The Role of Loop 6/7 in Folding and Functional Performance of Na,K-ATPase*

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Alanine substitutions were made for 15 amino acids in the cytoplasmic loop between transmembrane helices 6 and 7 (L6/7) of the human α-subunit of Na,K-ATPase. Most mutations reduced Na,K-ATPase activity by less than 50%; however, the mutations R834A, R837A, and R848A reduced Na,K-ATPase activity by 75, 89, and 66%, respectively. Steady-state phosphoenzyme formation from ATP was reduced in mutants R834A, R837A, and R848A, and R837A also had a faster E2P → E2 dephosphorylation rate compared with the wild-type enzyme. Effects of L6/7 mutations on the phosphorylation domain of the protein were also demonstrated by 18O exchange, which showed that intrinsic rate constants for P1 binding and/or reaction with the protein were altered. Although most L6/7 mutations had no effect on the interaction of Na+ or K+ with Na,K-ATPase, the E825A, E828A, R834A, and R837A mutations reduced the apparent affinity of the enzyme for both Na+ and K+ by 1.5-3-fold. 1-Bromo-2,4,6-tris(methylisothiouronium)benzene (Br-TITU3), a competitive antagonist of Rb+ and Na+ occlusion (Hoving, S., Bar-Shimon, M., Tijmes, J. J., Goldschleger, R., Tal, D. M., and Karlish, S. J. D. (1995) J. Biol. Chem. 270, 29788-29793), was used to test whether charged residues in L6/7 are involved in binding monovalent cations and cation antagonists. Br-TITU3 inhibited ouabain binding to wild type Na,K-ATPase with an IC50 of 30 μM. Oubain binding to the E825A, E828A, R834A, or R837A mutants was still inhibited by Br-TITU3, indicating that Br-TITU3 does not bind to charged residues in L6/7. This observation makes it unlikely that L6/7 functions as a cytoplasmic cation binding site in Na,K-ATPase, and together with the effects of L6/7 mutations on phosphate interactions with the enzyme suggests that L6/7 is important in stabilizing the phosphorylation domain and its relationship to the ion binding sites of the protein.

Na,K-ATPase, also called the sodium pump, is a heterodimeric protein consisting of α- and β-subunits. It pumps 3 Na+ out of the cell and 2 K+ into the cell during each transport cycle at the expense of ATP hydrolysis, and this activity maintains transmembrane gradients of the ions. Na,K-ATPase belongs to the P-type ATPase family in which the mechanism of action involves formation of a transient, covalently phosphorylated intermediate during the reaction cycle. Other P-type ATPases include sarcoplasmic reticulum and plasma membrane Ca-ATPase, H,K-ATPase found in stomach and colon, and several prokaryotic transport enzymes (1–3). These P-type ATPases have similar structural organization, and amino acid sequences near the sites of ATP binding, phosphorylation, and Mg2+ binding, and residues attributed to cation binding in the transmembrane domain are highly conserved (3, 4). The catalytic α-subunit of Na,K-ATPase is composed of 10 transmembrane helices (TM).† Proteolysis experiments (5, 6) and site-directed mutagenesis studies indicate that the cation occlusion sites are located within the transmembrane domain, and carboxyl and other oxygen-containing side chains of residues within TM 4, 5, 6, and 8 are crucial for occlusion of cations (7–12).

According to the crystal structure of Ca-ATPase (13, 14), the parts of the polypeptide that are exposed in the cytoplasm are organized into three interacting domains designated A, N, and P. The nucleus (phosphorylation) domain is composed of the intracellular loop between TM4 and TM5, with the N-terminal part (roughly Asn330–Asp365) connecting to TM4, and the C-terminal part (roughly Lys803–Asp857) connecting to TM5. The aspartate residue (Asp855) that accepts the γ-phosphate from ATP during ATP hydrolysis is situated in the C-terminal end of the central β-strand of domain P. The N (nucleotide binding) domain is inserted between the two parts of the P domain (roughly Gln666–Arg690). The A (anchor) domain is composed of the N terminus of the polypeptide and the loop between TM2 and TM3. It undergoes large conformational changes during the transport cycle. The cytoplasmic loop between TM6 and TM7 (L6/7, Phe869–Ser883) wraps around TM5 and is close to the P domain and the transmembrane domain.

The function of L6/7 has been investigated extensively in Ca-ATPase and is controversial. Faison and co-workers (15–17) replaced aspartate residues in L6/7 of Ca-ATPase with alanine, and found that the mutations reduced the Ca2+ affinity by 1000-fold. They proposed that L6/7 serves as an initial cation binding site in Ca-ATPase. Zhang et al. (18, 19) reported that mutations in L6/7 reduced enzyme phosphorylation from both ATP and Pi, and several aspartate to asparagine mutations (D813N/D815N/D818N) also reduced Ca2+ affinity. They

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‡ The abbreviations used are: TM, transmembrane helix; L6/7, the intracellular loop between transmembrane helices 6 and 7; WT, wild type; P, inorganic phosphate; EP, phosphoenzyme; Br-TTU3, 1-bromo-2,4,6-tris(methylisothiouronium)benzene; Br-TITU3, 1,3-dibromo-2,4,6-tris(methylisothiouronium)benzene; mXBG2, m-xylene bisguanidinium; pXBG2, p-xylene bisguanidinium; PNP, p-nitrophenylphosphate.
proposed that the effect of the mutations on Ca\(^{2+}\) affinity was probably because mutations in L6/7 influenced the correct positioning of TM6, and that L6/7 was important in stabilizing the phosphorylation domain and the cation binding domain in Na,K-ATPase. In the Na,K-ATPase crystal structure, L6/7 is close to the P domain and the transmembrane domain, in a good position to mediate communication between the phosphorylation site and the cation binding site, and Toyoshima et al. (13) suggested that L6/7 may function as a coordinator between these sites. In the Na,K-ATPase, Shainmaya et al. (20) measured the effects of chymotrypsin digestion of L6/7 on the release of a TM5/TM6 previously described (22). The data were fit to a modified Hill equation (22, 25).

The partition coefficient (Pc) were estimated from the isotopomer distribution of TM6, and that L6/7 was important in stabilizing the phosphorylation site and the cation binding site, and Toyoshima et al. (13) suggested that L6/7 may function as a coordinator between these sites. In the Na,K-ATPase, Shainmaya et al. (20) measured the effects of chymotrypsin digestion of L6/7 on the release of a TM5/TM6 previously described (22). The data were fit to a modified Hill equation (22, 25).

The amino acid changes in L6/7 of the H9251 and H11001 were purchased from ICN Biomedicals. All other chemicals were reagent grade.

Preparation of the yeast expression plasmids for human Na,K-ATPase a\(_1\)- and b\(_1\)-subunits, designated as YhN and GhN, has been described (22). Amino acid changes in L6/7 were introduced using the Stratagene QuikChange site-directed mutagenesis kit. The presence of the mutations was confirmed by DNA sequencing by the Microchemical Core Facility at USC. Transformation of Saccharomyces cerevisiae with Yhn1 and GhN1b, cell growth for expression of Na,K-ATPase 1- and 2-subunits, designated as YhN and GhN, has been described (22). Protein concentration was determined by the method of Lowry et al. (24).

**Na,K-ATPase Activity Measurement**—Ouabain-sensitive Na,K-ATPase activity was measured using a coupled optical assay as previously described (22, 25). Ouabain Binding Experiments—Equilibrium ouabain binding, inhibition of ouabain binding by KCl, inhibition of ouabain binding by NaCl, and NaCl concentration-dependent ouabain binding were performed as previously described (22). The data were fit to a modified Hill equation (11,26) from which were extracted values for K\(_d\), R\(_{50}\), and IC\(_{50}\). Phosphate Concentration Dependence of the Mg\(^{2+}\) and P\(^{-}\)-dependent Ouabain Binding—The apparent affinity of the Na,K-ATPase for P\(^{-}\) was measured by equilibrium P\(^{-}\) and Mg\(^{2+}\)-dependent ouabain binding in 20 nM [H\(_3\)O] ouabain, 4 mM MgCl\(_2\), 50 mM Tris/HCl (pH 7.4) with 0–10 mM P\(^{-}\). Data were fitted to a rectangular hyperbola to obtain the K\(_{50}\) for P\(^{-}\).

**18O Oxygen Exchange Measurements**—\(^{18}O\) exchange between P\(^{-}\) and water was measured as previously described (25) using yeast membranes that had been extracted with 0.1% SDS. The exchange rate and the partition coefficient (P\(_{P}\)) were estimated from the isotopeomer distribution at a single time point by non-linear least squares. Parameters relating exchange to metal cofactor and substrate binding and to the equilibrium between non-oxidative and covalently bound P\(^{-}\) were estimated by globally fitting the rate equation for ordered binding of Mg\(^{2+}\) before P\(^{-}\) to points of the exchange rate as a function of both Mg\(^{2+}\) and P\(^{-}\) concentration (25).

**Heat Inactivation of Ouabain Binding**—1 mg of crude yeast membrane protein was suspended in 0.4 ml of 25 mM imidazole/HCl, 1 mM EDTA (pH 7.4) and was heated at 50 °C for 90 s. The ouabain binding capacity of the sample was measured at 37 °C after heating and was compared with that of the control sample without heating. The experiments were done in triplicate.

**Steady-state Phosphorylation from [γ-\(^{32}\)P]ATP**—Steady-state phosphorylation of Na,K-ATPase was carried out in 200 mM of 20 mM Tris/HCl (pH 7.4), 3 mM MgCl\(_2\), 100 mM NaCl, and 2 mM [γ-\(^{32}\)P]ATP. The reaction was initiated by adding the [γ-\(^{32}\)P]ATP, and was continued for 10 s before being quenched by 0.8 ml of 1 M H\(_3\)PO\(_4\) (pH 2.4). After quenching, the tubes were immersed in ice for 10 min. After adding 100 μg of bovine serum albumin, the samples were pelleted in a microcentrifuge at 13,000 × g for 20 min at 4 °C. The pellets were washed twice by centrifugation with 1.1 ml of cold washing buffer (0.25 M H\(_3\)PO\(_4\), pH 2.4), once with 1.1 ml of cold distilled water, and were dissolved in 35 μl of sample loading buffer (prepared by adding 0.2 ml of 50% glycerol containing 1 mg/ml bromphenol blue and 10 μl of 2-mercaptoethanol to 0.8 ml of 10 mM Na,HPO\(_4\), 1% SDS) (18). The entire sample was applied to a 4% SDS-polyacrylamide gel, pH 6.2 (18, 27). After run-in at 100 mA for 30–60 min at 4 °C, the gels were stained overnight in a fume hood. Radioactive phosphorylases were detected by autoradiography, and the quantitation of the phosphorylases specific to Na,K-ATPase was obtained by liquid scintillation counting of slices corresponding to the a-subunit bands of the dried gel. Reactions carried out with 100 μM KCl replacing NaCl served as control for background phosphorylation.

**Time course of E\(_P\) → E\(_o\)-phosphorylation**—Phosphorylation at low concentrations of Na\(^{+}\) in the absence of added K\(^{+}\) will accumulate E\(_P\) (28). The time course of E\(_P\) → E\(_o\) can be obtained by measuring the disappearance of the phosphorylases after adding K\(^{+}\) to the enzyme (E\(_i\) + Na\(^{+}\) + ATP ⇄ E\(_P\) ⇄ E\(_o\) + K\(^{+}\) + E\(_o\)). Enzyme was phosphorylated in 50 μl of 20 mM NaCl, 20 mM Tris/HCl (pH 7.4), 3 mM MgCl\(_2\), and 1 μM [γ-\(^{32}\)P]ATP on ice for 15 s. Dephosphorylation was initiated by adding 10 μl of a chase solution to give a final concentration of 0.1 mM KCl and 10 mM EDTA, followed by adding 100 μl of quench solution (2 mM H\(_3\)PO\(_4\), pH 2.4) at serial time points. The reaction was carried out in 2 ml tubes with magnetic stirring. After adding an additional 0.8 ml of 1 M H\(_3\)PO\(_4\) (pH 2.4) containing 100 μg of bovine serum albumin, the reaction solutions were transferred to 1.5-ml tubes and were centrifuged at 13,000 × g for 20 min to pellet the protein. Reactions carried out in the presence of 20 mM KCl replacing NaCl served as control for background phosphorylation. Pellets were washed, proteins were separated by gel electrophoresis, and radioactive phosphorylase was quantified as described above. The data were fitted to a monoequponential equation: y = a\(_{\infty}\) + y\(_o\), where y\(_o\) is the amount of phosphorylated Na,K-ATPase, a is the maximum phosphorylation, y\(_o\) is the phosphorylase present at long times, k is the rate constant, and t is the chase time.

**Western Blot**—100 μg of SDS-extracted yeast membrane proteins were loaded onto 10% SDS-polyacrylamide gels. 100 ng of SDS-purified dog kidney Na,K-ATPase was used as positive control and membranes from non-transformed yeast served as a negative control. After electrophoresis samples were transferred to Immobilon P membranes and incubated with monoclonal antibody 5 (D. Fambrough, Johns Hopkins), which is specific for the α-subunit of Na,K-ATPase. Image detection was done using an Odyssey Infrared Imaging System (LI-COR). ImageJ 1.38x (Wayne Rasband, National Institutes of Health) and Microsoft Excel were used to analyze the data. Best fits for non-linear curve fitting are shown as lines in the figures, and the extracted parameters are given in the tables and figure legends. Standard deviations or standard errors are indicated for the means and for parameters extracted by non-linear curve fitting. Student’s t test was used for most statistical analysis. Free P\(^{-}\) and Mg\(^{2+}\) concentrations were calculated using Winmax v2.00, www.stanford.edu/~cpatton/maxc.html.

**Intracellular Loop 6/7 of Na,K-ATPase**

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RESULTS

Expression of Na,K-ATPase in Yeast—The amino acid sequences of L6/7 in human Na,K-ATPase α1-subunit and in rabbit sarcoplasmic reticulum Ca-ATPase are shown in Fig. 1. The effects of alanine substitutions of charged residues, prolines, and conserved residues in L6/7 of the human Na,K-ATPase α1-subunit were examined after expression of the protein in yeast cells. As shown in Fig. 2, all mutants were detected in membranes prepared from transformed yeast cells, although the triple mutant E825A/E828A/D830A and the E825A, E828A, and M832A mutants were expressed at lower levels than wild type and most other mutants. All mutants except for the E825A/E828A/D830A mutant bound ouabain in an Mg²⁺- and Pₐ-dependent equilibrium ouabain binding assay. Unlike the other mutants and wild-type enzyme, the E825A and E828A mutants lost the ability to bind ouabain after SDS extraction of crude microsomal membranes. The ouabain binding capacity (Bₘₐₓ) of the mutants in SDS-extracted yeast membranes varies, ranging from 3 to 29 pmol/mg protein (Table I). The dissociation constant for ouabain (Kₒ) for most mutants was similar to the wild-type value, ranging from 9 to 31 nM, except for R837A, which had a Kₒ of 218 nM, 12-fold higher than that of wild-type Na,K-ATPase, and for R837K, which had a Kₒ of 66 nM, a 3.6-fold increase.

Effects of Mutations on Na,K-ATPase Activity—The ouabain sensitive Na,K-ATPase activity was measured on SDS extracted yeast membranes. Without SDS extraction the fraction of ouabain-sensitive ATPase activity for M832A could not be accurately measured because of their loss of ouabain binding after SDS extraction, and ATPase activity for M832A could not be accurately measured because of its low expression level.

Effects of Mutations on Na⁺ and K⁺ Affinity of Na,K-ATPase—In the presence of saturating concentrations of ATP, the apparent affinity of Eₒ for Na⁺ can be measured by the Na⁺ dependence of ouabain binding (Eₒ + Na⁺ + Mg²⁺ + ATP ⇌ EP + ouabain ⇌ EP-ouabain). In this reaction, Eₒ binds to Na⁺ with high affinity and is phosphorylated in the presence of ATP and Mg²⁺ to EP, which binds ouabain to form a stable EP-ouabain complex. In the presence of Mg²⁺ and Pₐ the apparent affinity of Eₒ for K⁺ can be measured by K⁺ inhibition of ouabain binding (Eₒ (2K) ⇌ K⁺ + Eₒ + Mg²⁺ + P₀ ⇒ EP + ouabain ⇌ EP-ouabain). In this reaction, K⁺ binds to Eₒ and antagonizes ouabain binding by competing for Eₒ with P₀ and phosphoenzyme formation. Na⁺ also inhibits Mg²⁺- and Pₐ-dependent ouabain binding (EₒNa⁺ = Eₒ + Na⁺ + Eₒ + Mg²⁺ + P₀ ⇒ EP + ouabain = EP-ouabain). Both the Eₒ/Eₐ equilibrium and the affinity of Eₒ for Na⁺ affect the amount of EP formed from P₀ in the presence of Mg²⁺ and P₀, and affect the amount of ouabain bound and the value of IC₅₀ for Na⁺.

Most mutations did not change the apparent affinity of the pump for Na⁺ or K⁺. For Na⁺-dependent ouabain binding (Fig. 3), the Kₐ₅₀ value for Na⁺ ranged from 0.7–1.6 mM for wild-type and most mutants, which is close to the reported Kₐ₅₀ values of 0.7 mM and 0.6 mM measured by Na⁺-dependent steady-state phosphoenzyme formation (11, 12). The Kₐ₅₀ values of E825A and R837A for Na⁺ were increased by 2–3-fold. For K⁺ inhibition of ouabain binding (Fig. 4), the IC₅₀ for K⁺ of wild type and most mutants was 0.3–0.4 mM, consistent with the reported value of Kₐ₅₀ of 0.31 mM (12) and 0.77 mM (29). E825A, E828A and R837A displayed a 2-fold increase in IC₅₀ for K⁺. For Na⁺ inhibition of ouabain binding, the IC₅₀ value of wild type for Na⁺ was 20 ± 1 mM (Fig. 5). The IC₅₀ for Na⁺ inhibition of

![Fig. 1. Comparison of L6/7 sequence of human Na,K-ATPase α1-subunit and rabbit SERCA Ca-ATPase. The residues in Na,K-ATPase changed to alanine in this study are marked with an asterisk.](http://www.jbc.org/)

![Fig. 2. Expression of L6/7 mutants of Na,K-ATPase in yeast membranes. Western blot of SDS-extracted yeast membranes. 100 µg of membrane proteins were separated on 10% SDS-polyacrylamide gels and transferred to Immobilon P. Na,K-ATPase α1-subunits were detected with a monoclonal anti-Na,K-ATPase α1-subunit antibody. DKA, 0.1 µg of SDS-purified Na,K-ATPase from dog kidney; N.C., membranes from non-transformed yeast cells; WT, wild type human Na,K-ATPase; AAA, triple mutant E825A/E828A/D830A.](http://www.jbc.org/)
ouabain binding for most mutants was not different from wild type, but it was increased 1.5–3-fold for E825A, E828A, and R834A, and it was decreased 3-fold for R837A. The effects of E825A, E828A, and R837A on apparent cation affinity are summarized in Table II.

Effects of Br-TITU⁺ and La³⁺ on Ouabain Binding—Br-TITU⁺ and La³⁺ act as competitive cation antagonists and inhibit occlusion of both Rb⁺ and Na⁺ by Na,K-ATPase (20, 30). Br-TITU⁺ inhibits Mg²⁺- and Pᵢ-dependent ouabain binding to wild type Na,K-ATPase with an IC₅₀ of 30 μM (data not shown). Fig. 6 shows the extent of inhibition of ouabain binding to wild-type Na,K-ATPase and the E825A, E828A, R834A, and R837A mutants by 50 μM Br-TITU⁺. Inhibition of ouabain binding was similar in the wild type and the E828A mutant, was greater than wild-type in the E825A and R837A mutants, and was less than wild type in the R834A mutant. La³⁺ at concentrations up to 0.5 mM did not inhibit ouabain binding by wild type Na,K-ATPase, and 0.5 mM La³⁺ had no effect on the inhibition of ouabain binding by KCl (data not shown).

Heat Inactivation of Ouabain Binding—E825A and E828A lost ouabain binding after SDS extraction suggesting that these two mutations destabilize the enzyme. To test this hypothesis, crude yeast membranes containing the Na,K-ATPase were heated at 50 °C for 90 s and ouabain binding at 37° was compared in heated samples and unheated controls. As shown in Fig. 7, wild-type, D830A, M832A, R834A, and R837A lost less than 25% of ouabain binding capacity after heating, while the amount of ouabain bound by the membranes of E825A and E828A was reduced by 80–90%.

R837A Reduced the Apparent Affinity of Na,K-ATPase for Pᵢ—Relative to wild-type, R837A exhibited a 12-fold higher Kd value for ouabain in Mg²⁺- and Pᵢ-dependent equilibrium ouabain binding (Table I). One possible reason for the reduced ouabain binding affinity might be an effect of the mutation on the interaction between the enzyme and Pᵢ, since only phosphorylated enzyme has a high affinity for ouabain. To test whether R837A affects Pᵢ interactions, the Pᵢ concentration dependence of ouabain binding was measured. The results of these measurements showed that the Kᵢ₀.₅ for most mutants was not significantly different from wild type (Fig. 8), whereas the mutation R837A increased Kᵢ₀.₅ nearly 8-fold. At a total Mg²⁺ concentration of 4 mM, ouabain binding reached a plateau at 8–10 mM Pᵢ. Therefore, the measurement of ouabain Kᵢ was repeated with total Pᵢ concentration increased to 10 mM and total Mg²⁺ kept at 4 mM (2 mM free Mg²⁺ and 8 mM free Pᵢ). The Kᵢ for ouabain under these conditions was similar to the Kᵢ obtained in standard binding buffer, which contained 4 mM total Mg²⁺ and 4 mM Pᵢ (3 mM free Mg²⁺ and 3 mM free Pᵢ). The reduced affinity of R837A for ouabain, therefore, is not due...
specific ouabain binding procedures.

bound ouabain was determined as described under “Experimental Procedures.” After incubation, the amount of bound ouabain was determined as described under “Experimental Procedures.” A, lines through data are best fits of a modified Hill equation: Specific ouabain binding = a − [ax^n/(c^n + x^n)], in which x is the K^+ concentration, a is the maximum ouabain binding, c is IC_{50}(K), n is the Hill coefficient. Bars indicate mean ± S.D. (n = 4–6). *, p < 0.05.

solely to differences in the extent of saturation of P_i binding between the mutant and wild-type enzymes.

The observed increase in K_{0.5} for P_i activation of ouabain binding to R837A could mean that the mutation affected the intrinsic affinity of the enzyme for P_i. Oxygen exchange measurements were made to test that interpretation. Table III compares averaged exchange parameters for both purified and expressed wild-type Na,K-ATPase with the estimates for selected mutants. Only the parameter including the intrinsic constant for P_i dissociation from metalloenzyme (K_p) is statistically different (p < 0.01). Therefore, a decrease in affinity for P_i could semi-quantitatively explain why mutant R837A required higher concentrations of P_i than wild type for activation of both high-affinity ouabain binding (8-fold) and catalysis of 18O exchange between P_i and H_2O (5-fold). Phosphorylation of the enzyme by P_i is an intermediate step in both reactions.

Steady-state Level of Phosphorylation—During the catalytic cycle of P-type ATPases an acid-stable phosphoenzyme intermediate is formed from ATP. In Na,K-ATPase phosphorylation occurs in the presence of Na^+, Mg^{2+}, and ATP. K^+ decreases the amount of the phosphoenzyme intermediate by stimulating dephosphorylation and pulling the enzyme to the E_2 conformation. In Ca-ATPase, mutations to Lys^{515} and Arg^{522} resulted in lower levels of steady-state phosphoenzyme formation from both ATP (20% of wild-type) and P_i (20–35% of wild-type) without affecting dephosphorylation of the phosphoenzyme (18). For Na,K-ATPase the steady-state level of phosphoenzyme was measured in the absence of K^+ for wild type, R834A, R837A, and R848A. Phosphorylation with K^+ replacing Na^+ served as control for background phosphorylation, which was subtracted from all data points, and purified dog kidney Na,K-ATPase served as positive control. As shown in Fig. 9, the phosphoenzyme was formed in

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**TABLE II**

Effect of mutations on ion interactions with Na,K-ATPase

| Allele   | K_{0.5} Na^+ | IC_{50} Na^+ | IC_{50} K^+ |
|----------|--------------|--------------|-------------|
|          | mm           | mm           | mm          |
| WT       | 1.0 ± 0.1    | 20 ± 1       | 0.3 ± 0.1   |
| E825A    | 3.1 ± 0.4    | 35 ± 4       | 0.7 ± 0.1   |
| E828A    | 1.1 ± 0.2    | 65 ± 6       | 0.9 ± 0.2   |
| R834A    | 1.6 ± 0.1    | 39 ± 6       | 0.3 ± 0.1   |
| R837A    | 2.7 ± 0.2    | 62 ± 2       | 0.8 ± 0.1   |

*From Fig. 3, Na^+-dependent ouabain binding.
†From Fig. 5, Na^+ inhibition of ouabain binding.
‡From Fig. 4, K^+ inhibition of ouabain binding.

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the presence of Na\(^+\), however, it was negligible in the buffer with K\(^+\). In this experiment, all samples contained equal amounts of functional Na\(_2\)K-ATP\(_\Lambda\)se, determined as the same number of ouabain binding sites. The steady-state level of phosphoenzyme was reduced to 85, 67, and 58\% relative to wild type for R834A, R837A, and R837A, respectively.

**Time Course of Dephosphorylation of E\(_2\)P → E\(_3\)P—Phosphorylation at low concentrations of Na\(^+\) accumulates E\(_2\)P (28, 31).** Phosphoenzyme was formed in 20 mM NaCl, and E\(_2\)P → E\(_3\)P dephosphorylation kinetics were observed by chasing the reaction to a final concentration of 0.1 mM K\(^+\) and 10 mM EDTA, followed by quench of the reaction at different times. Background phosphorylation measured with KCl replacing NaCl was subtracted from all data points. As shown in Fig. 10, a good fit of a monoexponential decay function to the data could be obtained. The extracted \(t_{0.5}\) for wild type is 1.9 ± 0.2 s, while R834A and R848A have \(t_{0.5}\) values of 2.2 ± 0.2 s and 2.2 ± 0.1 s, respectively. R837A led to faster E\(_2\)P → E\(_3\)P dephosphorylation, with a \(t_{0.5}\) of 1.4 ± 0.1 s, which is significantly different from that of the wild type (\(p < 0.05\)).

![Figure 6. Br-TITU\(^{3+}\) inhibition of ouabain binding.](image)

![Figure 7. Heat inactivation of ouabain binding.](image)

![Figure 8. Pi-dependent backdoor ouabain binding.](image)

**TABLE III**

| Sample ID | \(K_{p}^{i}1+K_{p}^{i}2\) | \(K_{p}^{i}3\) | \(P_{i}^{i}\) |
|-----------|----------------|----------------|----------|
| WT        | 1.1 ± 0.7 | 2.5 ± 1.6 | 0.22 ± 0.04 |
| R837A     | 5 ± 1    | 0.4 ± 0.2 | 0.16 ± 0.04 |
| E825A     | 0.8 ± 0.4 | 1.1 ± 0.8 | 0.21 ± 0.04 |
| R834A     | 0.6 ± 0.2 | 1.5 ± 0.5 | 0.25 ± 0.01 |
| D830A     | 0.3 ± 0.2 | 3.4 ± 1.9 | 0.19 ± 0.02 |
| R834A     | 0.5 ± 0.2 | 2.9 ± 1.2 | 0.17 ± 0.02 |

\(a\) \(K_{p}^{i}\) is the intrinsic P\(_i\) dissociation constant from metalloenzyme and \(K_{p}^{i}1\) is the equilibrium constant between non-covalently and covalently bound P\(_i\) (25).

\(b\) \(K_{p}^{i}2\) is the intrinsic Mg\(^{2+}\) dissociation constant to reform holoenzyme.

\(c\) The partition constant (P\(_i\)) is the probability of forming covalently bound phosphate from the non-covalent (Michaelis) complex with P\(_i\).

\(d\) Average values for wild type were obtained from 4–5 preparations purified from renal medulla or expressed in yeast membranes.

\(*\) \(p < 0.01\).

**K\(^{+}\)-stimulated p-Nitrophenylphosphate Hydrolysis—**Since R834A and R837A greatly reduced Na,K-ATP\(_\Lambda\)se activity, and R837A affected dephosphorylation, the effects of these mutations on the phosphatase activity were measured. As shown in Fig. 11, R837A reduced the phosphatase activity to 50\% of the wild-type value, while R834A did not affect this activity.
Most mutations in L6/7 retained at least 50% of wild-type Na,K-ATPase activity, with the exception of R834A, R837A, and R848A. The ATPase activity of E825A and E828A could not be measured since they lost ouabain binding after SDS extraction. In Na,K-ATPase Arg834 corresponds to Lys819 (Arg819) in chicken (rabbit) Ca-ATPase, Arg848 corresponds to Trp832 (Fig. 1). Amino acid substitutions to both Lys819 and Arg822 in Ca-ATPase led to loss of over 50% of Ca-ATPase activity (18), similar to the effects of the R834A and R837A mutations in Na,K-ATPase. No amino acid substitutions have been reported for Trp825. In Ca-ATPase, the effect of these mutations on ATPase activity was similar to the inhibition of phosphoenzyme formation from ATP or P_i, and it was concluded that Lys819 and Arg822 play an important role in determining the functional integrity of the phosphorylation domain of the enzyme (18).

The observation that E825A and E828A cause the Na,K-ATPase to lose ouabain binding after SDS extraction or brief heating suggests that Glu825 and Glu828 are structurally important. Consistent with this hypothesis, the triple mutant E825A/E828A/D830A had low protein expression and no ouabain binding could be detected in yeast membranes from cells transfected with a plasmid containing this mutant. One possible explanation for these results is that the E825A/E828A/D830A mutation might elicit the unfolded protein response in yeast resulting in proteolytic degradation. The E825A and E828A mutations probably alter the conformation of L6/7, which in turn, disturbs global protein structure. Several observations are consistent with this hypothesis. Firstly, the helical turn involving residues 816–819 in L6/7 of Ca-ATPase was replaced by random coil after alanine substitution of Asp813, Asp815, Asp818, and Glu826 in a synthetic L6/7 peptide (13, 17). Secondly, in Ca-ATPase L6/7 interacts with phospholamban, and the mutants N810A and D813A (equivalent to E825A and E828A in Na,K-ATPase) resulted in diminished ability to interact with phospholamban (32). Thirdly, in the Ca-ATPase structure Asn810 and Asp813 are close enough to form hydrogen bonds with the intracellular loop connecting TM8 and TM9 (Ser915, Ser917, Asn930), and TM5 (Asn755, Asn756). Replacing

![FIG. 9. Phosphoenzyme formed by utilization of ATP. Steady-state phosphoenzyme was formed by incubating SDS-extracted yeast membranes in 20 mM Tris/HCl (pH 7.4), 3 mM MgCl₂, 2 μM [γ-32P]ATP, 100 mM NaCl (or 100 mM KCl as background) at 0 °C as described under “Experimental Procedures.” The amount of protein in each sample was determined by the maximum ouabain binding capacity (B_max) of the membrane preparation to yield the same pmol of Na,K-ATPase as 20 μg of yeast membranes containing wild-type enzyme. The samples were subjected to SDS-electrophoresis at pH 6.2, and the radioactive phosphoenzyme bands corresponding to the Na,K-ATPase α-subunit were excised and counted by scintillation counter. A, autoradiograph of the phosphoenzyme specific to Na,K-ATPase. B, relative amount of phosphoenzyme formed from ATP. The experiments were done in duplicate, and the mean ± S.E. of four experiments on two different membrane preparations are given. *, p < 0.05.](Image)

![FIG. 10. Dephosphorylation of the phosphoenzyme formed at 20 mM NaCl. SDS-extracted yeast membranes containing wild-type Na,K-ATPase or the indicated mutants were phosphorylated in the presence of 20 mM NaCl, 20 mM Tris/HCl (pH 7.4), 3 mM MgCl₂ and 1 μM [γ-32P]ATP on ice for 15 s. Dephosphorylation was initiated by adding chasing solution to give final concentration of 0.1 mM KCl, 10 mM EDTA followed by acid quench at serial time points. Reactions carried out in the presence of 20 mM KCl replacing NaCl served as background phosphorylation. The entire samples were subjected to electrophoresis. The radioactive bands corresponding to Na,K-ATPase α-subunit detected by autoradiography in dry gel slices were excised and counted by scintillation counter. A, autoradiograph of the phosphoenzyme specific to Na,K-ATPase. B, amount of phosphoenzyme remaining after chasing for serial times. The experiments were done as duplicates and the data from four experiments was fitted to monoexponential decay curve, and calculated t½ for wild type (WT: 1.9 ± 0.2 s), R834A (●): 2.2 ± 0.2 s, for R837A (○):1.4 ± 0.1 s (p < 0.05), for R848A (▲): 2.2 ± 0.14 s. Each time point in the figure represents mean ± S.E. (S.E. < 5%).](Image)

![FIG. 11. K⁺-stimulated p-nitrophenylphosphatase activity (PNPPase activity). Ouabain-sensitive ATPase activity (white bars) and PNPPase activity (black bars) were measured on the same SDS-extracted membrane preparations as described under “Experimental Procedures.” PNPPase activity data are mean ± S.D. of four measurements on two membrane preparations. Each measurement was done in duplicate. Data are shown as percent of wild-type activity.](Image)
Asn<sup>810</sup> and Asp<sup>813</sup> with alanine would disrupt this hydrogen bond network.

In Na,K-ATPase, it was suggested that negatively charged residues in L6/7 constitute a cytoplasmic cation entry port that controls access to the cation occlusion sites (20). In H,K-ATPase, the mutants E825A, E828A, and D839N (equivalent to Glu<sup>825</sup>, Glu<sup>828</sup>, and Asp<sup>830</sup> in Na,K-ATPase) could not form a phosphorylated intermediate from ATP (33). It was proposed that these residues might be essential for the enzyme to become phosphorylated if they were involved in H<sup>+</sup> binding. In Ca-ATPase, alanine substitution of Asp<sup>813</sup>, Asp<sup>815</sup>, and Asp<sup>818</sup> (D813A/D818A and D813A/D815A/D818A) reduced Ca<sup>2+</sup> affinity from micromolar to millimolar as detected by Ca<sup>2+</sup>-dependence of phosphoenzyme formation from ATP or Pi (15, 16). These observations led to the hypothesis that L6/7 functions as an initial cation binding site in Na,K-ATPase. A synthetic L6/7 peptide (Gly<sup>808</sup>–Pro<sup>827</sup>) was able to bind Ca<sup>2+</sup> and lanthanum, and this cation binding could be disrupted by alanine substitutions for all carboxylates (Asp<sup>813</sup>, Asp<sup>815</sup>, Asp<sup>818</sup>, and Glu<sup>820</sup>) (17). In contrast, replacing aspartate by asparagine (D813N/D818N) in Ca-ATPase also reduced the Ca<sup>2+</sup> affinity, yet the ATPase activity was only 25% of the wild type even in the presence of millimolar Ca<sup>2+</sup> (19). The persistence of low ATPase activity in the presence of high concentrations of Ca<sup>2+</sup> was interpreted to indicate that L6/7 in Ca-ATPase is important in linking Ca<sup>2+</sup> binding to catalytic activation, presumably by influencing the arrangement of TM6, rather than in direct Ca<sup>2+</sup> binding.

In the present study, most of the L6/7 mutations did not change the K<sub>D</sub><sub>Na</sub><sup>+</sup> or IC<sub>50</sub> values for either Na<sup>+</sup> or K<sup>+</sup> (Table II). Several points suggest that the changes that were observed for the E825A, E828A, and R837A mutants are due to indirect effects of the mutations on cation binding. First, the same residues whose substitution with alanine resulted in enzyme instability or a greater than 50% reduction in Na,K-ATPase activity are also responsible for changes in cation affinity. Second, the magnitude of the effects on cation interaction with the mutants is small. E825A, E828A and R837A showed only a 1.5–3-fold decrease in apparent affinity for Na<sup>+</sup> and K<sup>+</sup>, while mutations to intramembrane carboxyl groups known to coordinate cations in the cation occlusion site abolished K<sup>+</sup> occlusion or Na<sup>+</sup>-dependent phosphoenzyme formation (9–12). Third, the mutants are not consistently altered in their interaction with the cations. E828A had the largest increase in IC<sub>50</sub> for Na<sup>+</sup> in Na<sup>+</sup> inhibition of ouabain binding, yet showed an apparent affinity for Na<sup>+</sup> similar to the wild type in the Na<sup>+</sup>- and ATP-dependent ouabain binding assay. Whereas R837A showed a 2.7-fold increase in K<sub>D</sub><sub>Na</sub><sup>+</sup> in the Na<sup>+</sup>-dependent ouabain binding reaction, the IC<sub>50</sub> for Na<sup>+</sup> inhibition of ouabain binding was only one-third of wild type. Fourth, the apparent affinity for Na<sup>+</sup> was reduced by neutralization of both basic (Arg<sup>837</sup>) and acidic (Glu<sup>825</sup>) amino acid side chains. L6/7 is a cytoplasmic loop and does not participate in cation occlusion. Under physiological conditions and in the forward cycling of the pump, Na<sup>+</sup> binds to the enzyme from the intracellular side of the membrane and K<sup>+</sup> binds to the enzyme from extracellular side. The observations that E825A, E828A, and R837A affect both Na<sup>+</sup> and K<sup>+</sup> affinity may be understood if L6/7 of Na,K-ATPase is involved in cation binding indirectly, possibly by affecting the cation occlusion sites through its connections with TM5 and TM6. Both TM5 and TM6 have residues involved in Na<sup>+</sup> and K<sup>+</sup> occlusion, and L6/7 residues are hydrogen bonded with TM5 and are directly linked with TM6.

The most compelling evidence that L6/7 is not a cytoplasmic cation binding site comes from the effects of Br-TITU<sup>3+</sup> on ouabain binding. Br-TITU<sup>3+</sup> and other cation antagonists such as Ca<sup>2+</sup>, Mg<sup>2+</sup>, La<sup>3+</sup>, mXBG<sup>2+</sup>, pXBG<sup>2+</sup>, and Br<sub>2</sub>-TITU<sup>3+</sup> are competitive inhibitors of both Na<sup>+</sup> and Rb<sup>+</sup> occlusion. The cation antagonists are not themselves occluded, however, and it has been suggested that they inhibit occlusion by competing with occluded cations for negatively charged residues in L6/7 at the entrance to the occlusion sites (20, 30). As shown in Fig. 6, however, Br-TITU<sup>3+</sup> binds to wild-type Na,K-ATPase and to L6/7 mutants in which either negatively or positively charged amino acid side chains have been replaced with alanine. If Br-TITU<sup>3+</sup> were binding to negatively charged amino acids in L6/7, then replacement of these amino acids by alanine would be expected to reduce the affinity of the protein for Br-TITU<sup>3+</sup>, and, consequently, reduce the extent of Br-TITU<sup>3+</sup> inhibition of ouabain binding. For the E825A mutant, the opposite effect on ouabain binding is observed, and there is no difference in the extent of Br-TITU<sup>3+</sup> inhibition of ouabain binding by the E828A mutant and wild type. These results indicate that Br-TITU<sup>3+</sup> does not bind to charged residues in L6/7.

There is substantial evidence that aryliothioiuronium derivatives and other cation antagonists inhibit occlusion by competing with Na<sup>+</sup> and Rb<sup>+</sup> at cytoplasmic sites (34, 35). One interpretation of this result is that the antagonists and occluded cations bind to the same sites on the protein. In the two-step model for occlusion (20, 35), Na<sup>+</sup> and Rb<sup>+</sup> bind to sites at an entrance port or gate in the cytoplasmic domain of the protein before being transferred to the sites of occlusion within the transmembrane domain. Based on the competitive inhibition of occlusion by the cation antagonists, it was proposed that the cation antagonists inhibit occlusion of Na<sup>+</sup> and Rb<sup>+</sup> by competing for the binding sites at the entrance port. If this is an accurate description of the mechanism of inhibition of occlusion by the antagonists, then the results reported here indicate that the entrance port does not include charged residues in L6/7. An alternative interpretation of the competition between occluded cations and the antagonists is that they bind to different sites on the protein but that binding is mutually exclusive. An entrance port or initial binding site is not required in this interpretation because binding of the antagonists at some cytoplasmic site would prevent access of Na<sup>+</sup> or Rb<sup>+</sup> to the occlusion sites in the transmembrane domain of the protein. The cytoplasmic binding site for the antagonists is unknown, but the results of the investigations reported here again show that it cannot include charged residues of L6/7.

Br-TITU<sup>3+</sup> binds to and stabilizes the E<sub>1</sub> conformation of the enzyme (30). The increased inhibition of ouabain binding by Br-TITU<sup>3+</sup> seen for the E825A and R837A mutants can be explained if these mutations stabilize the E<sub>1</sub> conformation, and the reduced inhibition of ouabain binding seen for the R834A mutant can be explained if this mutation stabilizes the E<sub>2</sub> conformation. Support for this interpretation is provided by the observation that the p-nitrophenylphosphatase activity of the R837A mutant is reduced relative to wild type, whereas the p-nitrophenylphosphatase activity of the R834A mutant is similar to wild type. p-Nitrophenylphosphate hydrolysis is catalyzed by the Na,K-ATPase in the E<sub>2</sub> conformation.

R837A greatly decreased both the affinity for ouabain in a Mg<sup>2+</sup>- and P<sub>i</sub>-dependent assay at fixed [Mg<sup>2+</sup>] and [P<sub>i</sub>] (Table I), and the apparent affinity for P<sub>i</sub> at fixed Mg<sup>2+</sup> and ouabain concentrations (Fig. 8). Because the ouabain K<sub>D</sub> value did not change when the concentration of P<sub>i</sub> was increased in the titration with ouabain (not shown), it is possible that the mutation altered the conformation of the ouabain binding site. Although L6/7 is cytoplasmic and ouabain binds from the extracellular side of the membrane, ouabain binding induced a conformational change in L6/7 that exposed a cytoplasmic chymotrypsin cutting site that was not accessible in the absence of ouabain (20). Similarly, conformational changes induced in L6/7 by mutations can be
expected to affect the ouabain binding site. Residues affecting ouabain binding affinity have been identified in TM 5–7 and in the extracellular loops between TM 5–8 (36, 37). Any effect of mutations in L6/7 on the ouabain binding site probably occurs through changes in the TM6 conformation. The $^{18}$O exchange results show unequivocally that intrinsic rate constants for $P_i$ binding and/or reaction with the protein were altered by mutation because $^{18}$O exchange between $P_i$ and $H_2$O is not ouabain-dependent. The exchange parameter containing the intrinsic $P_i$ dissociation constant ($K_{p_i}$) and the equilibrium constant between non-covalently and covalently bound $P_i$ ($K_{p_i}^{(co)}$) was significantly larger (5-fold) for the R837A mutant than for wild-type enzyme (Table III). An increase in $K_{p_i}$ could semi-quantitatively explain both the exchange results and the decrease in apparent affinity for $P_i$ in the $Mg^{2+}$- and $P_i$-dependent ouabain binding assay. However, a remote effect of a mutation in L6/7 on phosphorylation and dephosphorylation rate constants is also possible. We have previously shown that specific effects on $P_i$ binding caused by mutating amino acids outside the active site can be detected by $^{18}$O exchange (25).

In Ca-ATPase, mutations of Lys$^{319}$ and Arg$^{222}$, residues equivalent to Arg$^{234}$ and Arg$^{237}$ in Na,K-ATPase, reduced steady-state phosphoenzyme levels from both ATP and $P_i$ without affecting dephosphorylation of the phosphoenzyme (18). In Na,K-ATPase, steady-state levels of phosphoenzyme formation from ATP in R834A, R837A, and R848A were also lower than the wild-type enzyme (Fig. 9). R834A and R848A do not affect the $E_2P \rightarrow E_2$ dephosphorylation rate, but R837A results in faster $E_2P \rightarrow E_2$ dephosphorylation. The steady-state level of phosphoenzyme formation depends on the phosphoenzyme formation rate and the dephosphorylation rate. Since the dephosphorylation rates of R834A and R848A were the same as wild type, the lower level of steady state phosphoenzyme for R834A and R848A suggests that these two mutations affect the phosphoenzyme formation rate. Attempts to measure the phosphoenzyme formation rate from ATP in these mutants directly using manual sampling were unsuccessful because the rate of phosphorylation was too fast (data not shown). In Ca-ATPase, the reduction of phosphoenzyme formation from ATP was interpreted as evidence for an important role for Lys$^{819}$ and Arg$^{822}$ in determining the functional integrity of the phosphorylation domain of Ca-ATPase, and the reduced overall Ca-ATPase activity was attributed to this effect (18). In Na,K-ATPase, R834A, R837A, and R848A inhibited overall ATPase activity to a greater extent than they inhibited phosphoenzyme formation, suggesting that the mutations interfere with other partial reaction steps as well. The observation that R837A also inhibits the $K^+$-stimulated phosphatase activity and shows an increase in the $E_2P \rightarrow E_2$ dephosphorylation rate is consistent with this suggestion. The effects of L6/7 mutations on multiple Na,K-ATPase reaction steps and protein stability, together with the small effect of these mutations on the apparent affinity for $Na^+$ and $K^+$, support a role for L6/7 in maintaining the functional integrity of the phosphorylation domain and its relationship to the ion binding domain. This is similar to the role suggested for L6/7 in Ca-ATPase by Zhang et al. (18).
