Specific Sites in the Cytoplasmic N Terminus Modulate Conformational Transitions of the Na,K-ATPase*

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The cytoplasmic N terminus of the Na,K-ATPase is a highly charged and flexible structure that comprises three predicted helical regions including H1 spanning residues 27 to 33 and H2 spanning residues 42 to 50. Previous deletion mutagenesis experiments showed that deletion of residues up to and including most of H2 shifts the E$_1$/E$_2$ conformational equilibrium toward E$_1$. The present study describes a clustered charge-to-alanine mutagenesis approach designed to delineate specific sites within the N terminus that modulate the steady-state E$_1$ ↔ E$_2$ and E$_1$P ↔ E$_2$P poise. Criteria to assess shifts in poise include (i) sensitivity to inhibition by inorganic orthovanadate to assess overall poise; (ii) K$^+$-sensitivity of Na-ATPase measured at micromolar ATP to assess changes in the E$_2$(K$^+$) + ATP → E$_1$-ATP + K$^+$ rate; (iii) K$^{\text{ATP}}$ for low-affinity ATP binding at the latter step; (iv) overall catalytic turnover, and (v) the E$_1$P → E$_2$P transition. The results of alanine replacements in H1 (31KKE) suggest that this site stabilizes E$_2$P and to a lesser extent E$_1$. In H2, residues within 47HRK have a role in stabilizing E$_2$, but not E$_1$P as revealed with double mutants 31KKE → AAA/47H → A and 31KKE → AAA/47HRK → AAA. Taken together, these observations suggest that sites 31KKE in H1 and 47HRK in H2 have distinct roles in modulating the enzyme’s conformational transitions during the catalytic cycle of the enzyme.

The Na,K-ATPase or sodium pump is essential for active transport of Na$^+$ and K$^+$ across the plasma membrane of mammalian cells and consequently for many key cellular functions including the maintenance of the membrane potential, cell volume, and secondary active transport systems. The Na,K-ATPase is an integral membrane protein complex consisting of a large catalytic α subunit (∼110 kDa) that spans the plasma membrane ten times and a smaller, highly glycosylated β subunit, which ensures the proper folding and anchoring of α at the cell surface. As a member of the family of P-type ATPases, during the course of its reaction cycle the α subunit of the Na,K-ATPase undergoes phosphorylation and dephosphorylation of a conserved aspartate residue within the large cytoplasmic loop between transmembrane segments 4 and 5 (L4/5). Both the dephosphorylated and phosphorylated forms of the enzyme undergo conformational transitions commonly referred to as E$_1$ ↔ E$_2$ and E$_1$P ↔ E$_2$P, respectively. At present, four isoforms of α and three isoforms of β have been described, which are distributed in a tissue- and developmentally dependent manner (for recent reviews, see Refs. 1, 2).

Analogous to the sarco-endoplasmic reticulum Ca-ATPase (SERCA)$^2$ of known crystal structure (3), conformational transitions of the Na,K-ATPase involve the interaction of the actuator (A) domain, comprising the cytoplasmic N terminus and the loop between transmembrane segments 2 and 3 (L2/3), with the loop between transmembrane segments 4 and 5 (L4/5) that encompasses the nucleotide binding (N) and phosphorylation (P) domains. (For a review of the nature of cytoplasmic domain interactions associated with conformational transitions, see Ref. 3). Although both P-type pumps share a high degree of homology, there are several distinguishing characteristics between these pumps. (For a comprehensive structural comparison, see Ref. 4). One notable difference between the two P-type pumps is their cytoplasmic N terminus. In both, the extended link to the first transmembrane domain as well as the preceding two helical regions are fairly well conserved. However, the N terminus of Na,K-ATPase is ∼40 residues longer than that of SERCA and forms a highly charged and flexible region that includes an additional α-helix. Thus, in Na,K-ATPase there are three putative helices: H1 spanning at least residues 27 to 33; H2 spanning at least residues 42 to 50; and H3 near the S1 stalk region of the enzyme (5). Interestingly, however, not all of the N terminus of Na,K-ATPase is essential to pump function as portions can be removed or substituted with a tag protein sequence without abrogating pump activity (5, 6).

In an earlier study (5), we showed that deletion of 32 or 40 residues, corresponding to disruption or loss of H1, progressively shifts the E$_1$ ↔ E$_2$ poise of the enzyme toward E$_1$ forms. Further removal of 46, 49, or 56 residues, corresponding to disruption or loss of H2, reverts this shift. The goal of the present study was to gain more definitive insight into the identity and role of residues or residue clusters involved in modulating conformational transitions. Based on the premise that intramolecular interactions via salt bridges underlie structural changes accompanying E$_1$ ↔ E$_2$ transitions (c.f. Ref. 2), a clustered charge-to-alanine mutagenesis approach has been used to identify and functionally characterize specific sites between residues 24

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$^2$ The abbreviations used are: SERCA, sarco-endoplasmic reticulum Ca-ATPase; WT, wild-type.
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and 56. This modification of conventional alanine scanning mutagenesis yields a small systematic set of mutants that can readily be analyzed, an approach that has been used successfully to study structure-function relationships of various proteins including plasminogen activators (7), vaccinia virus (8), staphylokinase (9), and cyclin proteins (10).

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Transfection, Clone Selection, and Cell Culture**—Alanine substitutions were introduced into the rat α1 cDNA within a modified pBlI shuttle vector using the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The full-length mutant cDNAs were sequenced to verify the presence of the desired substitutions and then released from the shuttle vector by digestion with HindIII. The mutated cDNAs were then ligated into pCDNA3.1(−) (Invitrogen) and their orientation determined by restriction analysis. Mutant sequences and designations are shown in Table 1.

HeLa cells were transfected with the pCDNA-rat α1 wild-type (WT) or mutant constructs using the Lipofectamine technique (Lipofectamine, Invitrogen), and cells expressing the relatively ouabain-resistant rat α1 enzyme and mutants thereof were selected as described previously (11). HeLa cells expressing the mutant α1 enzymes were amplified in Dulbecco’s modified Eagle’s medium plus 10% newborn calf serum, 100 units/mg penicillin G, 100 μg/ml streptomycin, and 1 μM ouabain as described previously (12).

**Membrane Preparation**—NaI-treated microsomal-rich membranes were prepared from the WT and mutant cells by the method of Jewell and Lingrel (11) as described by Lane (13). Protein content was determined with a detergent-modified Lowry assay (14). Prior to assay, membranes were treated for 10 min at room temperature with SDS (0.65 mg/ml) in the presence of bovine serum albumin (1%) followed by dilution 5-fold with ice-cold 0.3% bovine serum albumin as described by Forsbus (15).

**Assays of ATP Hydrolysis**—Na,K-ATPase activity was measured as the release of 32P, from [γ-32P]ATP as described previously (16). Briefly and unless indicated otherwise, the membranes were pre-incubated for 10 min at 37 °C with all reactants added except [γ-32P]ATP. The reaction was initiated by the addition of [γ-32P]ATP and stopped with ice-cold trichloroacetic acid-1 solution (5% trichloroacetic acid, 5 mM ATP, and 2.5 mM NaH₂PO₄). Final concentrations for Na,K-ATPase activity measurements were 100 mM NaCl, 10 mM KCl, 4 mM MgSO₄, 30 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM EGTA (pH 7.4), and 5 μM ouabain (Sigma). 5 mM ouabain was used to determine base-line hydrolysis activity. As in earlier studies, assays of Na,K-ATPase activity were carried out using 1 mM ATP to maintain close to saturating ATP concentration and also maximize sensitivity of assays of the relatively low-activity cultured cells (c.f. Refs. 16–18). Na-ATPase activity was measured at 1 μM ATP as described previously (12) in the presence of 20 mM NaCl, 4 mM MgSO₄, 30 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM EGTA, 5 μM ouabain, with varying amounts of added KCl and choline chloride to maintain constant chloride (40 mM) concentration; base-line activity was determined with NaCl omitted and 40 mM KCl added. For studies of vanadate sensitivity, inorganic orthovanadate solutions were prepared fresh, in water, prior to the experiment and added with the [γ-32P]ATP solution to initiate the reaction. Na,K-ATPase activities obtained at various vanadate concentrations 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 (19).

**Catalytic Turnover**—Catalytic turnover was estimated as the ratio $V_{\text{max}}/E_{\text{Pmax}}$ as described previously (5) with minor modifications. Briefly, for estimation of $V_{\text{max}}$, one aliquot of SDS-solubilized membranes was taken for estimation of Na,K-ATPase activity at 1 mM ATP as described above. For measurement of $E_{\text{Pmax}}$, another aliquot was pretreated for 10 min at 37 °C with 20 μM ouabain and then for 2.5 min at room temperature with either 50 mM NaCl and 0.2 mg/ml oligomycin to block $E_{\text{P}} \rightarrow E_{\text{P}}$ or without oligomycin (vehicle alone) and with 50 mM KCi replacing NaCl (base-line values). Phosphorylation was carried out for 30 s at 0 °C with 1 μM [γ-32P]ATP as described earlier (5).

**Rate of $E_{\text{P}} \rightarrow E_{\text{P}}$**—The enzyme was first phosphorylated by [γ-32P]ATP in the presence of high (600 mM) NaCl concentration to stabilize the enzyme in the $E_{\text{2P}}$ state (20). The $E_{\text{2P}} \rightarrow E_{\text{2P}}$ rate was then determined by measuring the rate of disappearance of total $E_{\text{2P}}$ following the simultaneous dilution of the salt concentration, reduction in [γ-32P]ATP specific activity (to allow “normal” relaxation of $E_{\text{2P}} \rightarrow E_{\text{2P}}$), and addition of KCi to catalyze rapid hydrolysis of $E_{\text{2P}}$ (20, 21). Accordingly, the enzyme was first phosphorylated for 30 s in medium containing 1 μM [γ-32P]ATP, 600 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, and 20 mM Tris-HCl (pH 7.4). Dephosphorylation was then initiated by 5-fold dilution with a “chase” solution containing final concentrations of 20 mM KCi, 10 μM unlabeled ATP, 1 mM EGTA, and 20 mM Tris-HCl (pH 7.4). Samples were taken for measurement of total $E_{\text{2P}}$ for periods up to 30 s. Background phosphoenzyme levels were obtained by allowing the chase to proceed for 60 s. Because the rate of K⁺-activated dephosphorylation of $E_{\text{2P}}$ is much faster than that of the preceding formation of $E_{\text{2P}}$ from $E_{\text{P}}$, the time course of $E_{\text{2P}}$ decay reflects primarily the $E_{\text{P}} \rightarrow E_{\text{P}}$ transition.

**RESULTS**

To identify sites in the cytoplasmic N terminus of the α1-subunit of the Na,K-ATPase responsible for its modulatory role in conformational transitions, a series of charge-to-alanine mutants were generated. As shown in Fig. 1 and detailed in Table 1, alanine replacements of clusters of up to three charged amino acids between residues 27–33, 42–50, and 61–68 are shown. Charged residues are shown in red, and those mutated to alanines are underlined. Numbering is based on the mature rat α1 amino acid sequence.
### TABLE 1
Summary of kinetic behavior of Na,K-ATPase mutants

| Enzyme | Max. K⁺ stim. Na-ATPase | K⁺<sub>ATP</sub> | I<sub>v</sub> (vanadate) | E<sub>f</sub> → E<sub>p</sub> | Turnover<sup>f</sup> | V<sub>max</sub><sup>e</sup> |
|--------|-------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| α1     | Inhibition               | 363 ± 36<sup>b</sup> | 0.99 ± 0.10     | 1.0 ± 0.08      | 10,200 ± 540    | 113 ± 15 (20)   |
| α1M27  | Inhibition               | 172 ± 13<sup>b</sup> |                | 5.85 ± 0.76<sup>b</sup> | 4420 ± 879     |
| α1M32<sup>a</sup> | 289 ± 16 | 145 ± 31<sup>b</sup> |                | 11.66 ± 0.79<sup>h</sup> | 3330 ± 30       |
| α1M40<sup>a</sup> | 423 ± 26 | 77 ± 32<sup>b</sup> | 891 ± 106<sup>b</sup> | 4.76 ± 0.73<sup>b</sup> | 8620 ± 610      |
| 24ERD → AAA | 165 ± 7.26 | 145 ± 12<sup>b</sup> | 1.72 ± 0.61     | 1.35 ± 0.43     | 101 ± 8 (7)    |
| 24ER → AA | Inhibition              | 397 ± 37        | 0.81 ± 0.31     | 287 ± 59 (11)   |
| 28DE → AA | Inhibition              | 198 ± 27        | 0.66 ± 1.4      | 54 ± 12 (7)     |
| 31HKE → AAA | 218 ± 26 | 30 ± 5<sup>b</sup> | 1980 ± 538<sup>h</sup> | 9.260 ± 230     |
| 31K → AA | 164 ± 16 | 54 ± 8<sup>b</sup> | 449 ± 114<sup>h</sup> | 2.62 ± 0.32<sup>h</sup> |
| 31K – A | 269 ± 9 | 99 ± 13<sup>b</sup> | 94 ± 14<sup>h</sup> | 211 ± 18 (10)   |
| 32K → A | 147 ± 3 | 177 ± 31<sup>b</sup> | 122 ± 10.3<sup>h</sup> | 178 ± 27 (9)    |
| 33E → A | 195 ± 5 | 79 ± 13<sup>b</sup> | 80 ± 15<sup>h</sup> | 463 ± 29 (8)    |
| 37DD → AA | Inhibition              | 301 ± 16        | 2.46 ± 0.64     | 211 ± 21 (10)   |
| 40K → A | Inhibition               | 277 ± 48        | 0.78 ± 0.44     | 204 ± 26 (7)    |
| 44DE → AA | Inhibition              | 301 ± 16        | 1.64 ± 0.61     | 233 ± 23 (4)    |
| 48RK → AA | 136 ± 7 | 119 ± 16<sup>b</sup> | 7.48 ± 0.88<sup>b</sup> | 6740 ± 70       |
| 53D → A | Inhibition               | 334 ± 116       | 0.84 ± 0.09     | 129 ± 19 (6)    |
| 56R → A | Inhibition               | 419 ± 168       | 0.73 ± 0.25     | 116 ± 21 (7)    |
| 47H → A | 128 ± 11 | 315 ± 14        | 1.40 ± 0.07     | 12000 ± 10      |
| 47HRK → AAA | Inhibition              | 274 ± 23        | 0.91 ± 0.23     | 10730 ± 27      |
| 31K – A/47HRK → A<sup>+</sup> | 425 ± 56 | 55 ± 9<sup>b</sup> | 1516 ± 258<sup>h</sup> | 6840 ± 40       |
| 31K → A/47HRK → AAA<sup>+</sup> | 158 ± 89 | 76 ± 8<sup>b</sup> | 4.73 ± 0.61<sup>h</sup> | 144 ± 19 (5) |

<sup>a</sup>Unless indicated otherwise, values shown are means ± S.E. of at least 3 separate experiments and on two different clones.

<sup>b</sup>Activities are maximal K⁺ stimulation presented as percent control activity measured in the absence of K⁺ (data from the averaged experiments shown in Fig. 3).

<sup>c</sup>K⁺<sub>ATP</sub> values taken from Fig. 4.

<sup>d</sup>See<sub>max</sub> values taken from Fig. 2.

<sup>e</sup>See<sub>v</sub> values calculated from the rate constants of averaged experiments shown in Fig. 5 and normalized to that of α1.

<sup>f</sup>Mean ± S.E. of at least two separate experiments for which See<sub>max</sub> values (nmol/mg/min) were 93 ± 6 (control α1), 68 ± 1 (31K → AAA), 70 ± 4 (31K → AA), 167 ± 22 (31K → A), 129 ± 15 (48RK → AA), 281 ± 16 (47HRK → AAA), 369 ± 23 (47HRK → AAA), 325 ± 5 (31K → AA/47HRK → A), and 319 ± 12 (31K → AA/47HRK → AAA).

<sup>g</sup>See<sub>v</sub> values are averages of the number of preparations indicated in brackets.

<sup>h</sup>Values significantly different from α1, p < 0.005.

<sup>i</sup>Unless indicated otherwise, values shown are means ± S.E. of at least 3 separate experiments and on two different clones.

<sup>j</sup>Compared to 31K → AA, p values for 31K → AA/47HR → A and 31K → AA/47HRK → AAA are 0.928 and 0.03 for K⁺<sub>ATP</sub>, respectively, 0.019 and 0.045 for maximal K⁺ activation, and with both mutants, p = 0.002 for I<sub>v</sub> vanadate. See<sub>v</sub>, E<sub>f</sub> → E<sub>p</sub> for the two mutants are not different from 31K → AA, i.e. p = 0.566 (31K → AA/47HR → A) and 0.322 (31K → AA/47HRK → AAA).

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**Changes in E<sub>f</sub>/E<sub>p</sub> Poise Caused by Charge-to-Alanine Mutations**—Results of the kinetic assays of the alanine mutants are summarized in Table 1 and Figs. 2–5.

**Vanadate Sensitivity**—A particularly discriminating measure of the E<sub>f</sub>/E<sub>p</sub> poise is sensitivity of the overall Na,K-ATPase activity to inhibition by inorganic orthovanadate, a transition state analogue of inorganic phosphate that binds to E<sub>f</sub> form(s) of the enzyme. I<sub>50(VAN)</sub> values for the control and mutant enzymes are shown in Table 1, and plots of those which differ significantly from the control are presented in Fig. 2. The most striking decrease in vanadate sensitivity is seen with 31K → AAA within H1; its I<sub>50(VAN)</sub> is ~2000-fold higher than that of the WT enzyme. As might be expected, the I<sub>50(VAN)</sub> of 31K → AA is similar to that of deletion mutant α1M32 reported earlier and also shown in Table 1: I<sub>50(VAN)</sub> values of both are ≈450-fold higher than WT α1. Increases in mutant I<sub>50(VAN)</sub> for the single charge-to-alanine replacements of residues 31KKE reflect the contributions of the individual charged residues, Lys<sup>31</sup>, Lys<sup>32</sup>, and Glu<sup>33</sup>, to the maintenance of the WT E<sub>f</sub>/E<sub>p</sub> conformational poise. In H2, a modest increase in I<sub>50(VAN)</sub> was seen for mutant 48RK → AA. Little or no effects of alanine substitutions in other “single site” mutants were observed.
K⁺ Sensitivity of Na-ATPase and K’_{ATP}—At micromolar ATP concentrations sufficient to saturate only the high-affinity phosphorylation site of the enzyme, the K⁺-sensitivity profile of Na-ATPase is, in general, a convenient and sensitive indication of mutant-specific changes in the E₂(K) → E₁K⁺ → E₁ reaction of the Na-K-ATPase reaction (for examples, see Refs. 12, 18). Thus, as shown previously and summarized in Table 1, at 1 μM ATP, K⁺ inhibits Na-ATPase activity of the control rat α₁ enzyme but stimulates up to ~400%, the activity of deletion mutants α1M32 and α1M40, reflecting their increased rate of the normally rate-limiting K⁺ deocclusion step of the above reaction sequence (5). As shown in Fig. 3 and also summarized in Table 1, charge-to-alanine mutations of residues in primarily two “sites”, 31KKE in H1 and 47HRK in H2, also resulted in K⁺ stimulation of Na-ATPase activity. In H1, all replacements (31KKE → AAA as well as replacements 31KK → AA, 31K → A, 32K → A, and 33E → A) resulted in K⁺ stimulations of similar magnitude, i.e. ranging from 147 ± 3% to 218 ± 26%. In H2, K⁺ stimulation is seen with mutants 48RK → AA and 47H → A. Although K⁺ activation is not seen with the triple mutant 47HRK → AAA, its K⁺-sensitivity profile indicates less K⁺ inhibition compared with the rat α₁ control. K⁺ activation caused by replacement 24ERD → AAA upstream from the H1 helical region is absent in 24ER → AA, suggesting that Asp^{26} at least in the context of 24ERD, also has a role, albeit minor, in modulating the E₂(K) → E₁ transition. The notably greater K⁺ activation of Na-ATPase seen in earlier studies with the deletion mutants α1M32 and α1M40 probably reflects the combined removal of charged residues 24ERD and 31KK.

Because the rate of K⁺ de-occlusion is increased almost two orders of magnitude by ATP binding with low affinity to E₂(K) via the pathway ATP + E₂(K) → ATP-E₂(K) → ATP-E₁K → ATP-E₁ + K⁺, it follows that a change in the E₁/E₂ poise affects the apparent K’_{ATP} for low-affinity ATP binding and vice versa. The effects of the N-terminal mutations on K’_{ATP} are summarized in Table 1, and plots of the data are presented in Fig. 4. There are several noteworthy observations vis à vis these data and the K⁺-sensitivity profiles described in Fig. 3. Thus, mutants 31KKE → AAA and 31KK → AA showed weaker K⁺ activation of Na-ATPase than either of the deletion mutants α1M32 or α1M40, yet both alanine-replacement mutants have lower K’_{ATP} values than the deletion mutants. Similarly, K’_{ATP} of mutant 24ERD → AAA, like that of deletion mutant α1M27, is lower than that of the WT α₁ enzyme, whereas unlike α1M27, K⁺ stimulates Na-ATPase of 24ERD → AAA. These distinctions probably reflect the extent to which K⁺ de-occlusion proceeds through E₂(K) → E₁K⁺ → E₁ + K⁺ or through the low-affinity ATP binding pathway or is the consequence of a change in rate of the subsequent E₁P → E₂P conformational transition as discussed below. Thus, it is when these analyses are taken together with assessments of vanadate sensitivity and rate of E₁P → E₂P that they provide clearer insight into this issue (see “Discussion”).

The Rate of E₁P → E₂P—Vanadate sensitivity reflects the relative abundance of E₂ forms and thus reflects the rates of the E₁P → E₂P and E₂(K) → E₁ transitions. To gain insight into the reason for the large increase in I_{V_{50, VANO}} of 31KKE → AAA and 31KK → AA but only modest changes in K⁺-sensitivity of Na-ATPase that presumably reflected the E₂(K) → E₁ rate, E₁P → E₂P rates of these mutants were determined. The results are shown in Fig. 5 and t₁/2 values normalized to the control α₁ enzyme given in Table 1. As shown previously (5) and reproduced in Fig. 5, E₁P → E₂P is slowed ~6-fold with both α1M32

![Image](URL)
and P remained unclear.

and α1M40 deletions. However, with mutants 31KKE → AAA and 31KK → AA, the rates are reduced ~12- and 5-fold, respectively, consistent with the pattern of change, relative to the control, in vanadate inhibition. Of the single-site mutants tested, these were the only ones with reduced rates of transition of E1P to E2P.

Catalytic Turnover—In general, those mutants with decreased E1P → E2P rates had lower catalytic turnovers. As shown in Table 1, the turnover decreased progressively from a value of 10,200 ± 540 min⁻¹ for the control α1 enzyme to 8,620 ± 610 min⁻¹ for 31KKE → AA and to 3,300 ± 30 min⁻¹ for the 31KKE → AAA mutant. The same holds true for the deletion mutants as shown previously (5) with data reproduced in Table 1.

Consequences of Combining Mutations in Putative Helical Regions H1 and H2 —To gain insight into possible intramolecular interactions between H1 and H2 that underlie the role of the N terminus in modulating the interaction of the A domain with the catalytic loop to effect E₁ ↔ E₂ and E₁P ↔ E₂P transitions, the consequences of combining alanine replacements in 31KKE and in 47HRK were tested. Initial experiments indicated that cells transfected with cDNA encoding either double mutant 31KKE → AAA/48RK → AA or 31KK → AA/48RK → AA failed to support growth in ouabain. However, double mutants 31K → AA/47H → A and 31KK → AA/47HRK did support growth in ouabain and were at least as active as the WT α1 enzyme stably transfected into HeLa cells. The kinetic behavior of these mutants is shown in Figs. 2–4 (lower right-hand panels) and Fig. 5 (right-hand panel) and is summarized in Table 1.

As shown in Table 1, I₅₀(VAN) and K⁺ activation of Na⁺-ATPase caused by combining either 47H → A or 47HRK → AAA with 31KK → AA are significantly increased above the values obtained with the single-site mutation 31KK → AA, consistent with a further shift in E₁/E₂ poise toward E₁. Interestingly, significant differences in the rate of E₁P → E₂P of either double mutant compared with 31KK → AA were not seen. Other parameters (Kₐ₅ₐ₅, catalytic turnover) were minimally altered.

**DISCUSSION**

E₁ ↔ E₂ transitions of the Na,K-ATPase involves the docking and undocking of the A domain comprising the cytoplasmic N terminus plus L2/3 loop onto the N and P domains located in the large L4/5 catalytic loop. As E₁ shifts to E₂, the A domain rotates vertically ~100° C, whereas the P domain rotates away from the N domain. Distinct tryptic cleavage patterns of the enzyme in E₁(2K) versus E₂(3Na) first described by Jorgensen (22) suggested that charged residues in the cytoplasmic N terminus have an important role in the E₁–E₂ transitions, probably by participating in salt bridge formation (23). The cleavage patterns suggest interactions between N and A domains such that in E₂ forms, the 440-RAVAGDA motif in the N domain interacts with the segment around Lys⁶⁰ in the cytoplasmic N terminus of the A domain. Interactions between P and A domains have been delineated primarily in the studies of Fe²⁺-catalyzed cleavages of Na,K-ATPase. Thus, in E₂(2K), association of 216ESE in the L2/3 loop of A associates with motifs “near 367CSDK, 608MVTGD, and 712VNDS” (pig enzyme numbering) have been observed (reviewed in Ref. 2) and are in fact, confirmed by the crystal structures of the related sarcoplasmic reticulum Ca-ATPase (24). Although marked E₁ shifts indicating synergistic effects of combining cleavage at Lys⁶⁰ (c.f. tryptic cleavage site T2 (23)) and mutation E233K (rat enzyme numbering) in L2/3 were also observed (25), the identification of specific motifs in the cytoplasmic N terminus and the mechanism underlying its role in modulating the docking and undocking of A onto N and P remains unclear.
N Terminus Modulation of Na,K-ATPase

The present clustered charge-to-alanine mutagenesis experiments have identified specific sites in the cytoplasmic N terminus of A that impact A/N-P interactions. Two sites have a particularly significant role in maintaining the wild-type E1/E2 poise. The site of primary importance is 31KKE in the first putative helical region, H1. Thus, mutation of these residues to alanines causes large shifts in the conformational poise toward E1 form(s), as seen by a marked decrease in sensitivity to inhibition by inorganic orthovanadate as well as changes in related kinetic characteristics, namely (i) an increased E2(K) → E1 rate apparent as K+ activation of Na-ATPase, (ii) decrease in K+ATP stimulated E2P, (iii) decreased rate of E2P → E2P, and (iv) an overall decrease in catalytic turnover. Furthermore, the extent of these changes in 31KKE → AAA reflect the consequences of replacing the individual residues by alanines because mutants 31KK → AA as well as 31K → A, 32K → A, or 33E → A shifted the poise toward E1 to progressively lesser extents. Overall, the kinetic changes resulting from these replacements suggest that 31KKE in H1 facilitates association of the A domain (cytoplasmic N terminus and loop 2/3) with N-P in the catalytic loop L4/5 during the E1P → E2P conformation change and to a somewhat lesser extent (relatively modest increases in the E2(K) → E1 rate) retards dissociation of A from N-P during E2(K) → E1 + K, effectively stabilizing the E2 conformation(s).

Residues in a second site, 47HRK in putative helical region H2, also modulate interaction of the first cytoplasmic loop L2/3 with L4/5; this interaction is particularly evident once E2P-promoting residues in site 31KKE are replaced by alanines. Thus, compared with mutant 31KK → AA, alanine replacements of either 47H or 47HRK in mutant 31KK → AA further decrease sensitivity to vanadate and increased E2(K) → E1 as evidenced by notably greater K+ activation of Na-ATPase. Compared with 31KK → AA, neither K+ATP nor the rate of E2P → E2P was significantly different in either mutant 31KK → AA/47H → A or mutant 31KK → AA/47HRK → AAA, suggesting that the increase in K+ activation of Na-ATPase reflects primarily a faster E2(K) → E1 and not a higher ATP affinity. Accordingly, the behavior of these double mutants suggests that the primary role of residues in the 47HRK site in H2 is to regulate the undocking of A from N-P that is associated with the E2(K) → E1 phase of the reaction cycle.

Two charged sites upstream from 31KKE had only minor effects on the catalytic cycle because a significant change in conformational poise as evidenced in the IsovCVD was not observed. Thus, despite the modest K+ stimulation of Na-ATPase and decrease in K+ATP caused by the 24ERD → AAA mutation, no change in sensitivity of Na,K-ATPase activity to vanadate inhibition was observed. The same holds true for mutant 28DE → AA, which has a somewhat higher apparent ATP affinity but otherwise behaves like the WT rat α1 enzyme.

Whereas alanine replacement of charged residues 44DE in helical region H2 does not change the E1P/E2P poise, their deletion by removal of 40MLSDE from the E1P-shifted deletion mutant M40 essentially reverses the shift toward that of the WT enzyme (5). One explanation for this difference is that removal of 40MLSDE from deletion mutant α1M40 dramatically decreases the predicted helical propensity of nearby downstream residues 47HRK, whereas as expected, alanine replacement of the charged residues in the full-length enzyme does not. Hence, it is likely that an E2P-promoting role of 47HRK is, in some way, enhanced by the decrease or loss (deletion mutants α1M46 and consequently further deletions α1M49 and α1M56; Ref. 5) in its helical structure. Overall, the behavior of the double mutants indicates that 47HRK acts in concert with 31KKE to regulate the E1/E2 poise. Unfortunately, the unstructured nature of the N-terminal extension has precluded our attempts to gain structural information by solution NMR of a recombinant N-terminal peptide (residues 1–85 of rat α1).

The present mutagenesis approach focuses on ionic interactions and does not exclude effects of hydrophobic interactions. The foregoing limitations notwithstanding, the present alanine scan has identified specific sites in the N terminus that have a major role in modulating conformational transitions and quite possibly conformation-based pump regulation and intracellular signaling as discussed below.

Physiological Implications—Regulation of conformational transitions of P-type ATPases by their cytoplasmic N and C termini has previously been described. Noting that the former is the least conserved region of these pumps, Palmgren and Axelsen (26) proposed that the non-conserved domains likely evolves into specialized functions for the different enzymes. In fact, examination of the primary structure of the N terminus of the Na,K-ATPase reveals several putative binding motifs including or juxtaposed to the conformationally relevant sites identified in the present study, i.e. ones which interact with 14-3-3-2 (48RKYGTDL), with PDZ-3 (27MDDEL, 41LDEL), as well as several putative PKC phosphorylation sites (EuKaryotic Linear Motif, ELM; elm.eu.org).

An example of direct interaction and regulation of pump kinetics via intermolecular N-terminal interaction is the phospholemman-like protein from shark or FXDYD10 regulation of the shark Na,K-ATPase activity (27). This member of the FXYD family of tissue-specific pump regulators appears to modulate its effect through direct interaction with the N terminus of the Na,K-ATPase. Mahmmoud et al. (27) showed that selective trypsinolysis of the pump N terminus at the conserved trypsinsensitive T2 site in KKE abrogates phospholemman-like protein from shark binding to the pump as well as any kinetic effect, a finding that may have significant impact on tissue-specific regulation of pump function.

The N terminus of Na,K-ATPase is reported to have an important role as a signal transducer, acting as a hub for the recruitment of several scaffolding proteins and signal peptides, to modulate transcription, cell growth, apoptosis, and cell motility. In their recent studies, Aperia and co-workers (28, 29) demonstrated that the N terminus binds directly to the N terminus of the inositol trisphosphate receptor (InsP3R), tethering it into a Ca2+-modulating complex that appears to be important for ouabain-induced Ca2+ oscillations. Furthermore, the interaction depends on the presence of residues 30-LKK in the N terminus of Na,K-ATPase. Whether alterations in the steady-state conformational poise of the enzyme impact its signaling function is an important issue that remains to be determined.

It is noteworthy that both 31KKE and 48RK of the α1 subunit of the Na,K-ATPase are perfectly conserved among species
including xenopus, bufo, torpedo, and artemia, as well as among the α2 and α3 isoforms of the rat. In fact, both the rat gastric and non-gastric H,K-ATPase (KKE and XK) as well as the human non-gastric H,K-ATPase (QKE and XK) show high degrees of sequence identity at these sites suggesting that they may well represent an intrinsic method of conformational transition regulation for these P-type pumps with extended N termini.

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