Allosteric Regulation of the Access Channels to the Rb\(^{+}\) Oclusion Sites of (Na\(^{+}\) + K\(^{+}\))-ATPase*

James Haseauer, Wu-Hsiung Huang, and Amir Askari‡

From the Department of Pharmacology, Medical College of Ohio, Toledo, Ohio 43699-0008

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Previous work on the role of occluded Rb\(^{+}\) (a K\(^{+}\) substitute) in the reaction cycle of (Na\(^{+}\) + K\(^{+}\))-ATPase has focused on the kinetics of the dissociation of the enzyme-Rb\(^{+}\) complex at 20–24 °C. Doing experiments at 4 °C, we have made the following observations on the equilibrium binding levels and the kinetics of binding and release of Rb\(^{+}\). 1) The plot of bound Rb\(^{+}\) as a function of [Rb\(^{+}\)] showed occupancy of high affinity sites, followed by binding to sites of lower affinity. The estimated number of Rb\(^{+}\) sites/active site was two to three, but a higher number was not ruled out. Release of bound Rb\(^{+}\) was slow and not monoexponential, the major portion being in a pool with a half-life of 4–5 h. Dissociation curves were identical at different levels of site occupancy. Rb\(^{+}\) binding also had fast and slow phases, requiring about 24 h to reach steady state at vastly different [Rb\(^{+}\)]. These data suggest that (a) Rb\(^{+}\) occlusion sites are confined within the protein matrix and connected to the medium by narrow access channels that are heterogeneous in size, and (b) channel heterogeneity is distinct from differences in occlusion site affinities. 2) ATP, at a low affinity allosteric site, had no significant effect on the maximal level of bound Rb\(^{+}\) at any [Rb\(^{+}\)], but it accelerated both the fast and the slow phases of Rb\(^{+}\) binding and release, and it increased the ratio of fast to slow phases. Evidently, ATP activates the channels (lowers the energy barrier for access) without altering binding site affinities. 3) Na\(^{+}\) was a competitive inhibitor of Rb\(^{+}\) at the occluded sites, but it also acted at an allosteric site to activate the access channels. Rb\(^{+}\) and K\(^{+}\) also had allosteric effects: although they did not affect the access channels directly, they blocked the allosteric effect of Na\(^{+}\). 4) Ouabain was an access channel inhibitor. It reduced the rates of binding and release of Rb\(^{+}\), blocked channel activation by ATP and Na\(^{+}\), but seemed to have no effect on the events at the occluded sites.

The existence of heterogeneous access channels to the ion transport sites and the demonstration of channel regulation by the physiologic ligands of the enzyme suggest the necessity of the inclusion of such allosteric mechanisms in the reaction cycle of (Na\(^{+}\) + K\(^{+}\))-ATPase.

(Na\(^{+}\) + K\(^{+}\))-ATPase catalyzes the coupled active transport of Na\(^{+}\) and K\(^{+}\) across the plasma membrane of most eucaryotic cells. During the pump cycle both ions are thought to be transiently occluded within the transmembrane domains of the protein so that they are not exposed to either the intracellular medium or the extracellular medium (1, 2). Although such states of the enzyme containing inexchangeable ions have not been demonstrated, a great deal about the role of occluded K\(^{+}\) in the reaction mechanism of the pump has been surmised from studies on the kinetics of the release of bound Rb\(^{+}\) (a K\(^{+}\) substitute) from the enzyme at 20–24 °C (1, 2).

Because the study of the kinetics of Rb\(^{+}\) binding has not been possible at this temperature and since many of the studies on the reaction mechanism of the enzyme have been done at lower temperatures (0–4 °C), we have examined the interaction of Rb\(^{+}\) with the enzyme at 4 °C in some detail. Here, we present the results of the experiments on the equilibrium binding plots of Rb\(^{+}\), the kinetics of binding and release of Rb\(^{+}\), and the regulation of these by several of the enzyme’s ligands. The new information obtained from these studies supports some previous conclusions but also questions the validity of several of the existing assumptions regarding the mechanisms involved in the occlusion and deocclusion of Rb\(^{+}\) and the role of occluded Rb\(^{+}\) (or K\(^{+}\)) in the reaction cycle of the pump.

EXPERIMENTAL PROCEDURES

The purified membrane-bound enzyme of the canine kidney medulla was prepared and assayed as described before (3). The specific activities of the various preparations used were in the range of 1,000–1,600 pmol of ATP hydrolyzed/mg of protein/h.

"Rb\(^{+}\) binding to the enzyme was measured by a modification of the method of Shani et al (4). The enzyme (75–160 μg/ml) was incubated at 4 °C in a medium containing 0.2 M sucrose, 12 mM histidine (pH 6.8), the indicated concentration of "RbCl, and other additions as specified. After an appropriate incubation period, 0.5-ml aliquots of the above suspension were passed through a cation exchange column and eluted with 1.5 ml of a solution containing 0.2 M sucrose, 12 mM histidine (pH 6.8). Unless stated otherwise, the flow rate was controlled so that the passage of the sample and the wash solution through the column was completed in 2 min. The entire procedure was carried out in a cold room at 4 °C, with the columns and the solutions also precooled to this temperature. The enzyme that was collected in the eluate and contained bound "Rb\(^{+}\) was counted by conventional procedures. The ion exchanger (3 ml of wet resin) was the Tri form of Dowex 50W-X8, 20–50 mesh, which was poured to a height of 7 cm in a disposable column (Econo-Column, Bio-Rad). Prior to use the column was washed with 1 ml of 25 mg/ml bovine serum albumen, followed by 2 ml of the above sucrose-histidine solution. Control experiments showed that when the native enzyme was passed through the ion exchange column according to the above protocol, 100% of the enzyme activity was recovered in the eluate. Based on the known bed volume of the resin and its void volume (35%), the transit time of the bulk of the enzyme through the column was estimated to be about 60 s. This value was used for the interpretation of the results of some experiments as specified under "Results."

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‡ To whom correspondence should be addressed: Dept. of Pharmacology, Medical College of Ohio, P. O. Box 10008, Toledo, OH 43699-0008. Tel.: 419-381-4183; Fax: 419-382-7395.

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To measure release of bound $^{86}\text{Rb}^+$, the enzyme (6-8 mg/ml) was first incubated at 4 °C with $^{86}\text{RbCl}$ in the sucrose-histidine solution for a specified period as indicated above. The sample was then diluted 100-fold with the above sucrose-histidine solution containing 20 mM unlabeled RbCl and other additions as indicated. In specified experiments the unlabeled Rb+ was omitted from the diluting solution. At appropriate intervals after the dilution, a 0.5-ml aliquot of the sample was removed and passed through the ion exchange column to determine the level of bound $^{86}\text{Rb}^+$. Unless indicated otherwise, all values presented in the figures are averages of duplicate determinations.

The maximal phosphorylation capacity of the enzyme was determined using 0.1 mM $\text{Pi}$, 2 mM Mg$^2+$, and 1 mM ouabain as described before (3).

The ouabain-complexed enzyme was prepared as described before (5) by incubating the enzyme with a saturating concentration of labeled or unlabeled ouabain in the presence of $\text{Pi}$, and Mg$^2+$. The complex was then washed cold and used for Rb+ binding and release experiments as indicated above.

Nucleotides, ouabain, histidine, RbCl, and the ion exchange resin were obtained from Sigma. $^{86}\text{RbCl}$, $^{32}\text{P}$, and $[\text{H}]$ouabain were purchased from Du Pont-New England Nuclear. Computer analysis of the equilibrium binding data was done using the program LIGAND (6) purchased from Biosoft.

**RESULTS**

**Stability of the Enzyme-Rb+ Complex on the Ion Exchange Column**—In the course of describing a simple manual assay for Rb+ binding to the enzyme, Shani et al. (4) noted that the enzyme-Rb+ complex was quite stable in an ice-cold sucrose solution, but they did not present much data on the kinetics of Rb+ release or binding at low temperatures. To study such kinetics, it was first necessary to examine the stability of the complex under conditions used for its assay, i.e. during the time that the complex is being separated from free Rb+ on the cation exchange column (see "Experimental Procedures").

The enzyme suspended in a sucrose-histidine solution at 4 °C was exposed to $^{86}\text{Rb}$ at several fixed concentrations and then passed through columns at different flow rates. Appropriate control samples containing $^{86}\text{Rb}$ but no enzyme, or $^{86}\text{Rb}$ plus enzyme that had been boiled for 1 min, were also passed through columns. At all tested Rb+ concentrations, identical data were obtained in the two controls. The results of such experiments with 3 and 200 µM Rb+ are presented in Fig. 1. These and similar data obtained with up to 2 mM Rb+ showed that with the column and the sample sizes used (a) about 60 s was required to separate free Rb+ from the complex, and (b) the complex was stable up to 140 s of elution time through the column. For the measurement of bound Rb+ in all experiments presented below, the enzyme and the appropriate control were passed through the column for 120 s under the standard conditions described under "Experimental Procedures." Bound Rb+ was then calculated from the difference between the counts of the eluate containing the native enzyme and those of the control eluate.

**Formation of the Enzyme-Rb+ Complex as a Function of the Rb+ Concentration**—In the experiments of Fig. 2 the enzyme was mixed at 4 °C with varying concentrations of Rb+, allowed to stand for 24 h to achieve maximal binding at each concentration (see below), and then passed through ion exchange columns to measure bound Rb+. The Scatchard plot of the data (inset to Fig. 2) is clearly concave upward, indicating either the presence of heterogeneous binding sites with undifferented affinities or the existence of negative interactions among identical sites. Nonlinear regression analysis of the data based on the assumption of only two classes of unidentical sites resulted in the following estimated parameters for the two hypothetical sites. Dissociation constants (± S.E.): $K_d1 = 21 ± 1.5 \mu\text{M}$; $K_d2 = 1.22 ± 0.23 \mu\text{M}$. Binding capacities (± S.E.): $B_1 = 2.07 ± 0.24 \text{nmol/mg}$; $B_2 = 5.21 ± 1.11 \text{nmol/mg}$.

To permit the estimation of the number of Rb+ binding sites/active site, the maximal level of the phosphoenzyme formed from P1 in the presence of ouabain was also determined in the various preparations used in Fig. 2 and was found to be $2.99 ± 0.16 \text{nmol/mg}$ (± S.E.; $n = 15$).

In separate experiments it was established that the enzyme incubated under the conditions of Fig. 2 retained more than 94% of its activity.

**Kinetics of Rb+ Release**—The enzyme was incubated with different Rb+ concentrations (5 µM to 5 mM) to obtain bound Rb+ at widely different degrees of saturation, and the time course of Rb+ release was then examined. At any degree of saturation, the dissociation curve was the same regardless of whether or not the dissociation medium contained 20 mM unlabeled Rb+ (Fig. 3). The dissociation curves were not monoeponential but identical at all degrees of saturation. A composite of the curves at different degrees of saturation

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**Fig. 1. Separation of enzyme-Rb+ complex from free Rb+ on a cation exchange column.** The enzyme was incubated with 3 µM $^{86}\text{Rb}^+$ (O) or 200 µM $^{86}\text{Rb}^+$ (■) as described under "Experimental Procedures." Aliquots (0.5 ml) of each mixture and 0.5-ml aliquots of controls containing $^{86}\text{Rb}^+$ (△, 3 µM A, 200 µM) but no enzyme were passed through columns followed by 1.5 ml of a wash solution as described under "Experimental Procedures." Flow rates were adjusted to obtain the indicated elution times for the 2-ml samples.

**Fig. 2. Binding of Rb+ to the enzyme as a function of Rb+ concentration.** The enzyme was incubated at 4 °C for 24 h with varying concentrations of $^{86}\text{Rb}^+$ (2.5 µM-2 mM) in the absence of ATP (O) and in the presence of 2 mM ATP (■). Incubation and assay conditions were as described under "Experimental Procedures." Each value is the mean of six determinations. When the standard error is larger than the size of the symbol, it is indicated by the vertical bar. The inset is the Scatchard plot of the binding data in the absence of ATP. The straight lines of this plot represent two populations of hypothetical binding sites fit by LIGAND to a two-site model as described under "Experimental Procedures" (p < 0.05 compared with a one-site model).
is shown in Fig. 3. The curve could be resolved into two exponentials, showing that the major portion of the bound Rb+ is in a highly stable pool with a half-life of about 4.5 h (legend to Fig. 3). The identity of the dissociation curves at high and low degrees of saturation indicates that the rapidly dissociating and the slowly dissociating pools are not related to the low and the high affinity sites that are indicated by the binding data of Fig. 2.

Kinetics of Rb+ Binding.—The time course of Rb+ binding was also examined over a wide range of Rb+ concentrations. Binding was slow at all concentrations, requiring about 24 h to reach steady-state levels. Examples of the binding curves at two widely different Rb+ concentrations (5 μM and 2 mM) are shown in Fig. 4. At all Rb+ concentrations binding was multiphasic, i.e. relatively rapid initial binding followed by slower phases of binding. This is most clearly evident by the nonlinearity of the binding data as plotted on logarithmic scales in insets to Fig. 4, a and b. That these curves at such widely different Rb+ concentrations are nearly superimposable indicates that the rapid and the slow phases of the binding kinetics are also not related to the high and the low affinity sites of Fig. 2. Another aspect of the data on binding kinetics, however, is clearly related to the equilibrium binding data of Fig. 2. Even a casual comparison of the data of Fig. 4a with those of Fig. 4b is sufficient to indicate that the increase in the initial rate of Rb+ binding that is observed when Rb+ concentration is raised 400-fold (from 5 μM to 2 mM) is far too small to be consistent with the independence of the association rate constant (kass) from Rb+ concentration. This, coupled with the identical dissociation rates at all degrees of saturation (Fig. 3), indicates that kass decreases with increasing Rb+ concentration and that this is the cause of the decreasing affinity of the binding sites with increasing Rb+ concentration (Fig. 2).

Release of bound Rb+ was accelerated by 2 mM ATP. The data of Fig. 3 show that (a) the ATP effect on dissociation is the same regardless of the degree of saturation of the Rb+ binding sites, and (b) in the presence of ATP, as in its absence, the dissociation curve is not monoeponential. As indicated in legend to Fig. 3, the dissociation curve in the presence of ATP could also be resolved into two exponentials. Comparison of the characteristics of the two hypothetical pools of bound Rb+ in the presence and absence of ATP suggests that (a) ATP increases the rate of release from both pools, and (b) a major effect of ATP is to shift bound Rb+ from the slowly dissociating pool to the rapidly dissociating pool.

ATP also increased the rate of binding of Rb+ to the enzyme at all Rb+ concentrations. As examples, the effects of 2 mM ATP on the time courses of binding at 3 μM Rb+ and 200 μM Rb+ are shown in Fig. 5. Rb+ binding in the presence of ATP had a decelerating time course (Fig. 6), as it did in the absence of ATP (insets to Fig. 4). The binding plots in the presence of ATP, but not those in the absence of ATP, could be resolved into two distinct components. In the nine experiments of Fig. 6, the size of the rapidly binding component was about 60% of the total (see legend to Fig. 6).

The effects of 2 mM ATP on binding levels measured after
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Fig. 5. Effects of ATP on the rates of Rb\(^+\) binding to the native and the ouabain-complexed enzyme. Panel a, 3 \(\mu\)M Rb\(^+\): ○, native enzyme; ●, native enzyme in the presence of 2 mM ATP; △, ouabain-complexed enzyme; ▲, ouabain-complexed enzyme in the presence of 2 mM ATP. Panel b, 200 \(\mu\)M Rb\(^+\): ○, native enzyme; △, native enzyme in the presence of 2 mM ATP. Other conditions were as indicated under “Experimental Procedures.”

Fig. 6. Decelerating time course of Rb\(^+\) binding to the enzyme in the presence of 2 mM ATP. Experiments were done as in Fig. 5. \(B_{\text{max}}\) is the level of binding at 24 h, and \(B_t\) is that of binding at the indicated times. The data are means ± S.E. of nine experiments done at three different Rb\(^+\) concentrations: three experiments at 5 \(\mu\)M, three at 25 \(\mu\)M, and three at 200 \(\mu\)M. Least square regression analysis resolved the data into two exponentials: \(k_1 = 0.88 \text{ min}^{-1}\); \(k_2 = 35.8 \text{ min}^{-1}\); ratio (fast/slow) = 62/38.

24 h at various Rb\(^+\) concentrations are shown in Fig. 2. It was established that at the end of these experiments more than 95% of the ATP remained intact. At each Rb\(^+\) concentration, measured bound Rb\(^+\) in the presence of ATP was 5–18% lower than the level of bound Rb\(^+\) in the absence of ATP (Fig. 2). Considering that ATP is in contact with the enzyme during its 60-s transit time through the cation exchanger resin (see “Experimental Procedures”) and knowing the effect of ATP on the rate of Rb\(^+\) release (Fig. 3), it may easily be calculated that the apparent reduction of bound Rb\(^+\) by ATP as noted in Fig. 2 is entirely a result of the accelerated release of bound Rb\(^+\) in the course of the assay. Evidently, ATP has no significant effect on the maximal level of bound Rb\(^+\) at any Rb\(^+\) concentration.

The experiments of Fig. 7 suggested that the ATP simulation of the binding and dissociation rates were because of ATP binding at a low affinity site (\(K_{0.5} \approx 0.5 \text{ mM ATP}\)). Limited experiments with other nucleotides indicated that AMPPCP, ADP, and CTP had effects similar to those of ATP on the binding rate, but that AMP, GTP, and UTP were ineffective at the concentrations used (Fig. 7). The specificity of the nucleotide effect on the dissociation rate was similar to the nucleotide specificity for the binding rate (data not shown).

\(Na^+\) Effects on Rb\(^+\) Binding and Release—When the time course of Rb\(^+\) binding to the enzyme was studied at a fixed Rb\(^+\) concentration, the addition of \(Na^+\) reduced both the rate of Rb\(^+\) binding and the maximal level of bound Rb\(^+\) (Fig. 8). A more detailed examination of the \(Na^+\) effect on maximal binding levels at several Rb\(^+\) concentrations suggested that \(Na^+\) is a competitive inhibitor of Rb\(^+\) binding with the \(K_i\)

Fig. 7. Stimulatory effects of varying concentrations of ATP on Rb\(^+\) binding and release. The enzyme was incubated with 5 \(\mu\)M Rb\(^+\) for 24 h, and the effects of indicated ATP concentrations on release of bound Rb\(^+\) after 15 min were determined (○). In separate experiments, the enzyme was incubated with 3 \(\mu\)M Rb\(^+\), and the effects of the indicated nucleotide concentrations on binding level after 15 min were measured: ●, ATP; △, AMPPCP; ▲, ADP or CTP; □, UTP or GTP; ■, AMP. Other conditions were as described under “Experimental Procedures.”

Fig. 8. Effect of \(Na^+\) on the time course of Rb\(^+\) binding. The Rb\(^+\) concentration was 0.2 mM. Other conditions were as described under “Experimental Procedures.”

\(^1\) The abbreviation used is: AMPPCP, adenosine 5'-\((\beta,\gamma\text{-methylene})\text{triphosphate}\).
value of about 2.5 mM Na⁺ (Fig. 9).

Na⁺ increased the rate of spontaneous release of bound Rb⁺ (Fig. 10), clearly indicating the simultaneous bindings of Na⁺ and Rb⁺ to the enzyme. In the presence of Na⁺, the Rb⁺ dissociation curve remained multiphasic (Fig. 10). When experiments similar to those of Fig. 10 were done and the effects of several Na⁺ concentrations up to 100 mM on Rb⁺ release after 15 min were compared, the half-maximal stimulation of release was obtained at 2 mM Na⁺ (data not shown). The similarity of this value with the K⁺ value of Na⁺ as an inhibitor of Rb⁺ binding (Fig. 9) suggests that the apparent competitive nature of this inhibition may be caused, at least in part, by the Na⁺-induced increase in the dissociation rate constant (kₐ) of the enzyme-Rb⁺ complex.

Effects of Rb⁺ and K⁺ on Rb⁺ Release—Since the lack of effect of medium Rb⁺ on Rb⁺ release (Fig. 3) was in dramatic contrast to the stimulation of Rb⁺ release by Na⁺ (Fig. 10), the effect of the simultaneous presence of Rb⁺ and Na⁺ on Rb⁺ release was examined. An addition of 20 mM Rb⁺ to the medium was sufficient to block the stimulation of Rb⁺ release by 5 mM Na⁺ (Fig. 10). The experiments of Fig. 11 showed that this effect of Rb⁺ was exerted at a site with an apparent affinity of 1.5 mM Rb⁺. Experiments similar to those of Figs. 10 and 11 showed that K⁺ also blocked the effect of Na⁺ on Rb⁺ release with an affinity about the same as that of Rb⁺ (data not shown).

Effects of Choline and Tris on Rb⁺ Binding and Release—Because previous studies on cation-induced conformational transitions of the enzyme had indicated Na⁺-like effects of choline and Tris (9, 10), the effects of these cations on Rb⁺ binding and release were examined. Both had effects similar to those of Na⁺ and ATP in accelerating the release of bound Rb⁺. The effects of several choline concentrations on Rb⁺ release are shown in Fig. 12. The results with the same concentrations of Tris were nearly identical to those shown for choline. Stimulatory effects of choline and Tris on Rb⁺ release were blocked by the presence of unlabeled Rb⁺ in medium (data not shown). In this respect, therefore, choline and Tris resembled Na⁺ rather than ATP.

In contrast to the Na⁺ effects shown in Fig. 8, up to 50 mM concentrations of choline and Tris had no significant effects on the maximal levels of bound Rb⁺ (measured at 3 and 200 μM Rb⁺) achieved after 24 h of incubation (data not shown). Both organic cations, however, had weak stimulatory effects on the initial rate of Rb⁺ binding (Fig. 13).

Effects of Ouabain on Rb⁺ Binding and Release—Since there have been conflicting reports on whether or not K⁺ and Rb⁺ bind to the ouabain-complexed enzyme (5, 7, 11, 12), the issue was reexamined. When the time courses of Rb⁺ binding to the ouabain-complexed enzyme and the native enzyme were compared at various Rb⁺ concentrations, it became evident that ouabain did not prevent Rb⁺ binding but reduced the rate of Rb⁺ binding to the enzyme (Fig. 14).
The enzyme was incubated with 5 μM [3H]Rb⁺ in the control sucrose-histidine solution (pH 6.8) or in the same solution containing 50 mM choline chloride or 50 mM Tris-HCl (pH 6.8) or 2 mM ATP (Tris salt, pH 6.8). Bound [3H]Rb⁺ was measured after 5 min.

FIG. 13. A comparison of the stimulatory effects of ATP, Tris, and choline on the rate of Rb⁺ binding to the enzyme. Equal concentrations of the native (○, △) and the ouabain-complexed enzyme (●, ▲) were used to measure [3H]Rb⁺ binding at 5 μM Rb⁺ (panel a) and 1 mM Rb⁺ (panel b). The incubation medium was either the standard sucrose-histidine solution (○, ●) or the same solution containing 50 mM Na⁺ (△, ▲). Other conditions were as described under “Experimental Procedures.”

FIG. 14. Comparisons of Rb⁺ binding rates, and the inhibitory effects of Na⁺ on Rb⁺ binding, in the native and the ouabain-complexed enzymes. Equal concentrations of the native (○, △) and the ouabain-complexed enzyme (●, ▲) were used to measure [3H]Rb⁺ binding at 5 μM Rb⁺ (panel a) and 1 mM Rb⁺ (panel b). The incubation medium was either the standard sucrose-histidine solution (○, ●) or the same solution containing 50 mM Na⁺ (△, ▲). Other conditions were as described under “Experimental Procedures.”

The ATP stimulation of the Rb⁺ binding rate that is observed in the native enzyme was not observed in the ouabain-complexed enzyme (Fig. 5a). Inhibition of Rb⁺ binding by Na⁺, however, was obtained in both the native and the ouabain-complexed enzymes (Fig. 14b). In the experiments of Fig. 15 the characteristics of Rb⁺ release from the native and the ouabain-complexed enzyme were compared. The rate of spontaneous release was significantly lower in the ouabain-complexed enzyme. Also, the ATP-induced and the Na⁺-induced increases in Rb⁺ release that occur in the native enzyme were not observed in the ouabain-complexed enzyme.

Temperature Effects—The following limited experiments, for which data are not presented, were done to examine the effects of temperature on occluded Rb⁺. Because binding of Rb⁺ to the enzyme was too fast at 24–37 °C, we were unable to measure binding rates at these temperatures with our techniques. When the enzyme was incubated with varying concentrations of Rb⁺ at 37 °C for 15 min, cooled rapidly to 4 °C, and assayed for bound Rb⁺ at 4 °C, the resulting binding plot was the same as that shown in Fig. 2. Longer incubation periods at 37 °C did not change the shape of the plot, suggesting that at this temperature maximal binding levels were achieved within 15 min. When the enzyme was incubated at 37 °C with several different Rb⁺ concentrations in the range of 0.003–2 mM and Rb⁺ release was then measured at 4 °C, the resulting dissociation curves were identical to those shown in Fig. 3. These findings indicate that the multiple affinities of the Rb⁺ sites (Fig. 2) and the heterogeneous pools of bound Rb⁺ with different dissociation rates (Fig. 3) are not artifacts of the long incubation periods of the binding experiments at 4 °C.

DISCUSSION

Multiplicity of the Occlusion Sites—The Rb⁺ binding sites that are characterized here are those that release Rb⁺ so slowly (Fig. 3) that they remain occupied with Rb⁺ while the enzyme-Rb⁺ complex is passing through a cation exchange column for a period just long enough to separate the complex from free Rb⁺ (Fig. 1). Although the occupied sites do not exhibit uniform dissociation rates, the major affinity requirement to release their Rb⁺ to the medium at 4 °C (Fig. 3). Clearly, these sites must be located deep within the protein matrix, occluding Rb⁺ in a confined space. The data show that Rb⁺ approaches the binding sites slowly (Fig. 4), indicating that the empty sites are also confined and difficult to reach.

To determine the affinities of these sites for Rb⁺ it is necessary to allow sufficient time for equilibration of free Rb⁺ with the binding sites. When this is done, the data clearly indicate the existence of binding sites with different affinities (Fig. 2). Such data alone do not tell us whether the different affinities preexist in different populations of sites or that they are induced because of negative interactions among identical sites. Comparison of the initial rates of binding at widely different Rb⁺ concentrations (Fig. 4), however, suggests that kₐ decreases with increasing Rb⁺ concentration, indicating that the sites with higher Kₚ (kₐ/kₘ) are induced upon progressive occupancy of the binding sites.

To estimate the number of Rb⁺ binding sites/active site (the phosphorylation site), it is convenient to assume two classes of independent Rb⁺ sites and calculate their maximum capacities based on the data of Fig. 2. These calculations (see “Results”) suggest the value of 2.4 Rb⁺ sites/active site. Considering the theoretical limitations of such calculations (13), based on these data it is not possible to say whether there are two or three (or even more) Rb⁺ sites/active site. Previous
studies have concluded the existence of two (4, 8, 14, 15) or three sites (7, 16, 17). Because there have been significant variations among the assay conditions of the previous studies (see below "Comparison with Previous Findings") and in view of the limitations of our own data, we believe that the exact number of Rb⁺ occlusion sites/active site remains to be determined.

Heterogeneity of the Access Channels—At first sight it may seem that the multiplicity of the Rb⁺ occlusion sites exhibiting different affinities must be related to the rapid and the slow phases that are observed in Rb⁺ dissociation (Fig. 3) and in Rb⁺ binding (Fig. 4). That this is not the case is indicated by the identity of dissociation curves at all degrees of site occupancy (Fig. 3) and by the fact that the ratio of rapid binding phase(s) to slow binding phase(s) seems to be the same at widely different Rb⁺ concentrations (Figs. 4, a and b). The most straightforward explanation for the rapid and the slow phases of Rb⁺ release and binding is that the access channels leading to the binding sites are heterogeneous in size and that this heterogeneity coexists with the induced or preexisting differences in the binding site affinities. Put in other words, a channel of certain width may lead to a site of either low affinity or high affinity.

Ligand-induced Regulation of the Access Channels—Perhaps the most important conclusion to be drawn from these studies is that the regulation of the channels that connect the binding sites to the medium is a process distinct from the events occurring at the binding sites. This is most readily evident from the experiments with ATP. Binding of the nucleotide at a site with low affinity exerts little or no effect on Rb⁺ site affinities (Fig. 2), whereas it accelerates the release of Rb⁺ (Fig. 3) and its approach to the binding sites (Fig. 5). Evidently, all access channels are "widened" by ATP, although the channels remain heterogeneous in the presence of ATP (Figs. 3 and 6).

Na⁺ also has a significant regulatory effect on the access channels as evident by its stimulation of the rate of Rb⁺ release (Fig. 10). We suspect that the rate of approach of Rb⁺ to the binding sites is also increased by Na⁺ but that it is difficult to demonstrate this in Rb⁺ binding experiments because Na⁺ also competes for Rb⁺ at the binding sites (Figs. 8 and 9). Although the affinity of Na⁺ for the binding sites (Fig. 9) seems to be about the same as the apparent affinity of Na⁺ at the site that stimulates Rb⁺ release (Fig. 10 and "Results"), the effects of Tris and choline suggest that the Na⁺ effects are exerted at two distinct sites. Both organic cations have weak Na⁺-like effects in widening of the channels (Figs. 12 and 13) but no inhibitory effects on the equilibrium binding levels of Rb⁺ ("Results"). That there are two separate Na⁺ effects is also supported by the finding that ouabain blocks the Na⁺ effect on Rb⁺ release (Fig. 15) but does not interfere with Na⁺ inhibition of Rb⁺ binding (Fig. 14b).

A most interesting regulatory effect on the access channels to the Rb⁺-occlusion sites is that of Rb⁺ itself. Whereas medium Rb⁺ has no effect on the rate of Rb⁺ release, it blocks the stimulatory effects of Na⁺, choline, and Tris on Rb⁺ release (Fig. 10 and "Results"). The simplest explanation is that Rb⁺ competes with Na⁺ and the organic cations at the allosteric site that regulates channel width but that Rb⁺ binding to this site does not induce the same conformational transition that leads to the widening of the access channels. K⁺ also has effects similar to those of Rb⁺ at the allosteric site. These highly specific effects of K⁺ and Rb⁺ also argue against the notion that the channel stimulatory effects of Tris and choline, which are obtained at relatively high concentrations, may be simple ionic strength effects.

Our data clarify the previous uncertainties about the effects of ouabain on Rb⁺ occlusion by identifying the effects of ouabain on the access channels. First, by "narrowing" the channels ouabain slows both the approach of Rb⁺ to the binding sites and the release of Rb⁺ from the occluded sites (Figs. 14 and 15). The second effect of ouabain binding to the enzyme is the blockade of the regulatory effects of ATP and Na⁺ on the access channels (Figs. 5a and 15). Beyond these, ouabain seems to have no significant effects on Rb⁺ binding at the occluded sites or on the competitive blockade of the Rb⁺ binding to these sites by Na⁺ (Fig. 14b and "Results").

Comparison with Previous Findings—There are some apparent discrepancies between our data and previous findings on bound Rb⁺ and K⁺. Although it is not practical to discuss all of these, it is appropriate to consider an example that will point to the potential causes of such discrepancies. In previous work the shape of the plot of bound Rb⁺ or K⁺ as a function of K⁺ concentration has been reported to be either hyperbolic (4, 8, 14, 16), or positively cooperative (7, 12, 15), or with apparent negative cooperativity (17) similar to our data of Fig. 2. In addition to differences in enzyme preparations and compositions of the incubation media, in the cited studies the highest cation concentration used to obtain each binding plot has ranged from 0.1 to 10 mM; the incubation and the assay temperatures (in the range of 0-37 °C) have been the same in some studies and different in others; and the incubation time (presumably to allow maximal binding at each cation concentration) has ranged from 0.5 to 30 min. Also, in some of the cited studies occluded or bound cation has been considered as that fraction which is readily released by a nucleotide and in some, that part which is prevented by the prior binding of ouabain to the enzyme. As our data show, the use of these criteria may underestimate the amount of bound cation.

Other aspects of the present work that need to be discussed in the context of previous findings are our data on the rapid and the slow phases of Rb⁺ binding and release. Based on experiments at 20-24 °C on the rate of Rb⁺ release from the enzyme in the presence of Mg²⁺ + P, (18, 19) Forbus (2, 19) has postulated two Rb⁺ sites in tandem that release Rb⁺ in order, one rapidly and one slowly, upon enzyme phosphorylation. The relation of these two pools of Rb⁺ in the phosphorylated enzyme to the rapid and slow phases of Rb⁺ binding and release that we have identified in the unphosphorylated enzyme is not clear. Forbus (8) did notice that
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![Diagram of the K⁺ transport segment of the Albers-Post cycle.](image)

FIG. 16. The K⁺ transport segment of the Albers-Post cycle.

Rb⁺ dissociation from the unphosphorylated enzyme deviated from a monoeponential in some experiments; however, he concluded that this heterogeneity was not related to the hypothetical tandem sites in the phosphorylated enzyme (8, 19). We are now in the midst of studies on the kinetics of binding and release of Rb⁺ in the phosphorylated forms of the enzyme at 4 °C. Completion of these studies should assist in the clarification of the above uncertainties. Meanwhile, it is important to note that there is nothing in the data presented here to indicate how many occluded sites are located at the end of each access channel.

Implications for the Reaction Mechanism of the Enzyme—Because variations of the Albers-Post scheme (e.g. Ref. 1) are most commonly used to describe the reaction mechanism of the enzyme, it is appropriate that we consider the implications of our findings in its context. In this scheme, Na⁺ and K⁺ transports occur consecutively by two distinct segments of the cycle. The present studies are related to the K⁺ transport segment of the cycle which is shown in Fig. 16. In this sequence, extracellular K⁺ binds with high affinity to the E₂P conformation of the phosphoenzyme accelerating its decomposition, and forming E₂(K⁺), i.e. the dephosphorylated enzyme containing two occluded K⁺ ions which are inaccessible to the extra- and the intracellular sides. The spontaneous conversion of E₂(K⁺) to E₁(K⁺), however, binding to the inside and the binding of intracellular Na⁺ to E₁, is slow in the absence of ATP. In the sequence of Fig. 16, however, binding of ATP to a low affinity site on E₂(K⁺) accelerates the E₁ to E₂ conformational change. Compared with E₁, E₂ has a lower affinity for K⁺ and a higher affinity for ATP.

Against the above background, and with the reasonable assumption that the properties of occluded K⁺ and occluded Rb⁺ are qualitatively the same, the relation of our data to the reaction mechanism may now be summarized.

1) In a medium of low ionic strength, such as the sucrose-histidine solution used here, the enzyme is in the E₂ state (10, 20). Our experiments of Figs. 2–4, therefore, describe the properties of E₂(K⁺), suggesting that there may be more than 2K⁺ occluded per E₂ and that there are different pools of E₂(K⁺) because of heterogeneity in the size of the access channels. 2 This suggests the necessity of the inclusion of pathways with slow and rapid kinetics within the K⁺ transport sequence of Fig. 16. It is important to note that the existence of slow reacting and rapid reacting components of E₁ and E₂ in other segments of the cycle, involving phosphorylation and dephosphorylation reactions, have also been reported by Froehlich and Fendler (21) and Suzuki and Post (22); and it has been suggested (21) that these pools may be because of heterogeneity in the restricted access of ions to the binding sites perhaps through the voltage-sensitive channels that have been suggested by studies on the electrogenicity of the pump (23, 24). These findings, taken together with our data, suggest that kinetic heterogeneity may need to be introduced throughout the Albers-Post scheme.

2) As discussed already, our data show that the apparent size of the access channels, and their distribution among different pools, are allosterically regulated by the physiological ligands of the enzyme. Although an allosteric effect of ATP, albeit incomplete, is included in Fig. 16, those of Na⁺ and K⁺ are not. Skou and Esmann (10, 25) also identified allosteric effects of Na⁺ and K⁺ on E₂ to E₁ conversion as detected by eosine fluorescence; Klodos and Ottolenghi (26) detected allosteric effects of Na⁺ on E₂ to E₁ conversion by phosphorylation studies; and Robinson and Pratap (27) described choline effects on enzyme activity that can best be explained by assuming an allosteric Na⁺ effect on the rate of K⁺ deocclusion. Recognition of the existence of these allosteric effects on the access channels leads to the conclusion that the kinetic heterogeneity of the reaction mechanism as discussed in the previous section must be regulated by the physiological ligands of the enzyme. Since it is established that kinetic heterogeneity of a pathway may indeed cause cooperativity (28), regulated changes in the heterogeneity would be expected to affect this cooperativity. It is tempting, therefore, to speculate that ligand-induced regulation of the ratio fast phase to slow phase in the various segments of the Albers-Post cycle may be related to the 3Na⁺/2K⁺ stoichiometry of the Na⁺/K⁺ exchange and the flexible stoichiometry of the Na⁺/Na⁺ exchange (29).

3) Our findings on the effects of ATP indicate that some of the common assumptions about reactions 3–5 of Fig. 16 may not be justified. According to this scheme, ATP accelerates E₁ to E₂ conversion, and the acceleration of K⁺ release follows because E₂ has much lower affinity than E₁ for at least one of the two K⁺ ions (4, 20). Our data show the existence of an E₂K⁺ ATP species that releases (and binds) K⁺ more rapidly than E₂(K⁺) but cannot be called E₁ because its binding sites for K⁺ have the same affinities as those of E₂(K⁺). The problem can be resolved in part by designating this species as E₂K⁺ATP, and inserting it at step 4 to precede E₂K⁺ATP. But since the affinity of ATP for E₁ is about 3 orders of magnitude higher than its affinity for E₂, why is it that the high concentrations of ATP used in our experiments of Fig. 2 do not cause significant reductions in the steady-state levels of bound K⁺ (Rb⁺)? Evidently, the conversion of E₂K⁺ATP to E₂K⁺ATP is blocked. Perhaps the conversion requires an allosteric effector (Na⁺?) that is not included in Fig. 16 and is not present under our experimental conditions of Fig. 2. The alternative is that E₂K⁺ATP is never converted to
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$E_K$ - ATP because $E_1$ and $E_2$ are in fact two distinct coexisting entities as suggested by studies of Schoner’s laboratory (30, 31). In this case, the $K^+$ transport (outside to inside) segment of the cycle ends on $E_2$, and the Na$^+$ transport segment begins on $E_1$, with the continuity of the cycle being provided by physical contact and interactions between $E_1$ and $E_2$.

Structural Bases of the Allosteric Interactions — The abundant evidence indicating that (Na$^+$ + K$^+$)-ATPase is an oligomer of $\alpha$-heterodimers and that there are cooperative interactions among the ligand binding sites of the identical or quasi-identical protomers was discussed by one of us in 1988 (32). Since then a good deal of additional work, using a variety of different experimental approaches, has provided further evidence in support of the oligomeric structure of the membrane-bound enzyme and the functional consequences of subunit interactions (30, 31, 33–37). As in most other multimeric assemblies, it is reasonable to assume that changes in free energies of subunit interactions are linked to changes in binding energies of multiple ligand binding sites of this protein and in the regulation of its function. It is evident from the present results, however, that regulation of pump function may also be achieved by allosteric interactions among ligand binding sites and different population of channels that are free energy barriers to the approach of ligands to their binding sites. That the two distinct allosteric mechanisms may be operating simultaneously under certain experimental conditions suggests that the two may be exerted at different hierarchies of structure, i.e. one may be nested within the other (38). Since several transmembrane helices of the $\alpha$-chain seem to be involved in cation occlusion (39), perhaps one mechanism is at the level of intrasubunit helix-helix interaction.

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