New Insights into the Role of the N Terminus in Conformational Transitions of the Na,K-ATPase*

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The deletion of 32 residues from the N terminus of the α1 catalytic subunit of the rat Na,K-ATPase (mutant α1M32) shifts the E1/E2 conformational equilibrium toward E1, and the combination of this deletion with mutation E233K in the M2-M3 loop acts synergistically to shift the conformation further toward E1 (Boxenbaum, N., Daly, S. E., Javaid, Z. Z., Lane, L. K., and Blostein, R. (1998) J. Biol. Chem. 273, 23086–23092). To delimit the region of the cytoplasmic N terminus involved in these interactions, the consequences of a series of N-terminal deletions of α1 beyond Δ32 were evaluated. Criteria to assess shifts in conformational equilibrium were based on effects of perturbation of the entire catalytic cycle (ii) sensitivity to vanadate inhibition, (ii) K⁺ sensitivity of Na-ATPase measured at micromolar ATP, (iii) changes in Kₐcat, and (iv) catalytic turnover), as well as estimates of the rates of the conformational transitions of phospho- and dephosphoenzyme (E1P → E2P and E2(K⁺) → E1 + K⁺). The results show that, compared with α1M32, the deletion of up to 40 residues (α1M40) further shifts the poise toward E1. Remarkably, further deletions (mutants α1M46, α1M49, and α1M56) reverse the effect, such that these mutants increasingly resemble the wild type α1. These results suggest novel intramolecular interactions involving domains within the N terminus that impact the manner in which the N terminus/M2-M3 loop regulatory domain interacts with the M4-M5 catalytic loop to effect E1 ↔ E2 transitions.

The Na,K-ATPase or sodium pump is a ubiquitous integral membrane protein that catalyzes the glycose sensitive, ATP-coupled exchange of three intracellular Na⁺ for two extracellular K⁺ ions across the plasma membrane of all animal cells. The sodium pump is essential to the maintenance of the electrochemical gradients of Na⁺ and K⁺ across the cell membrane, providing the driving force for the transport of nutrients into the cell and maintaining the cellular resting membrane potential. It comprises two essential subunits: a large catalytic α subunit (~100 kDa), containing the ligand binding and phosphorylation sites and a smaller, highly glycosylated β subunit (~35–55 kDa), which acts as a chaperone for α (for review see Refs. 1 and 2). A third subunit, γ (~7 kDa), was found in the kidney where it functions as a regulator of the pump (see Refs. 3 and 4).

The sodium pump is a member of a family of transporters known as P-type ATPases that are directly phosphorylated and dephosphorylated on a conserved aspartate residue within their cytoplasmic domain during the course of the reaction cycle. Both the phosphorylated and dephosphorylated forms of the enzyme can exist in at least two states that undergo conformational transitions (E1P → E2P and E2 → E1) that are coupled to the ion-translocating steps. Definitive evidence for the existence of distinct E1 and E2 conformational states and a role of the N terminus in effecting E1 ↔ E2 transitions was first obtained in 1975 by Jorgensen (5, 6) using tryptic cleavage of the renal enzyme in the presence of different ligands. Later confirmatory evidence was obtained using N-terminal deletion mutants expressed in cultured cells (7). This study showed that whereas deleting up to and including the lysine-rich cluster is without effect, removal of 32 residues (mutant α1M32) alters the enzyme kinetics by shifting the E1/E2 conformational equilibrium toward E1 forms. Interestingly, a similar shift toward E1 is caused by Glu233 → Lys substitution in the first (M2-M3) cytoplasmic loop. Furthermore, the combined removal of the N-terminal 32 residues and replacement of Glu233 → Lys (mutant α1M32E233K) results in a remarkably synergistic shift in poise toward E1 state(s) as evidenced in analyses of several characteristic properties including, for examples, extraordinary insensitivity to vanadate, high affinity for ATP (5 μM), and slow (~500 min⁻¹) catalytic turnover of α1M32E233K. These findings were interpreted to indicate that interactions between these two cytoplasmic regions and the M4-M5 catalytic loop are critical for conformational coupling (8).

The present study was carried out to define more precisely the role of the N terminus in conformational transitions. Accordingly, we investigated the consequences of further deletions of the N terminus of the Na,K-ATPase beyond the first 32 residues to near the beginning of the first transmembrane segment, using several criteria to assess shifts in the steady-state E1 ↔ E2 poise. The results of this analysis reveal a progressively increased shift in the E1/E2 poise toward E1, followed by reversal toward the wild type α1 E1/E2 distribution. Combined with secondary structure predictions of the N terminus, this behavior identifies a self-regulatory domain within the N terminus of the Na,K-ATPase that modulates conformational transitions via novel intramolecular interactions.

EXPERIMENTAL PROCEDURES

Determination of Helicity—Predictions of the secondary structure of the N-terminus sequence were evaluated using the following algorithms: PREDATOR (9), Sspro (10), SOPMA (11), GOR IV (12), Deleage

* This work was supported by operating grants from the Canadian Institutes of Health Research (Grant MT-3876) and the Quebec Heart and Stroke Foundation (Grant HL 49204) (to L. K.), and a predoctoral fellowship from the National Institutes of Health Research (Grant MT-3876) and the Quebec Heart and Stroke Foundation (to R. B.), the National Institutes of Health (Grants HL 44501 and HL 49204). This paper is available on line at http://www.jbc.org

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and Roux (13), LeVitt (14), Chou and Fasman (15), COILS v2.1 (16), and PSA (17). Putative helical regions were then ascribed to those sequences for which the above algorithms concur on helicity.

Mutagenesis, Transfection, Selection, and Cell Culture—The desired mutations were introduced into the 5′ SacI–SacII restriction fragment of the rat α1 cDNA as described previously (7). The mutant cassettes were then ligated into the rat Na,K-ATPase cDNA in the place of the wild type SacI–SacII cassette. The full-length mutant cDNAs were released form the shuttle vector by digestion with HindIII, ligated into the expression plasmid pCDNAS3.1 (Invitrogen), and orientation of the cDNAs was confirmed by restriction analysis. HeLa cells were transfected with the pCDNA-α1 mutant constructs using either the calcium phosphate method (18) or the LipofectAMINE technique (Invitrogen), and cells expressing the relatively ouabain-resistant rat α1 enzymes and mutants thereof were selected as described previously (19, 20). HeLa cells expressing the mutant α1 enzymes were amplified in Dulbecco’s modified Eagle’s medium plus 10% newborn calf serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 1 μg/ml ouabain as described previously (7). The medium was immediately supplemented with an additional 15 mM KCl (20 mM total; see Ref. 21), which was removed after several passages.

Membrane Preparation—NaI-treated microsomal membranes were prepared from the mutant cells as described earlier (19, 20). Protein content was determined with a detergent-modified Lowry assay (22).

Enzyme Assays—Na,K-ATPase activity was measured as the release of 32P, from [γ-32P]ATP as previously described (23). Briefly and unless indicated otherwise, the membranes were preincubated 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. Final concentrations for Na,K-ATPase activity measurements were 100 mM NaCl, 10 mM KCl, 3 mM MgSO4, 20 mM histidine (pH 7.4), 5 mM EGTA, 1 mM MgSO4, and 1 μM ouabain (SIGMA). 5 mM ouabain was used to determine baseline hydrolysis activity. As in earlier studies and unless indicated otherwise, assays of Na,K-ATPase activity were carried out using 1 mM ATP to maintain close to saturating ATP concentration and also maximize sensitivity of assays of the relatively low activity cultured cells (see Refs. 20, 24, and 25). Na,K-ATPase activity was measured at 1 μM ATP as described previously (7), with varying amounts of added KCl and choline chloride to maintain constant KCl (40 mM) concentration, and baseline activity was determined with 40 mM KCl. For studies of vanadate sensitivity, inorganic orthovanadate (Fisher) solutions were prepared 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 and expressed as percentage of that obtained in the absence of vanadate, were analyzed by fitting the data to a one-compartment model using a non-linear least-square analysis of a general logistic function, as described elsewhere (26).

Phosphoenzyme Determination—Phosphoenzyme levels were determined as described earlier (25). Briefly, membranes (~0.01 mg/assay) were preincubated with ouabain (20 μM ouabain, 0.4 mM MgSO4, and 10 mM glucyglycine-Tris (pH 7.4) for 30 min at 37 °C to inhibit endogenous HeLa Na,K-ATPase. The suspension was then treated with either oligomycin (20 μg/ml) for 1 min at room temperature or with vehicle alone (ethanol) for baseline measurements (see below). Phosphorylation was then carried out for 10 s at 0 °C in a final volume of 150 μl in medium comprising (final concentrations) 100 mM NaCl, 10 mM glucyglycine-Tris (pH 7.4), 5 mM EGTA, 1 mM MgSO4, and 1 μM [γ-32P]ATP (specific activity 10,000–20,000 cpm/pmol). Baseline EP levels were determined by replacing 100 mM NaCl with 50 mM KCl and 50 mM choline chloride, and phosphorylating the enzyme in the absence of oligomycin.

Formation of the E1P, from E1P(K+).—The rate of K+ deassociation (E1P(K+)) → E1P+) was measured indirectly by determining the rate of E1P formation from E1P(K+) as described elsewhere (25), with the modifications described by Therien and Blosestein (27). Rate of E2P → E2P. —Formation of E2P in the presence of high chloride concentration (28), the rate of E2P → E2P was determined by measuring the rate of disappearance of total phosphoenzyme following rapid dilution of the salt (to allow normal relaxation of E2P → E2P) plus addition of KCl to catalyze rapid hydrolysis of E2P (28, 29). Accordingly, the enzyme was first phosphorylated in medium containing 600 mM NaCl to stabilize E2P, 1 mM MgCl2, 1 mM EGTA, and 20 mM Tris-HCl (pH 7.4) with 1 μM [γ-32P]ATP for 30 s at 0 °C to obtain maximal phosphoenzyme. Dephosphorylation was then initiated by 6-fold dilution into a medium containing final concentrations of 20 mM KCl, 10 mM unlabeled ATP, 1 mM EGTA, and 20 mM Tris-HCl (pH 7.4), which simultaneously lowered the NaCl concentration to 100 mM. Samples were taken for measurement of E2P for periods of up to 30 s. Background phosphoenzyme levels were obtained by allowing the chase to continue for 60 s.

At least two different membrane preparations obtained from at least two different clones were assayed. The data presented are representative of at least three independent experiments. Each value shown is the mean ± S.D. of triplicate determinations.

RESULTS

To determine more precisely the structural basis and mechanism underlying the role of the cytoplasmic N terminus of the α1 catalytic subunit of the Na,K-ATPase on the steady-state E1 → E2 conformational poise, we first considered the nature of the secondary structure of this 85-residue N-terminal domain. This region is a highly charged, flexible structure with a high degree of helicity. A comparison of the results of several computational analyses (see “Experimental Procedures”) reveals the presence of three putative α-helical domains encompassing residues 27–33, 42–50 and 61–68 (Fig. 1). To remove or disrupt these regions, wild type HeLa cells were transfected with N-terminal deletion mutants of the α1 subunit of the rat Na,K-ATPase corresponding to either disruption or loss of the first putative helix (α1M32 and α1M40, respectively) and disruption (α1M46 and α1M56) of the second helix (Fig. 1). All five of the mutant constructs yielded functional enzyme capable of sustaining HeLa cell growth in 1 μM ouabain.

Several criteria were used to analyze shifts in the E1/E2 distribution; some that reflect the operation of the overall catalytic cycle and others that reflect partial reactions relevant to the major E1 ↔ E2 and E1P ↔ E2P transitions. The former include: (i) sensitivity of Na,K-ATPase activity to inhibition by vanadate, (ii) response of Na,K-ATPase to varying K+ concentrations relevant to the normally rate-limiting E2P(K+) → E1P + K+, (iii) catalytic turnover, and (iv) K+ ATP requirement to low affinity ATP binding to E2P(K+). The latter include: (v) rate of K+ deassociation (E2P(K+)) → E1P + K+ (see above), and (vi) rate of E1P → E2P as outlined in “Experimental Procedures.”

Functional Consequences of Deletion Mutants: (i) Analysis of the Overall Catalytic Cycle under Steady State Conditions—Compared with the wild type α1 enzyme, the poison in E1 ↔ E2 of the α1M32 deletion mutant is notably shifted toward E1 forms of the enzyme (7, 25). To gain insight into the effects of further
deletions on conformational equilibrium, we investigated the effect of vanadate on Na,K-ATPase activity. Inorganic orthovanadate is a transition state analog of inorganic phosphate that binds to P-type ATPases in the E2 conformation (30). Consequently, sensitivity of an enzyme to inhibition by vanadate is a measure of the proportion of enzyme in the E2 conformation. As shown by the representative experiment in Fig. 2 and summarized in Table I, a1M32 and a1M40 are both less sensitive to vanadate inhibition than is a1, suggesting that the E2/E1 equilibrium of these enzymes progressively shifts toward E1 as up to 40 residues are deleted from the N terminus. However, this shift is reversed to a more wild type-like sensitivity by deleting ≥46 residues (mutants a1M46, a1M49, and a1M56).

At micromolar ATP concentrations, sufficient to saturate only the high affinity phosphorylation site, the response of Na-ATPase to K⁺ is a sensitive means to characterize mutant-specific differences in the K⁺ deocclusion pathway of the reaction cycle (E2(K⁺) → E1 + K⁺), which becomes rate-limiting under these conditions (7, 31). Thus, as shown previously and in Fig. 3, K⁺ inhibits the Na-ATPase activity of a1 but stimulates that of a1M32. The deletion of 40 residues, a1M40, results in an even greater K⁺ stimulation, up to ∼400%. Interestingly, further deletion, i.e. a1M46, a1M49, and a1M56, yields a1-like K⁺ inhibition. These results, summarized in Table I, suggest a progressive shift in the E2/E1 conformational equilibrium toward E1 upon removal of up to 40 residues from the N terminus, which is reversed by deleting ≥46 residues.

The catalytic turnover of the Na,K-ATPase is estimated as the ratio of $V_{\text{MAX}}$ to $E_{\text{P MAX}}$, the latter measured at 0 °C in the

![Table I](https://example.com/table1.png)

**Table I**

Summary of kinetic behavior of Na,K-ATPase mutants during steady-state catalysis

| Enzyme | $I_{50}$ vanadate | Effect of 1 mM K⁺ on Na-ATPase | Turnover $V_{\text{MAX}}/E_{\text{P MAX}}$ | $K'_{\text{ATP,L}}$ |
|--------|------------------|-------------------------------|------------------------------------------|-----------------|
| a1     | 0.86 ± 0.03      | 60.1 ± 4.8                    | 8295 ± 886 (14)                          | 382 ± 71 (6)    |
| a1M32  | 436 ± 71         | 289 ± 16                      | 4420 ± 879 (12)                          | 145 ± 31° (3)   |
| a1M40  | 891 ± 106        | 423 ± 26                      | 1832 ± 380 (17)                          | 76.9 ± 32.2° (9) |
| a1M46  | 0.56 ± 0.08      | 77.9 ± 3.5                    | 6136 ± 1221 (4)                          | 336 ± 24 (2)    |
| a1M49  | 0.73 ± 0.05      | 60.2 ± 19                     | 5807 ± 1004° (5)                         | 284 ± 7 (2)     |
| a1M56  | 0.46 ± 0.09      | 65.4 ± 5.4                    | 5609 ± 1024° (11)                        | 151 ± 50° (5)   |

Columns: a, b, c, d

a $I_{50}$ values taken from the representative experiment shown in Fig. 2.
b Activity with 1 mM K⁺ presented as percent of control activity without K⁺ (data from representative experiment in Fig. 3).
c Catalytic turnover was determined with 100 mM NaCl, 1 mM KCl, and 1 mM ATP as described under "Experimental Procedures." Data are presented as percent Na,K-ATPase activity with 1 mM KCl, and 1 mM ATP (control) measured in the absence of vanadate. Results shown are from a representative experiment; shown are mean ± S.D. of triplicate determinations. Symbols are: ☐, a1 (dashed line); ☐, a1M32; ☐, a1M40; ☐, a1M46; ☐, a1M49; ☐, a1M56.

d $K'_{\text{ATP,L}}$ values are means ± S.D. of the number of experiments shown in brackets.

e Significantly different from a1, $p < 0.005$. 

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**Fig. 2.** Vanadate sensitivity of N-terminal deletion mutants. ATP hydrolysis at varying vanadate concentrations was determined with 100 mM NaCl, 10 mM KCl, and 1 mM ATP as described under "Experimental Procedures." Data are presented as percent Na,K-ATPase (control) measured in the absence of vanadate. Results shown are from a representative experiment; shown are mean ± S.D. of triplicate determinations. Symbols are: ☐, a1 (dashed line); ☐, a1M32; ☐, a1M40; ☐, a1M46; ☐, a1M49; ☐, a1M56.
presence of ATP, Na⁺, and oligomycin to trap the enzyme in the (Na⁺)E₁P state (see “Experimental Procedures”) (32). Table I shows the catalytic turnover of α1 and the mutant enzymes. As previously shown, α1M32 has ~50% reduction in turnover, consistent with a shift in the E₁ ↔ E₂ poise toward E₁. α1M40 has a significantly lower turnover, only 20% that of α1. In contrast, deletion of ≥46 residues restores the catalytic turnover to near that of α1.

Differences in K⁺ sensitivity of Na-ATPase at low ATP concentration (Fig. 3) suggest a change in the rate of a step in the K⁺ deocclusion phase of the Albers-Post reaction mechanism, which can be modeled by a branched pathway (see Scheme 1 in Ref. 33). In one branch, pathway A, ATP first binds with low affinity denoted by K⁺ATP (L), followed by rapid deocclusion (ATP-E₁K⁺ → ATP-E₂). In the other branch, pathway B, the slow release of K⁺ to ATP-E₁K⁺ → ATP-E₂K⁺.

FIG. 3. 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. Results shown are from a representative experiment. Values are the mean ± S.D. of triplicate determinations. Symbols are as in the legend to Fig. 2.

FIG. 4. Effect of N-terminal deletions on ATP dependence of Na,K-ATPase activity. ATP hydrolysis was assayed in the presence of 100 mM NaCl, 10 mM KCl, 3 mM MgSO₄, and varying ATP concentrations as described under “Experimental Procedures,” and normalized to 100% VMAX. Results are taken from a representative experiment; shown are the average ± S.D. of triplicate determinations. Symbols are as in the legend to Fig. 2.
from E₂(K⁺) via E₂(K⁺) → E₁P → E₁ + K⁺ is followed by high affinity ATP binding to E₁. Accordingly, a mutation causing a change in K⁺ stimulation of Na-pump at micromolar ATP concentration may reflect a change in K⁺ATPL values in Table I. In contrast, αM46 and αM49 have K⁺ATPL values similar to that of the wild type α1 enzyme, consistent with the observed reversal of the K⁺ effect on Na-pump at 1 μM ATP. It was noted, however, that the K⁺ATPL of αM56 is lower than that of α1 even though this enzyme is inhibited by K⁺ at low ATP. This characteristic of αM56 is considered further in the “Discussion.”

(ii) Analysis of Partial Reactions Relevant to Conformational Transitions—In another series of experiments we compared the slow release of K⁺ from E₂(K⁺) via the branch E₂(K⁺) → E₁P → E₁ + K⁺ for each of the mutants. As in our earlier studies (25, 27), we used an indirect approach whereby the relatively slow rate of E₁ formation from E₂(K⁺) is estimated at various periods following dilution of preformed E₂(K⁺) in Na⁺ medium containing [γ-³²P]ATP at 10 °C. Assuming that the ensuing phosphorylation of E₁ is rapid, the amount of phosphoenzyme (presumably Na-E₁P since oligomycin is present to trap this intermediate) is thus a measure of E₁ formed from E₂(K⁺). As shown in Fig. 5, the changes in E₂(K⁺) deduced from the increases in E₁P, fit well to single exponentials. The rate constants for αM32 and αM40 shown in Table II are significantly higher than that of α1. (Rate constants are similar to our previously published values for α1 and αM32, Ref. 25). This result suggests that for both αM32 and αM40 the E₂(K⁺) → E₁P → E₁ + K⁺ poise is shifted to the right. On the other hand, values for αM46, αM49, and αM56 are similar to, or even lower than, those for αM32 and αM40, suggesting that deletion of ≈46 residues reverses the above right-shift to a more wild type-like E₁E₈p distribution. It should be noted, however, that whereas the K⁺ sensitivity of Na-pump at 1 μM ATP reflects overall turnover via both branch pathways of K⁺ deocclusion, with the contribution of pathway A increasing as K⁺ATPL decreases, the time course of formation of E₁ from E₂(K⁺) reflects only deocclusion via pathway B. It is not known whether changes in each of the two pathways are quantitatively equivalent. They may not be equivalent. Thus, K⁺ stimulation of the Na-pump is notably greater for αM40 compared with αM32 despite their similar rates of E₂(K⁺) → E₁; K⁺ inhibition profiles of αM46 and αM49 are similar to that of α1, yet their rates of E₂(K⁺) → E₁ are slower than that of α1. Despite these issues and the fact that this reaction was analyzed at 10 °C, the generally consistent pattern of increasing followed by decreasing rates of E₂(K⁺) → E₁ associated with progressive deletions, is noteworthy.

To investigate the contribution of the conformational transition of the phosphoenzyme to the E₁ → E₂ shifts observed above, we examined the E₁P → E₂P transition at 0 °C as previously described (28, 29). Accordingly, the enzyme is first phosphorylated by [γ-³²P]ATP at high salt concentration (600 mm NaCl) to stabilize E₁P. Dephosphorylation is then initiated by a downward jump in NaCl concentration to 100 mm with the simultaneous addition of 20 mm KCl and 10 μM unlabeled ATP.

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**FIG. 5. Rate of formation of E₁ from E₂(K⁺).** The rate of K⁺ deocclusion was measured indirectly as the rate of formation of E₁ from E₂(K⁺) at 10 °C as described under “Experimental Procedures.” Results shown are taken from a representative experiment; each value is the mean ± S.D. of triplicate determinations. Symbols are as in the legend to Fig. 2.

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**TABLE II**

Summary of conformational transition rates of Na,K-ATPase mutants

| Enzyme | Reaction measured | E₂(K⁺) → E₁ | E₁P → E₂P | k⁺ |
|--------|------------------|-------------|------------|-----|
|        | sec⁻¹ | sec⁻¹ | sec⁻¹ | sec⁻¹ |
| α1     | 0.021 ± 0.007 (3) | 1.05 ± 0.27 | 0.66 |
| αM32   | 0.075 ± 0.025 (3) | 6.14 ± 0.80⁶ | 0.11 |
| αM40   | 0.063 ± 0.007 (3) | 5.83 ± 0.51⁶ | 0.12 |
| αM46   | 0.006 ± 0.000 (2) | 0.88 ± 0.22 | 0.79 |
| αM49   | 0.009 ± 0.002 (3) | 1.08 ± 0.02 | 0.64 |
| αM56   | 0.031 ± 0.013 (4) | 1.51 ± 0.50 | 0.46 |

a Rate constants shown are means ± S.D. of the number of experiments shown in brackets.
b Data taken from Fig. 6.
c Rates were calculated from the T₁/2 values shown.
d Significantly different from α1, p < 0.005.
Since the rate of K\(^{+}\)-activated dephosphorylation of E\(_2\)P from E\(_1\)P is much faster than that of the preceding formation of E\(_2\)P from E\(_1\)P, the time course of the E\(_1\)P decay reflects primarily E\(_1\)P \(\rightarrow\) E\(_2\)P. As shown in Fig. 6 and summarized in Table II, compared with the wild type \(\alpha\) enzyme, the rate of this transition is \(\sim\)6-fold slower for \(\alpha\)M32 and \(\alpha\)M40. Mutants \(\alpha\)M46, \(\alpha\)M49, and \(\alpha\)M56 have similar or only slightly slower dephosphorylation rates relative to the wild type enzyme. Taken together, these partial reaction analyses of the mutants and wild type enzyme reflect the behavior seen in assays of the overall catalytic cycle summarized in Table I.

**DISCUSSION**

The primary sequences of the N and C termini are the least conserved domains among the various P-type ATPases found in lower organisms and throughout the plant and animal kingdom. These extramembranous termini contain regions that inhibit pump activity, as well as binding motifs for regulatory proteins that may release pump inhibition by these auto-inhibitory sequences (34). Examples include the plasma membrane Ca\(^{2+}\)-ATPase, which contains auto-inhibitory domains in its N terminus in plants or in its C terminus in animals (35–37). Despite their low homology, a structural paradigm does exist for the N terminus of P-type ATPases, whereby the region comprises two domains. These are: (i) a variable domain, which differs markedly in sequence and length among the ATPases and (ii) a homologous domain that continues into the S1 stalk region of the N terminus (38).

The N terminus of the Na,K-ATPase is a highly charged and flexible structure, with a high degree of helicity (see Fig. 1). Although the primary sequence of this region diverges greatly among isoforms and species, the following features persist: (i) a lysine-rich cluster of about 5–9 residues and (ii) a highly conserved 10 amino acid sequence circumscribed by two methionines (M(D/E)ELKKE(I/V)(S/T)M) (41) and containing a proteolytically sensitive lysine residue (5).

In his landmark studies, Jorgensen showed that the lysine-rich domain of the N terminus of the Na,K-ATPase precedes a proteolytically sensitive site (T2) that can be selectively cleaved by trypsin when the enzyme is in the E\(_1\) conformation (5, 6). Cleavage at this residue increases K\(^{+}\) and decreases K\(^{+}\)ATP, with associated effects on the phosphorylation and dephosphorylation reactions of the enzyme (42–44), providing evidence for a shift in the E\(_1\)/E\(_2\) equilibrium toward E\(_1\) forms (45, 46).

Several studies have demonstrated that although the lysine cluster is not essential to pump function (47–51), the N terminus may play a role in Na\(^{+}\) sensitivity (52), K\(^{+}\) affinity, and in the dependence on membrane potential (53, 54) likely resulting from changes in rates of Na\(^{+}\) translocation (41) and K\(^{+}\) deocclusion reactions (55).

\(^1\) The abbreviation used is: SERCA, sarcoplasmic reticulum Ca\(^{2+}\)-ATPase.
We have previously shown that deletion of up to 27 residues preceding the first putative helix has no effect on the conformational equilibrium of the enzyme (7, 8, 25). Only upon deletion of 32 residues, which corresponds to T2 described above, is there a shift toward E1 (8, 25). Here we show that disruption (α1M32) or loss (α1M40) of H1 results in a progressive shift toward E1 forms of the enzyme. We also show that disruption (α1M46 and α1M49) or loss (α1M56) of H2 (as well as H1) reverses the shift to a more wild type-like E1 ↔ E2 equilibrium.

The aforementioned progressive changes notwithstanding, the behavior of α1M56 is slightly peculiar. Unlike α1M46 and α1M49, α1M56 retained a lower K_{ATP(L)} even though all three mutants resemble wild type α1 with respect to the following: (i) vanadate sensitivity, (ii) sensitivity to K+ inhibition at low ATP, (iii) rate of E1 formation from E2K+, and (iv) rate of E1P → E2P. These observations may indicate some change or destabilization of structure that occurs with extensive truncation. For example, truncation beyond the first 49 residues may perturb a heretofore undefined interaction of the N terminus with another region, which may cause ligand-specific effects independent of the conformational transitions, such as an increased access of nucleotide substrate to the binding site.

**Cytoplasmic Interactions Involved in Conformational Transitions**—Insights into cytoplasmic region(s) that interact with the N terminus were first obtained by Jorgensen and Collins (56) who proposed salt-bridge formation between the N terminus and other cytoplasmic domains. In a later study (8), the remarkable synergistic effects of the Δ52 deletion and the E233K mutation implicated the N terminus/M2-M3 loop in such a salt-bridge formation. It is noteworthy that the cytoplasmic region encompassing the N terminus/M2-M3 loop is analogous to the Activator or A domain identified in the crystal structure of SERCA1a (57). In the Na,K-ATPase, interaction of the A domain with the catalytic loop involves large domain movements (57). The emerging picture is that the structural changes underlying conformational transitions are generally similar for SERCA and the Na,K-ATPase, and much can be extrapolated about their analogous structures (for a detailed comparison, see Ref. 59).

**The Distinct Modulatory Role of the N Terminus of the Na,K-ATPase**—The present mutagenesis study reveals a specific role of the N terminus. The model shown in Fig. 7 depicts a region of the N terminus that acts as an autoregulatory domain modulating the E1/E2 conformational transitions. In the case of the wild type enzyme (Fig. 7, panel A), helical region H2 can interact with H1 either through helix-helix interactions and/or salt-bridge formation in the E2 conformation, allowing the M2-M3 loop and the catalytic loop to come together (shaded ovals). H2 can also interact with a domain of the M2-M3 loop in E1 to keep it apart from the catalytic loop as in the E2 conformation. When H1 is disrupted or lost (α1M32 or α1M40, see Fig. 7, panel B),

![Figure 7](image-url)

**FIG. 7. Hypothetical model for N-terminal regulation of conformational equilibrium.** Based on evidence from earlier work and the present study, a model is proposed whereby an autoregulatory region of the N terminus modulates the poise in E1 ↔ E2. For details see "Discussion."
H2 preferentially interacts with the M2-M3 loop, placing a constraint on the latter, thereby weakening the M2-M3/M4-M5 interaction and consequently stabilizing the E1 conformation. The result is a shift in the conformational equilibrium in favor of E1. Lastly, the disruption of αM1α46 and αM49 or loss of H2 (αM56) (see Fig. 7, panel C) alleviates the constraint on the M2-M3 loop allowing it once again to freely interact with the large catalytic M4-M5 loop, thus resembling the wild type α1 enzyme.

It is also noteworthy that the α2 and α3 isoforms of the catalytic subunit of the rat Na,K-ATPase have similar helical structures in their N termini. Furthermore, analogous disruptions of the first helix of α2 and α3 (mutants α2M30 and α3M26, respectively)\(^2\) shift the conformational equilibrium for each enzyme toward E1 forms, suggesting that the N terminus of the Na,K-ATPase may carry an autoregulatory domain present in all three isoforms.

In conclusion, we have identified an autoregulatory domain within the N terminus of the sodium pump, which modulates conformational transitions, suggesting novel intramolecular interactions within the cytoplasmic domains of the enzyme. Studies are currently underway to further pinpoint the residues in the N terminus that are involved in these interactions.

Acknowledgments—We thank Zahid Z. Javaid for preparation of the αM40 and αM56 constructs and Dr. Alex Therrien for helpful discussions and critical reading of the manuscript. The excellent technical assistance of Rosemarie Scanzano is gratefully acknowledged.

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\(^2\) L. Segall, S. E. Daly, and R. Bluestein, unpublished observation.