Evidence That Ser<sup>775</sup> in the α Subunit of the Na,K-ATPase Is a Residue in the Cation Binding Pocket

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Substitution of alanine for Ser<sup>775</sup> in a ouabain-resistant α1 sheep isoform causes a 30-fold decrease in apparent affinity for K<sup>+</sup> as an activator of the Na,K-ATPase, as well as an increase in apparent affinity for ATP (Argüello, J. M., and Lingrel, J. B (1995) J. Biol. Chem. 270, 22764–22771). This study was carried out to determine whether Ser<sup>775</sup> is a direct cation-ligating residue or whether the change in apparent affinity for K<sup>+</sup> is secondary to a conformational alteration as evidenced in the change in ATP affinity, with the following results. Kinetics of K<sup>+</sup>(Rb<sup>+</sup>) influx into intact cells show that the change is due to a change in K<sup>+</sup> interaction at the extracellular surface. The K<sup>+</sup> dependence of formation of K<sup>-</sup>-occluded enzyme (E<sub>2</sub>(K)) and of the rate of formation of deoccluded enzyme from E<sub>2</sub>(K) indicate that the Ser<sup>775</sup>→Ala mutation results in a marked increase (≥30-fold) in rate of release of K<sup>-</sup> from E<sub>2</sub>(K). The high affinity Na<sup>+</sup>-like competitive antagonist, 1,3-dibromo-2,4,6-tris-(methylisothiouronium)benzene (Br<sub>3</sub>TIUU), which interacts with the E<sub>1</sub> conformation and blocks cytoplasmic cation binding (Hoving, S., Bar-Shimon, M., Tijmes, J. J., Tal, D. M., and Karlish, S. J. D. (1995) J. Biol. Chem. 270, 29788–29793), inhibits Na<sup>+</sup>-ATPase of the mutant less than the control enzyme. With intact cells, Br<sub>3</sub>TIUU acts as a competitive inhibitor of extracellular K<sup>+</sup> activation of both the mutant and control enzymes. In this case, the mutant was more sensitive to inhibition. With vanadate as a probe of conformation, a difference in conformational equilibrium between the mutant and control enzymes could not be detected under turnover conditions (Na<sup>+</sup>-ATPase) in the absence of K<sup>+</sup>. These results indicate that the increase in apparent affinity for ATP effected by the Ser<sup>775</sup>→Ala mutation is secondary to a change in intrinsic cation affinity/selectivity. The large change in affinity for extracellular K<sup>+</sup> compared with cytoplasmic Na<sup>+</sup> and to Br<sub>3</sub>TIUU binding supports the conclusion that the serine hydroxyl is either part of the K<sup>-</sup>-gate structure or a direct cation-ligating residue that is shared by at least one Na<sup>+</sup> ion, albeit with less consequence on rate constants for Na<sup>+</sup> binding or release compared with K<sup>+</sup>.

The Na,K-ATPase is a heterodimeric protein comprised of a catalytic α subunit and a smaller heavily glycosylated β subunit (for review, see Ref. 1). The enzyme catalyzes the exchange of three cytoplasmic sodium ions for two extracellular potassium ions coupled to the hydrolysis of one molecule of ATP. It is a member of the family of P-type ion pumps (reviewed in Ref. 2) that are characteristically phosphorylated and dephosphorylated at an aspartyl residue. Its catalytic α subunit comprises probably 10 transmembrane helices and shows considerable homology to that of the other P-type pumps such as the gastric H<sub>1</sub>,K<sub>-</sub>ATPase and the Ca-ATPases of the sarcoplasmic reticulum and plasma membrane.

During transport, both sodium and potassium ions are occluded within the Na,K-ATPase (for review, see Ref. 3). The nature of the cation binding and occlusion sites is largely unknown. However, studies of the reaction mechanism underlying cation exchange and functional consequences of structural perturbations support the notion of a cation binding and occlusion pocket occupied consecutively by both Na<sup>+</sup> and K<sup>+</sup> ions during their translocation from the cytosol to extracellular milieu and vice versa (4). The structure of the binding/occlusion region must accommodate the highly distinctive cation selectivities such that Na<sup>+</sup> binds with high apparent affinity at cytoplasmic sites, and K<sup>+</sup> binds at extracellular sites.

A critical issue concerns the identification of amino acids that comprise the cation binding and occlusion sites of the Na,K-ATPase. To date, a number of functionally important carboxyl and hydroxyl containing amino acid residues have been identified by chemical modification and site-directed mutagenesis of residues in transmembrane regions. Thus, substituting polar residues by mutagenesis of carboxyl- or hydroxyl-containing amino acids in transmembrane segments M4, M5, M6, M8, and M9 has resulted in either inactive or functionally altered enzymes with altered apparent affinities for Na<sup>+</sup> and/or K<sup>+</sup>. Of these, one mutation localized to M5 (Glu<sup>779</sup>→Gln; see Ref.5) alters (decreases) only Na<sup>+</sup> affinity. An Asn<sup>326</sup>→Leu mutation in M4 increased the apparent affinity for Na<sup>+</sup> but decreased it for K<sup>+</sup> (6). Other transmembrane substitutions resulting in relatively moderate decreases in apparent cation affinities for activating Na,K-ATPase are<sup>1</sup> Glu<sup>221</sup> in M4 (7–9), Glu<sup>779</sup> in M5 (10, 11), Thr<sup>207</sup> in M6 (10), Asp<sup>353</sup> in M8 (12), and Asp<sup>353</sup> and Asp<sup>353</sup> in M9 (13).

In contrast to the relatively modest effects of the foregoing mutations, non-conservative substitutions of Asp<sup>353</sup> and Asp<sup>353</sup>
in M6 markedly disrupted K⁺-enzyme interactions as evidenced in K⁺-ouabain antagonism (14, 15), and substitution of Ser²⁷⁵ with either alanine or cysteine decreases apparent K⁺ affinity dramatically, 31- and 13-fold in the case of Ser²⁷⁵ → Ala and Ser²⁷⁵ → Cys, respectively (16). Of these three mutants, only those with substitutions of Ser²⁷⁵ are functional. Because of the remarkable change in apparent affinity for K⁺ caused by the replacement of Ser²⁷⁵ with alanine, we have carried out experiments to determine whether Ser²⁷⁵ is a direct ligand involved in K⁺ binding and/or occlusion or whether the change in apparent K⁺ affinity is secondary to an alteration in the conformational equilibrium.

EXPERIMENTAL PROCEDURES

Mutagenesis, Cloning, Tissue Culture, and Transfection—HeLa cells were transfected with sheep Na,K-ATPase α₁ subunit cDNA modified by two mutations (Gln¹²⁳ → Arg and Asn¹²² → Aasp) to encode an α₁ form with low affinity for ouabain (RD) and with a Ser²⁷⁵ → Ala mutant (S775A) of RD. The mutagenesis and cloning of these constructs, and the transfection and culture of HeLa cells, were described previously by Arguello and Lingrel (16).

Membrane Preparations and Enzyme Assays—Membranes were isolated, and assays of Na,K-ATPase and Na-ATPase were carried out as described previously (17) in medium containing 5 mM EGTA, 1 mM MgCl₂, and the indicated concentrations of ATP, Na⁺, and K⁺. Unless indicated otherwise, assays of ATP hydrolysis at pH 7.4 were carried out using 20 mM histidine-Tris, and those at pH 8.0 used 20 mM Tris-HCl. Prior to assay, the membranes were preincubated for 10 min at 37 °C with 5 μM ouabain in medium containing 1 mM MgCl₂ and 20 mM Tris-HCl, pH 7.4. K⁺ occlusion and the rate of K⁺ deocclusion were measured by the indirect assays described by Daly et al. (15). Ouabain-sensitive K⁺ (86Rb⁺) influx into transfected HeLa cells was measured as described by Munzer et al. (19) using 24-well Falcon plates and with medium containing 10 mM NaCl, the indicated concentrations of KCl, and choline chloride to maintain a constant (150 mM) chloride concentration. Values of ouabain-sensitive K⁺ (86Rb⁺) influx are differences between two sets of triplicate determinations, one set with 5 μM ouabain and the other with 10 μM ouabain present during the preincubation and flux assay. For each set of triplicates, coefficients of variation were ≤5%.

Materials—All materials were of the highest purity available. Choline chloride was purchased from Syntax AgriBusiness and recrystallized twice from hot ethanol. Br₂TITU was synthesized as described by Tal and Khalish (20), dissolved in 2 mM Tris-HCl, pH 7.4 or pH 8.0, and stored at −20 °C as a 1 mM stock solution. [γ⁻³²P]ATP was synthesized by a modification (21) of the method of Glynn and Chappell (22) and stored at −20 °C. [³²P]ATP was from Amersham Life Science, Inc.

RESULTS

When HeLa cells transfected with the RD Na,K-ATPase are grown in Dulbecco’s modified Eagle’s medium containing 10–6 M ouabain, expression of the exogenous enzyme enables cell growth. In contrast, mutation of Ser²⁷⁵ → Ala in this enzyme results in suppression of growth unless the K⁺ concentration is raised to at least 20 mM (16). This behavior is associated with a marked decrease in apparent affinity of the mutant enzyme (S775A) for K⁺, as evidenced in studies of K⁺-activation of Na,K-ATPase activity (16). With sided preparations (intact cells suspended in buffered choline chloride and containing 10 mM Na⁺ with monensin present to maintain a constant intracellular Na⁺ concentration as described in Munzer et al. (19)), a marked decrease in apparent affinity for extracellular K⁺ is also observed (Fig. 1). The difference (30-fold; average of five separate experiments, with a cooperative 2-site model used to fit the data) between the S775A and RD enzymes is similar to that observed previously with unsided membranes (16). It is evident also that the Vₘₐₓ of the mutant enzyme is higher than that of the control RD enzyme (5-fold in the representative experiments shown). This probably reflects the higher expression of S775A mutant compared with control RD protein observed previously (16).

Although this finding lends further support to the conclusion that Ser²⁷⁵ is a cation-ligating residue, the question remains as to whether the decrease in apparent affinity for extracellular K⁺ is due to an intrinsic decrease in K⁺ binding and/or occlusion in the cation binding “pocket” or reflects a change in the E₃/En conformational equilibrium during steady-state catalysis. Several experimental approaches were used to gain further insight into the function of Ser²⁷⁵. First, the Na,K-ATPase reaction was studied at very low ATP concentration since the K⁺-deocclusion reaction sequence (E₃(K) → E₁ + K⁺), which follows the K⁺-dependent dephosphorylation, is rate-limiting.

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2 The abbreviations used are: RD, ouabain-resistant sheep α₁ isoform; Br₂TITU, 1,3-dibromo-2,4,6-tris-(methylisothiouronium)benzene; E₃(K), K⁺-occluded enzyme.
at concentrations of ATP sufficient to saturate only the low affinity binding site (23). In fact, as shown earlier (17, 18, 24) and in the experiment described in Fig. 2 above, the response of Na-ATPase to K+ at micromolar ATP concentration is a simple and sensitive means of characterizing isoform- or mutant-specific differences in the K+ deocclusion pathway of the reaction.

As shown in Fig. 2, marked stimulation by K+ is observed in the mutant S775A enzyme, whereas the control RD enzyme is characterized by inhibition by K+ at micromolar ATP concentration. With both enzymes, inhibition by K+ at concentrations above 10 mM is probably due to K+ antagonism at cytoplasmic Na+ activation sites. This change in K+ response profile at low ATP concentration caused by the mutation suggests a change in rate of a reaction step following dephosphorylation of the K+-sensitive form of phosphoenzyme commonly referred to as E2P in the Albers-Post mechanism. According to a branched pathway of K+ deocclusion, which follows K+-activated dephosphorylation of E2P, namely E2P → ATP → ATP,E2(K) → ATP,E3(K) → ATP,E3(K) → ATP,E2(K) → ATP,E1 + ATP → ATP,E1 + K+ (pathway a), or E2(K) → E2,K → E1 + ATP → ATP,E1 + K+ (pathway b), an increase in either ATP,E2(K) → ATP,E3,K or E2(K) → E1 + K+ (pathway b) could be evidenced in a change from K+ inhibition to K+ activation at low ATP concentration.

Consistent with this prediction is the increase in apparent affinity for ATP of S775A compared with RD shown previously (16) and confirmed in this study. In fact, when the analysis is carried out with the ATP concentration varied from 1 to 500 µM and the data points fitted to a 2-component model describing the aforementioned pathways, one in the range 20–1000 µM ATP and the other in the range 0.5–10 µM ATP, the kinetic constants for the apparent affinities for ATP at low and high affinity sites, designated K′L and K′H, respectively, are 47.9 ± 6.4 and 3.65 ± 1.40 for S775A and 504 ± 191 and 6.01 ± 1.75 for RD. Maximum velocities via pathways a (V′a) and b (V′b) were also derived from the plots, and the relative values, expressed as the ratio V′a/V′b, are 0.05 for RD and 0.34 for S775A. These results indicate that the mutation increases the affinity for ATP via pathway a and increases the relative activity via the high affinity pathway b. At face value, the behavior shown in Fig. 2 and the kinetic changes effected by the Ser775 → Ala mutation are not remarkably different from those resulting from certain mutations in cytoplasmic regions (viewed in Ref. 25). However, fundamental differences are revealed by further analysis as described below.

**K+ Occlusion and Deocclusion**

Experiments aimed to obtain direct information about the effect of the Ser775 → Ala mutation on K+ binding, occlusion, and/or deocclusion were carried out as described previously. For K+ occlusion, the enzyme was equilibrated with varying concentrations of K+ (K+ → E1 + E2(K)), and the resulting decrease in E1 was measured as described by Daly et al. (18). In this assay, the enzyme is first equilibrated at room temperature (i) without and (ii) with varying amounts of K+. The amount of K+-occluded enzyme is reflected by the decrease in phosphoenzyme (E2P) formed during rapid phosphorylation with [γ-32P]ATP at 0 °C. The reduction in E32P resulting from preincubation with K+ (E2P) is a measure of the amount of E2P(K).

The results shown in Fig. 3 indicate that K+ occlusion in the RD enzyme can be described by a simple hyperbolic relationship (Amax[|S|]/(Kdis + |S|)), with a Kdis approximately an order of magnitude lower than that of the S775A mutant. In contrast, K+ occlusion showed sigmoid behavior in all experiments (n = 4) with the mutant enzyme. When the data for the RD and S775A enzymes are fitted to a cooperative n-site model, values of n are 0.8 for RD and 1.6 for S775A. The sigmoid behavior of S775A has important implications regarding the effect of the mutation on the individual rate constants for occlusion and/or deocclusion of the first followed by the second K+ ion as discussed below. The presence of a fraction of enzyme (25% of RD; 50% of S775A), which can be phosphorylated but cannot form E2P(K) from E1, at least following preincubation with up to 8 mM K+, suggests the existence of transfectected enzyme that is functionally altered (see "Discussion").

Fig. 4 shows that when the mutant enzyme is first preincubated with K+ to obtain maximal E2P(K), subsequent deocclusion at 10 °C is extremely rapid, precluding quantitation of the rate constant with the manual method used (18). However, with ≥90% of E2P(K) disappearing within the first 4 s, the rate constant must be ≥0.6 s−1, which is ≥30 times that (≈0.02 s−1) of the RD enzyme.
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Catalytic Turnover

Previous experiments suggested that the Ser^{775} → Ala mutation markedly reduced the catalytic turnover of the enzyme. We have reexamined this characteristic for the following reasons. First, assays of hydrolytic activity and phosphoenzyme formation using commercially available [γ-32P]ATP are problematic: high base-line values and variable levels of purity of commercial [γ-32P]ATP have precluded reproducible measurements of phosphoenzyme. Second, to obtain maximal estimates of phosphoenzyme, $E_P^{max}$, oligomycin is now added routinely to trap the phosphoenzyme as $E_P$. Using [γ-32P]ATP synthesized as described under “Experimental Procedures,” values for turnover calculated as the ratio of $V_{max}$ (data from assays carried out at 100 mM NaCl and varying K concentration fitted to a cooperative 2-site model $E_P^{max}$ (min^{-1}) are 7600 and 4700 for RD and S775A, respectively (experiment not shown). The lower turnover of the mutant compared with the control enzyme may be due, at least partly, to underestimation of $V_{max}$. Thus, with unsided preparations, it is likely that K^+, at the high concentration needed to activate S775A at extracellular sites, inhibits cytoplasmic Na^+ activation sites.

Effects of the Competitive Sodium Antagonist Br$_2$TITU

This member of a novel family of aromatic isothiouronium derivatives developed by Tal and Karlish (20) acts as a very high affinity Na^+-like competitive antagonist ($K_i = 0.32$ μM) that interacts with the $E_3$ conformation and blocks cytoplasmic cation binding and occlusion; at much higher concentration ($K_i \sim 10$ μM), particularly at low ionic strength, it affects cation interactions with the $E_2$ conformation. As discussed earlier (26), this family of antagonists should be uniquely useful in distinguishing between mutations that directly affect cation binding as distinct from those that have indirect effects due to alterations of rate constants of the reaction cycle.

Effects on Na,K-ATPase—The experiment shown in Fig. 5 compares the inhibitory effect of Br$_2$TITU on Na,K-ATPase of the RD and S775A enzymes assayed with saturating (1 mm) ATP, 100 mM Na^+ and sufficient K^+ (50 mM) to achieve approximately 75% $V_{max}$ of the mutant enzyme (16). Under these conditions, the S775A mutant is more sensitive to inhibition by Br$_2$TITU. The experiment shown in Fig. 6 was carried out at 20 mM KCl and indicates that inhibition by Br$_2$TITU (20 μM) decreases as the Na^+ concentration increases. In this representative experiment, the pH was raised to pH 8.0 to increase the effectiveness of Br$_2$TITU (26). Because of the low apparent affinity of the mutant enzyme for K^+, it was not possible to compare the effects of Br$_2$TITU on RD and S775A as a function of varying Na^+ at low K^+ concentration and, conversely, the effects of varying K^+ at low Na^+ concentration.

Inhibition of Na,K-ATPase can occur by binding of Br$_2$TITU at cytoplasmic sites with high affinity or at extracellular sites with lower affinity or by both. There are several possible explanations for the greater inhibition of S775A by Br$_2$TITU in the conditions of Figs. 5 and 6. First, the balance of the $E_2$ and $E_3$ equilibrium could be shifted to $E_2$, which binds Br$_2$TITU with a higher affinity than $E_3$. Second, the large decrease in K^+ affinity at the extracellular surface could reduce the ability of K^+ to compete with Br$_2$TITU on $E_3$ and make it a better competitor in S775A than in RD. Third, the intrinsic Br$_2$TITU binding might have been improved by mutation. A combination of these factors is also possible. Evidence in favor of the second...
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Motivation was obtained in experiments with intact cells, with Br2TITU present in the extracellular medium. Under these conditions, passive permeation of the compound is likely to be extremely slow in accord with its low (1:300) octanol:water partition (not shown) and triple positive charge. Thus, with both enzymes assayed at the same (2 mM) extracellular K+ concentration, Br2TITU inhibition of ouabain-sensitive (86Rb)K+ influx is weak with the RD enzyme and markedly increased with the S775A mutant (not shown). When the kinetics of extracellular K+ activation were compared in the absence versus presence of 100 μM Br2TITU as described in Fig. 7, it is clear that Br2TITU is a competitive inhibitor. The apparent K₁ for Br2TITU obtained by fitting a 2-site non-cooperative model to the data indicates that K₁ for Br2TITU inhibition is reduced only moderately by the mutation. As indicated in the legend to Fig. 7, values of K (in micromolar) for Br2TITU as an inhibitor were 0.23 mM for RD and 0.16 mM for S775A. Open circles, RD enzyme; closed circles, S775A enzyme.

Effects on Na-ATPase—In the absence of K+, the Na-ATPase activity requires only micromolar ATP and Na+ activation reaches saturation with ~1 mM Na+. This initial phase of activation is due to Na+ binding at high affinity cytoplasmic sites; it is followed by a plateau or slight inhibition as Na+ is further increased to ~10 mM (27–29). This inhibitory phase due to Na+ ions acting at high affinity extracellular sites (28, 29) is followed by a further increase in activity as the Na+ concentration is raised above ~20 mM due to Na+ acting as K+ congeners at extracellular activation sites (30). The increase effect by 100 mM Na+ is 2–3-fold for RD but less than 0.2-fold for the S775A mutant. This behavior (experiments not shown) suggests that the mutation affects the binding of both Na+ and K+ at extracellular sites.

Another aim of these experiments was to determine whether the Ser775 → Ala mutation affects a ligand common to the binding of Na+ at cytoplasmic sites as well as Na+ and K+ at extracellular sites. In earlier experiments aimed to assess the Na+ dependence of Na,K-ATPase under Vmax conditions, a small change in the activation profile was evidenced as a decrease, in the mutant, in the Hill coefficient obtained by using a cooperative n-site model to fit the data (16). In the present study, efforts to determine K′,Na in assays of Na-ATPase with varying Na+ (initial phase of activation) were confounded by substantial activity present in the absence of added Na+ due, most likely, to traces of residual (tightly bound?) Na+ in the membrane preparations (experiments not shown). However, the following condition was found in which the two enzymes show a significant difference in Br2TITU binding at cytoplasmic Na+ sites.

In the representative experiment shown in Fig. 8, carried out with Na+ present in concentrations sufficient to saturate only high affinity cytoplasmic sites (0–1 mM), both enzymes are sensitive to inhibition by Br2TITU at low (5 μM) concentration. Inhibition diminishes as Na+ is further increased. This may be due to Na+ counteracting inhibition by Br2TITU at the high affinity cytoplasmic Na+ -activation sites. The particularly interesting observation is that Na-ATPase of the mutant enzyme is less sensitive to inhibition by Br2TITU than the RD control. In other experiments (not shown), inhibition is diminished as the pH is decreased from pH 8.0 to 7.4, particularly with the mutant enzyme. These contrasting effects of Br2TITU on Na,K-ATPase versus Na-ATPase are difficult to explain on the basis of a difference in conformational equilibrium between the two enzymes at very low Na+ concentration. The most economical explanation is that intrinsic Br2TITU binding at cytoplasmic sites is altered by mutation of serine 775 to alanine. The difference in affinity is clearly much less than that of K+. In an experiment carried out at pH 8.2 (not shown), with Br2TITU varied up to 5 μM, the difference in IC50 for Br2TITU was approximately 2-fold.

Sensitivity to Vanadate

In the experiment shown in Fig. 9, vanadate was used as a probe of the E1/E2 conformational equilibrium (cf. Ref. 31) under conditions of turnover in the absence of K+ and with a Na+ concentration sufficient to saturate only high affinity cytoplasmic sites (Na+-ATPase). The results indicate that the sensitivities to vanadate inhibition of the mutant and wild-type enzymes are identical. Similar experiments with conformational mutants of α1, such as a Glu233 → Lys mutation of rat α1, or the α2 isoform, indicate that these enzymes are at least an order of magnitude less sensitive to vanadate compared with α1.3

3 N. Boxenbaum, S. Daly, L. Lane, and R. Blostein, unpublished observations.
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**DISCUSSION**

The most dramatic functional alteration effected by a site-specific mutation of the catalytic α subunit of the Na,K-ATPase is the substitution of serine 775 with alanine. This mutation causes a 30-fold decrease in apparent affinity for extracellular K⁺. The experiments described in this paper have addressed the question of whether this alteration reflects either (i) a role of serine 775 as a direct cation-ligating residue, or a component of a gating structure close to the K sites, or (ii) a change in apparent affinity for K⁺ secondary to a change in the poise of the E₂,₇⁻E₁ conformational equilibrium. The results provide several points of evidence in support of the conclusion that Ser775 is a K⁺-ligating residue.

The first point of evidence is that a decrease in apparent affinity for K⁺ (increase in Kₘ) is observed not only at saturating ATP concentration (see Fig. 2 of Ref. 16 and Fig. 1 of this study) but also at micromolar ATP concentration under which condition of lowering the concentration of ATP results in a decrease in Kₘ. Thus, changes in Kₑ,₇, ATP, the apparent affinity for ATP at its low affinity binding site, are complicated functions of changes in rate constants of steps leading to the sequential binding, occlusion, and deocclusion of two K⁺ ions as in the following sequence of partial reactions.

\[
E₄P.K \rightarrow E₄P.K + ATP \rightarrow ATP.E₄(K) \rightarrow ATP.E₄(K) + K⁺ \rightarrow ATP.K⁺ + E₁ \rightarrow E₁ + ATP.K⁺
\]

**REACTION 1**

According to the earlier analysis of Eisner and Richards (32), a greater than 5-fold increase in Kₘ was observed as ATP was increased from 1 μM to 1 mM. In the present study, the estimated Kₑ,₇ for K⁺-dependent activation of Na⁺-ATPase of S775A at 1 μM ATP is ~3 mM (2-site cooperative model fitted to the K⁺-activation curve shown in Fig. 2), which is still at least an order of magnitude higher than that of the control RD enzyme measured at saturating ATP concentration.

The dramatic changes in K⁺ dependence of occlusion and in the kinetics of K⁺-deocclusion provide further evidence for changes in the cation-ligating domain effected by the Ser775 → Ala mutation. Whereas the kinetics of K⁺ occlusion in the control RD enzyme are characteristically hyperbolic, those of the mutant are sigmoidal. This alteration may be explained by assuming that the intrinsic dissociation constant for release of the first K⁺ ion is normally very much lower than that of the second and that the sigmoidal kinetics of the S775A mutant indicates a marked increase in the dissociation constant for release at the first (high affinity) site.

Further evidence for a direct role of Ser775 in cation binding was obtained in assays of Na⁺-ATPase in the absence of K⁺ and at sufficiently low Na⁺ concentration to interact at only cytoplasmic transport sites. Under these conditions, in which the reaction cycle reflects ATP-dependent Na⁺ efflux that is not coupled to the influx of either K⁺ or Na⁺, the S775A mutant is approximately 2-fold less sensitive than the control RD enzyme to the high affinity Na⁺ competitor, Br₂TITU. This behavior suggests an alteration in intrinsic Na⁺ binding and is consistent with a small difference in the kinetics of Na⁺-activation of Na,K-ATPase documented previously (see Fig. 3 of Ref. 16). In fact, a change in Na⁺-activation kinetics was confirmed in this study. With the data fitted to a 3-site cooperative model, a significant, albeit modest (1.5-fold), decrease in Kᵥ,₅ for Na⁺ was observed.

The marked (at least 30-fold) increase in the rate of formation of E₄ from E₄(K) is particularly diagnostic of an alteration in the K⁺-occlusion pocket, with secondary changes in the conformational equilibrium and apparent affinity for ATP. The rationale for this argument is based on the distinctive behavior of S775A compared with that of "conformational" mutants described recently (25). Thus, there is a striking and important difference between the reciprocal changes in Kₑ,₇ and Kₑ,₇ observed with this mutant and those of mutants that are due to alterations in cytoplasmic regions and that have been characterized as E₄/E₄ conformational mutants. In particular, the Glu233 → Lys mutation in the cytoplasmic M2/M3 loop results in a shift in the poise of the E₂/E₄ equilibrium toward E₄. Although this mutant (E233K) resembles S775A in its higher (6-fold) apparent affinity for ATP at its low affinity site, a...
concomitant change in apparent affinity for K⁺ under conditions of high (1 mM) ATP concentration was minimal (1.6-fold), and the rate of formation of $E_1$ from $E_2(K)$ is only ~4-fold greater than that of the RD control enzyme. In the experiments with vanadate as a probe of the $E_2/E_3$ conformational equilibrium, the identical sensitivities to vanadate of the S775A mutant and control RD enzymes under conditions of turnover in the absence of K⁺ and with Na⁺ concentration sufficient to saturate only high affinity cytoplasmic sites (Na⁺-ATPase) is an important argument against the notion that S775A is a conformational mutant.

With both the control RD and mutant S775A heterologous enzymes, the maximal amount of $E_2(K)$ formed from $E_1$ is consistently lower than $E_{max}^{per}$ suggesting enzyme which is impaired in its catalysis of the reaction cycle. With the rat α1 enzyme and mutants thereof transfected into the same (HeLa) cells, little, if any, such functionally impaired enzyme was detected (18). With the ouabain-resistant RD sheep enzyme, the shortfall in $E_2(K)$ is relatively modest (25% of $E_{max}^{per}$), which is similar to that observed earlier with the heterologous rat α2 isoform (18). It may reflect newly synthesized enzyme that has not matured completely, perhaps due to the limitation of endogenous β subunits. However, with the S775A mutant, the greater shortfall may be due to the even higher expression. Alternatively, it may be, at least partly, a consequence of the mutation, per se. Thus, it is plausible that K⁺ is released from the altered binding pocket, not only to the cytoplasm from $E_2(K)$ (the normal route), but also to the extracellular side, from $E_2(K)$ according to the pathway $E_2(K) \rightarrow E_1K \rightarrow E_3 + K_{ext}$. This behavior would also explain the apparent paradox that the Ser⁷⁷⁵ → Ala mutation accelerates the $E_2(K) \rightarrow E_1$ process without altering the $E_2 \rightarrow E_1$ conformational transition, in spite of evidence that they are tightly coupled (33). Presumably, participation of such a low affinity binding pathway, is minimal during the normal forward operation of the complete reaction cycle. Otherwise, K⁺ release to the side (extracellular) from which it bound would result in uncoupling of K⁺ influx from ATP hydrolysis, resulting in a discordance between the decrease in apparent affinity for K⁺ observed in transport versus Na,K-ATPase assays of the S775A mutant. This was not the case; both were decreased ~30-fold (compare Fig. 1 in this paper with Fig. 2 in Ref. 16). This conclusion infers that release of $K_{ext}$ from $E_2(K)$ is counteracted by ATP binding to $E_2(K)$ whereby the forward reaction process $E_2(K) \rightarrow E_1KATP \rightarrow E_4KATP \rightarrow E_4ATP + K_{ext}$ predominates.

How does the change in Ser⁷⁷⁵ alter $K_{1C}$? One explanation derives from the model of Forbush (34) and assumes that (i) K⁺ ions are occluded at two distinct sites, (ii) the binding of the first K⁺ ion at the extracellular surface is followed by its repositioning closer to the cytoplasmic surface with concomitant binding of the second extracellular K⁺ ion, and (iii) subsequent release of the first ion into the cytoplasm is controlled by the characteristics of the exit or gate of the occlusion pocket. Accordingly, if the flickering-gate model for K⁺ release to the extracellular side (34) is generally applicable to release to the cytoplasmic side, then release of the first ion into the cytoplasm would depend on the opening time constant of the release gate. It is possible that one or more K⁺-ligating residues form(s) is/are associated with such a gate structure, and in particular, that Ser⁷⁷⁵ is one such residue; its replacement by alanine markedly increases the rate of release of the first K⁺ ion as mentioned above. Assuming, furthermore, that Ser⁷⁵ is a ligating residue common to both Na⁺ and K⁺, the small effect of the mutation on apparent affinity for Na⁺ would be consistent with the conclusion that a change in the time constant of the gate is not of rate-limiting consequence for the subsequent binding and occlusion of Na⁺.

The selective effect of the Ser⁷⁵ → Ala mutation on K⁺ versus Na⁺ affinity might be reconciled by a model of the cation binding cavity with certain overlapping cation sites and additional oxygen-rich groups that ligate K⁺ ions as formulated recently by Argüello and Lingrel (16) and by Karlish (35). Thus, such an additional group is Ser⁷⁵. In the absence of K⁺, the cavity space diminishes but still provides a snug fit for Na⁺. Of particular relevance is the high degree of alkali cation specificity as a consequence of an alteration in the structure of a coordinating serine in the ligating cavity of the K⁺-dependent enzyme dialkyglycine decarboxylase (36). With that enzyme, the exchange of Na⁺ for K⁺ reduces the average metal-ligand distance from 2.73 to 2.33 Å.