Considering, for example, the following sequence of reactions that constitute the low affinity pathway of K^{+} transport, it is likely that the various transmembrane residues which coordinate K^{+} ions probably do so with distinct affinities and/or selectivities.

\[ V_{ATP} = \frac{E_{P} \cdot E_{P} \cdot E_{K}}{E_{2}(K) \rightarrow ATP \cdot E_{1} \cdot K^{+} \cdot ATP \cdot E_{1}} \]

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Accordingly, distinct mutations may differentially alter the rate of specific reaction steps involved in K^{+} binding, occlusion, and deocclusion, with associated secondary changes in apparent affinity for ATP.

The relationships between cation and ATP affinities of the cytoplasmic regions of the α1 subunit in the H2-H3 loop alter its interaction(s) with other regions of the α subunit resulting in a change in the conformational equilibrium, such that the apparent affinity for ATP is increased. Changes in apparent affinity for K^{+} that were observed at suboptimal ATP concentrations are probably secondary changes to K_{ATP} as well as to steps involved in the conversion of conformational forms.

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