Are the States That Occlude Rubidium Obligatory Intermediates of the \( \text{Na}^+/\text{K}^+\)-ATPase Reaction?*

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In the Albers-Post model, occlusion of K\(^+\) in the \( E_2 \) conformer of the enzyme (\( E \)) is an obligatory step of \( \text{Na}^+/\text{K}^+\)-ATPase reaction. If this were so the ratio (\( \text{Na}^+\)/\( \text{K}^+\)-ATPase activity)/(concentration of occluded species) should be equal to the rate constant for deocclusion. We tested this prediction in a partially purified \( \text{Na}^+/\text{K}^+\)-ATPase from pig kidney by means of rapid filtration to measure the occlusion using the K\(^+\) congener Rb\(^+\). Assuming that always two Rb\(^+\) are occluded per enzyme, the steady-state levels of occluded forms and the kinetics of deocclusion were adequately described by the Albers-Post model over a very wide range of [ATP] and [Rb\(^+\)]. The same happened with the kinetics of ATP hydrolysis. However, the value of the parameters that gave best fit differed from those for occlusion in such a way that the ratio (\( \text{Na}^+\)/\( \text{K}^+\)-ATPase activity)/(concentration of occluded species) became much larger than the rate constant for deocclusion when [Rb\(^+\)] <10 mM. This points to the presence of an extra ATP hydrolysis that is not \( \text{Na}^+\)-ATPase activity and that does not involve occlusion. A possible way of explaining this is to posit that the binding of a single Rb\(^+\) increases ATP hydrolysis without occlusion.

The active transport of \( \text{Na}^+ \) and K\(^+\) across cell membranes is mediated by the \( \text{Na}^+/\text{K}^+\)-pump, which is an intrinsic membrane transport system, identical with the \( \text{Na}^+/\text{K}^+\)-ATPase (for a recent review and references see Ref. 1). There is abundant evidence (2–9) that during the pump cycle and under adequate conditions, K\(^+\) or its congeners become bound to the \( \text{Na}^+/\text{K}^+\)-ATPase in such a way that their release is slow, even if the enzyme that holds them is exposed to cation exchange resins (3) or submitted to extensive washings (6, 9). This type of binding is called occlusion because it is believed that the ions are trapped in a domain of the enzyme that restricts their access to the bulk of the solvent. Occlusion of K\(^+\) through the direct route (where, presumably, K\(^+\) enters the enzyme from the cytoplasmic side) can be obtained by just mixing enzyme and K\(^+\) in the absence of \( \text{Na}^+ \) and ATP and was first described by Beaugé and Glynn (3). Since then, the properties of the species occluded through this process have been studied extensively (see reviews by Forbush (7), Glynn and Richards (10), and Glynn and Karlish (11)). The release of K\(^+\) or its congeners from their occluded states (deocclusion) is considerably accelerated by binding of ATP at a site with low affinity relative to the active site and where ATP does not seem to undergo hydrolysis (6, 12). This process is the cause of most of the increase in \( \text{Na}^+\)/\( \text{K}^+\)-ATPase activity with increasing ATP concentration (13). Occlusion of Na\(^+\) has also been detected (5, 14), and there is evidence that the occlusion site for Na\(^+\) is in the same domain but in a different conformation, as the K\(^+\) site (7, 15). Na\(^+\) occlusion seems to occur at a stage of the ATP hydrolysis cycle that is different from that which occludes K\(^+\), suggesting that occlusions of the two cations are mutually exclusive events.

In the present study we expand the information about the \( \text{Na}^+\)/\( \text{K}^+\)-ATPase reaction, using the congener \(^{86}\text{Rb}^+\) as a probe for K\(^+\) and performing direct measurements of the steady-state concentration levels of the intermediates containing occluded Rb\(^+\) (Rb\(_{\text{occl}}\)). In these experiments Rb\(_{\text{occl}}\) was formed through the so-called physiological route, i.e. during the turnover of the \( \text{Na}^+\)/\( \text{K}^+\)-ATPase. In this condition K\(^+\) enters the enzyme presumably from the extracellular side to activate the catalysis of the hydrolysis of ATP (16). Brief reports of measurements of this type of occlusion have been given by Glynn and Richards (4), Glynn et al. (5), and Forbush (6). Rossi and Nerby (9) have studied the pre-steady-state kinetics of Tl\(^+\) occlusion via the physiological route using a similar technique as that in the present paper.

The measurements we are reporting here were made possible by the development of a new technique (see “Experimental Procedures” and Ref. 17) for the isolation in the millisecond time scale and quantitative determination of the intermediates containing occluded cations. The method is used during both steady-state turnover and in transient state experiments with the \( \text{Na}^+\)/\( \text{K}^+\)-pump. With the same enzyme preparations and under the same experimental conditions, we have also measured the steady-state ATPase activity, \( v_i \), and the rate coefficient, \( k_{\text{deoccl}} \), for deocclusion of Rb\(^+\) from Rb\(_{\text{occl}}\). These can be analyzed as part of the Albers-Post model; a simplified version is shown in Fig. 1.

The results presented here show that the kinetics of the species containing occluded Rb\(^+\), i.e. \( E_{\text{occl}} \) and \( E_{\text{occl}}\text{ATP} \), could be quantitatively described by the scheme in Fig. 1. We showed that the intermediates holding occluded Rb\(^+\) were only kinetically obligatory for the ATPase reaction at saturating concentrations of Rb\(^+\). We found, however, that even at concentrations of Rb\(^+\) that were sufficiently high as to drive the reaction totally away from the pathway that generates \( \text{Na}^+\)-ATPase activity, a significant part of the hydrolysis bypassed the oc-
steady state the overall rate (the terminal phosphate of ATP to the enzyme. Occluded RbE enzyme forms with ATP. k_{b,i} is the conformer of the enzyme that binds noncovalently ATP to the active site to form the enzyme-ATP complex, E_{occ}. E_{ATP} and E_{P} are two conformational states of a phosphoenzyme formed by the covalent binding of the terminal phosphate of ATP to the enzyme. Occluded Rb is held in enzyme forms with (E_{occ-ATP}) and without (E_{occ}) noncovalently bound ATP. K_{diss} is the equilibrium constant for the dissociation of ATP from E_{occ-ATP}, and K_{1b} and K_{2b} are the equilibrium constants for the dissociation of Rb from E_{PRb} and E_{PRb_2}, respectively. The lower-case ks are rate constants for the elementary steps indicated by the neighboring arrows. For the analysis of the behavior of the model, Na\(^+\)-ATPase will be considered as the activity that takes place through the dephosphorylation of E_{P}. As [Rb\(^+\)] increases, Na\(^+\)-ATPase activity tends to 0, whereas the reaction is shifted toward pathways involving the dephosphorylation of E_{PRb} and E_{PRb_2} (Na\(^+\)/Rb\(^+\)-ATPase activity). Only the dephosphorylation of E_{PRb_2} leads to the formation of E_{occ}.

**Substrate(s) \( \rightarrow \) A \( \rightarrow \) B \( \rightarrow \) Product(s)**

**Scheme 1**

cluded forms. We show that a possible reason for this is that the phosphoenzyme containing only one Rb\(^+\) (E_{PRb}) dephosphorylates, without leading to occlusion of Rb\(^+\), with a rate coefficient whose value lies between those for dephosphorylation of E_{P} and E_{PRb_2}.

At this point, it seems convenient to include the theoretical grounds that underlie the experiments in which we tested whether the enzyme species containing occluded Rb\(^+\) are obligatory intermediates in the reaction cycle.

To illustrate this, let us take the elementary reaction A \( \rightarrow \) B in Scheme 1 as one of the steps of the enzyme reaction. The possibility that the step A \( \rightarrow \) B can be bypassed with the rate \( v_{\text{other}} \) during the overall reaction is important for the argument to follow, whereas the order of addition of substrate(s) and the release of product(s) are not.

If \( n \) pathways participate in the A \( \rightarrow \) B transition, then in steady state the overall rate \( (v_i) \) of the reaction catalyzed by the enzyme can be written as Equation 1.

\[
v_i = \sum_{i=1}^{n} k_{f,i}[A] - \sum_{i=1}^{n} k_{b,i}[B] + v_{\text{other}} \quad (\text{Eq. 1})
\]

where \( A \) and \( B \) are each of the \( n \) states of \( A \) and \( B \), and \( k_{f,i} \) and \( k_{b,i} \) are the rate constants for their transitions. If the conversion of \( A \) to \( B \) were irreversible, then \( \sum k_{b,i} [B] = 0 \), and Equation 1 is reduced to Equation 2,

\[
v_i = \sum_{i=1}^{n} k_{f,i}[A] + v_{\text{other}} \quad (\text{Eq. 2})
\]

which, dividing both sides by \( [A] = \Sigma [A] \) becomes Equation 3:

\[
v_i = \sum_{i=1}^{n} \frac{k_{f,i}[A]}{[A]} + \frac{v_{\text{other}}}{[A]} \quad (\text{Eq. 3})
\]

Equation 3 shows that the ratio between the steady-state overall velocity, \( v_i \), and the total concentration, \( [A] \), of the intermediates at this step will be a linear, weighted combination of the rate constants involved in their transformation, plus a term related to alternative reaction pathways, \( v_{\text{other}}/[A] \).

Equations 1–3 provide a criterion to test if a family of intermediates (here, \( A_S \)) are obligatory participants of the enzyme reaction. If this were the case, then \( v_{\text{other}} \) would be 0 and the ratio \( v_i/[A] \) would be equal to the rate coefficient for the reaction \( A \rightarrow B \).

The experimental investigation of this criterion will be in many cases hampered by difficulties in the measurement of the concentration of the enzyme intermediates and in the direct determination of the rate constants of their reactions. In the case of the intermediates that hold occluded K\(^+\) or its congeners, these difficulties can be circumvented because newly available procedures (Refs. 9 and 17 and “Experimental Procedures” of this paper) make it possible to estimate with accuracy their steady-state levels and to follow the time courses of their release of occluded Rb\(^+\).

The ratio between steady-state ATPase activity \( (v_i) \) and the total concentration of the conformer(s) of the enzyme holding the occluded Rb\(^+\), \( [E_{occ}] = [E_{occ}] + [E_{occ-ATP}] \), would be given by (see Fig. 1 and Equation 3) Equation 4:

\[
v_i = \frac{v_{occ}}{[E_{occ}]} - \frac{v_{other}}{[E_{occ}]} \quad (\text{Eq. 4})
\]

where \( v_{occ} \) is the activity through the occlusion producing pathway, and under irreversibility conditions, it can be calculated as the sum of the rates of the deocclusion pathways as shown in Equation 5:

\[
v_{occ} = k_{1}[E_{occ}] + k_{2}[E_{occ-ATP}] \quad (\text{Eq. 5})
\]

From the definition of \( [E_{occ}] \) and considering that ATP binds to \( E_{occ} \) to form \( E_{occ-ATP} \) with an apparent dissociation constant \( K_{ATP} = [ATP]/[E_{occ}]/[E_{occ-ATP}] \), Equation 4 becomes Equation 6:

\[
v_i = \frac{k_{1}K_{ATP} + k_{2}[ATP]}{K_{occ}} + \frac{v_{other}}{[E_{occ}]} \quad (\text{Eq. 6})
\]

A well known example of the eventual contribution to \( v_i \) of the pathways that do not include \( E_{occ} \), \( v_{other} \) in Equation 6 is the Na\(^+\)-ATPase activity of the Na\(^+\)/K\(^+\)-ATPase (see e.g. Ref. 13). In the absence of Rb\(^+\), hydrolysis of ATP proceeds with a rate that is about 5–10% of \( V_{\text{max}} \) for Na\(^+\)/Rb\(^+\)-ATPase. Referring to Fig. 1 and the conditions of the present study, this rate will be \( v_{other} = v_i = k_{40} \times [E_{P}] \) and almost all the enzyme will be in the form of \( E_{P} \). At subsaturating concentrations of Rb\(^+\) the species \( E_{PRb} \) is also present, and \( v_{other} \) must be expressed as Equation 7:

\[
v_{other} = k_{42}[E_{P}] + k_{44}[E_{PRb}] \quad (\text{Eq. 7})
\]
At saturating Rb⁺ concentrations, ν_{oc} = 0 because both [E_P] and [E_P,Rb] become 0, and all the reaction flux proceeds through the formation and breakdown of the occluded forms. Under this condition (see Equation 8),

$$\frac{v_t}{v_{oc}} = \nu_{oc} \frac{k_{ATP} + k_{c[ATP]} + k_{c[ATP]}}{k_{ATP} + k_{c[ATP]}} \quad (\text{Eq. 8})$$

Thus, at saturating [Rb⁺]⁻, ν_{oc}/v_{oc} as a function of [ATP] should be a rectangular hyperbola, increasing from k₄ to k₅ as [ATP] goes from 0 to ∞, respectively, with half-maximal effect for [ATP] = K_{ATP}. Note that K_{ATP} will be larger than or equal to the equilibrium constant for the dissociation of ATP from E₃,ATP, depending on whether rapid equilibrium holds or not for this reaction.

A preliminary report of some of the results given here has been published (18).

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**Experimental Procedures**

**Enzyme—**Na⁺/K⁺-ATPase was partially purified from pig kidney (19). The specific activity at the time of preparation was 23–25 (μmol Pi) min⁻¹, measured under optimal conditions (150 mM NaCl, 20 mM KCl, 3 mM ATP, and 3 mM MgCl₂, 25 mM imidazole HCl, pH 7.4, at 37 °C). This corresponds to an ADP-binding site concentration of 2.4–2.7 μmol (mg of protein)⁻¹.

**Reagents and Reaction Conditions—**[γ-³²P]ATP was synthesized using the procedure of Glynn and Chappell (20), except that no unlabeled orthophosphate (Pᵢ) was added. Carrier-free [³²P]Pi, was from the Comisión Nacional de Energía Atómica (Argentina).

[³²Rb]RbCl was from NEN Life Science Products. ATP, enzymes, and reagents for the synthesis of [γ-³²P]ATP were from Sigma. All other reagents were of analytical grade.

All incubations were performed at 25 °C in media containing 150 mM NaCl, enough MgCl₂ to give a final concentration of free Mg²⁺ of 0.5 mM, 25 mM imidazole–HCl, pH 7.4 (at 25 °C), and the amounts of RbCl and ATP indicated for each experiment. In all cases before starting a reaction, the components to be mixed were diluted in these media.

**Determination of ATPase Activity—**ATPase activity was determined according to Schwarzbaum et al. (21), measuring the amount of [³²P]Pi released from [γ-³²P]ATP. The enzyme concentration was never lower than 4 μg/ml in order to approach that used in the determination of occluded rubidium ions. For this reason incubation times were short (between 30 s and 1 min) to avoid the hydrolysis of more than 10% of the ATP during the assay and to ensure initial rate conditions. Control experiments showed that activity was independent of the enzyme concentration from 4 to 200 μg/ml and that it was unaffected by the passage of the enzyme through the rapid mixing apparatus described below. Na⁺/Rb⁺- and Na⁺/ATPase activities were calculated as the difference between total activity and the activity either in the same media but with 1 mM ouabain or in media in which all the Na⁺ was replaced by K⁺. The latter procedure was applied when the reaction times were less than 1 min because at high [Rb⁺⁻], the onset of the inhibition by ouabain is not fast enough to ensure complete inhibition. The inorganic phosphate detected under these conditions was practically identical to that measured in the reagent blanks indicating that the concentration of ATPases other than the Na⁺/K⁺-ATPase was negligible.

**Determination of Phosphorylated Intermediates—**This was performed according to Schwarzbaum et al. (21).

**Determination of Rubidium Occlusion—**The technique was similar to that developed by Rossi and Narby (9) except that [³²Rb]⁻ was used instead of [³²O]⁻ and by Rossi et al. (17). The arrangement that is described in detail in Ref. 17 is shown in (Fig. 2). It includes a rapid mixing apparatus (RMA) connected to a quenching-and-washing chamber (QWC) through a suitable polyethylene tubing. Reactions took place at 25 °C in the RMA and are quenched after the appropriate time by injecting the reaction mixture into the QWC at a flow rate of 1–5 ml s⁻¹. During the injection process, the fluid was mixed with an ice-cold washing solution flowing at a rate of 30–40 ml/s and then filtered through a Milipore filter (AA, 0.8 μm pore size) placed in the QWC in

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**TABLE I**

| Quantity | Equation | Eq. No. |
|----------|----------|---------|
| | | |
| v_{oc} | | |
| | | |
| D | | |
| | | |
| D_{1,0} | | |
| | | |
| D_{1,1} | | |
| | | |
| D_{1,2} | | |
| | | |

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**Fig. 2.** A scheme of the arrangement used to measure occluded Rb⁺. S1–S4, syringes 1–4; M1–M3, mixers 1–3; DL1–DL3, delay lines. DL3 is an external tube that has the additional function of transferring the reaction mixture from the RMA into the QWC. The rapid mixing apparatus was an SFM4 from Bio-Logic (France), with four syringes, each of its plungers being driven individually by a stepped motor. This allows us to perform precise and accurate dilution in the machine by changing the relative velocity of the plungers (mixing ratios). Additionally, the flow can be interrupted for several seconds (or minutes) to permit the evolution of the reactions in the delay lines. The RMA was thermostated at 25 °C. The QWC is made of Plexiglas and consists of two pieces, the chamber piece and the funnel piece. The funnel piece holds a 55-mm-diameter Millipore filter. A spherical joint pinch clamp keeps the chamber and funnel pieces together, with the Millipore filter sandwiched in between, providing the sealing of the chamber. The pressure difference (about 745 mm Hg) driving the flow through the filter is created by a water-jet pump. A detailed description of the QWC and its properties has been published separately (17).

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1 The abbreviations used are: RMA, rapid-mixing apparatus; QWC, quenching-and-washing chamber.
order to retain the enzyme-containing membrane fragments. To ensure that the initial temperature in the QWC was 1–2 °C and that the flow was constant, about 50 ml of washing solution was allowed to run through the filter prior to the injection of the reaction mixture, and 240 ml of washing solution was applied to the filter from that moment. The composition of the washing solution was 50 mM KCl, 20 mM imidazole-HCl, pH 7.4 at 0 °C. The procedure is devised to slow down instantaneously the reactions by means of the sudden drop in both the temperature and the concentration of ligands, like ATP, that might accelerate deocclusion. The quenching time was 3–4 ms, even when the reactions were performed in media with 2.5 mM ATP (17). Furthermore, the deocclusion. The quenching time was 3–4 ms, even when the reactions were performed in media with 2.5 mM ATP (17). Furthermore, the washing effectively removes the unbound \(^{86}\text{Rb}^+\). After the washing solution was drained, the filter was removed, dried under an incandescent lamp, and counted for radioactivity in a \(\beta\)-scintillation counter.

Blanks were estimated from the amount of radioactive \(^{86}\text{Rb}^+\) retained by the filters in experiments where occlusion was prevented by omitting ATP during the enzyme reactions. Otherwise the conditions were the same. As shown by Rossi et al. (17), the blanks were linearly related to the \(^{86}\text{Rb}^+\) concentration from 0 to 10 mM; they rarely exceeded 10% of the amount of occluded \(^{86}\text{Rb}^+\) (and generally were much smaller) and were independent of the mass of enzyme employed.

Determination of Steady-state Levels and the Rate of Release of Occluded Rubidium Ions—The steady-state amount of occluded \(^{86}\text{Rb}^+\) was estimated by mixing equal volumes of a suspension of \(\text{Na}^+/\text{K}^+\)-ATPase in a buffer containing 150 mM NaCl, 0.5 mM free Mg\(^{2+}\), 2.5 mM imidazole-HCl, pH 7.4 (at 25 °C), with the same buffer containing ATP and \(^{86}\text{Rb}^+\). Generally, the reaction mixture was incubated for 3 s before being injected into the QWC. Control experiments using aging times of 5–10 s showed no variation in the level of occluded rubidium with respect to the results obtained at 3 s. To measure the rate of release of occluded \(^{86}\text{Rb}^+\), \(\text{Na}^+/\text{K}^+\)-ATPase containing occluded rubidium ions was formed as described above. It was then mixed with enough of a similar solution, but lacking \(^{86}\text{Rb}^+\), to cause a 20-fold isotopic dilution of \(^{86}\text{Rb}^+\). After the appropriate aging times, the resulting mixture was injected into the QWC, and the occluded \(^{86}\text{Rb}^+\) remained in the enzyme was measured as described above. To attain the isotopic dilution of \(^{86}\text{Rb}^+\), 1 volume of \(^{86}\text{Rb}^+\)-containing enzyme was mixed either with 20 volumes of a solution with unlabeled \(^{86}\text{Rb}^+\) at the same concentration or with 1 volume of a solution with 20 times higher concentration of unlabeled \(^{86}\text{Rb}^+\). Both procedures gave similar results for the rate of deocclusion, provided that the rest of the conditions remained unchanged. Note that 150 mM NaCl was present in all solutions.

Steady-state Solutions for the Model in Fig. 1—These were obtained using the Solve routine of Mathematica, version 2.2.1 for Windows®.

Data Analysis by Nonlinear Regression—Where indicated, results of steady-state ATPase activity and steady-state occluded rubidium were adjusted by nonlinear regression using commercial programs (Excel 5.0 for Windows and Sigma-Plot 2.0 for Windows). Equations were derived from the solutions for \([E_{\text{occ}}]\) and \(v_1\) in Table I (Equations 9–11). These are shown in Equations 18 and 19.

\[
\text{Rb}_{\text{occ}} = \frac{a_1[Rb]^2 + a_2[Rb][ATP]}{d_0 + d_1[Rb]^2 + d_2[Rb][ATP] + d_3[ATP][Rb^+][ATP][Rb^+] + [ATP][Rb^+]} \tag{Eq. 18}
\]

for occluded rubidium and

\[
v_1 = \frac{b_0 + b_1[Rb]^2 + b_2[ATP] + b_3[ATP][Rb^+][ATP][Rb^+]}{d_0 + d_1[Rb]^2 + d_2[Rb][ATP] + d_3[ATP][Rb^+][ATP][Rb^+]} \tag{Eq. 19}
\]

for ATPase activity. Notice that the mathematical form of these equations corresponds to that of Equations 9 and 10, respectively, after dividing both numerator and denominator by \(D_{\text{oc}}\) and rearranging the numerators by lumping \(E_p\) with rate and equilibrium constants into coefficients \(a_1\) and \(b_1\). Additionally, in Equation 18 it is assumed that \(Rb_{\text{occ}}\) is strictly proportional to \([E_{\text{occ}}]\), i.e., that rubidium is occluded into the enzyme with a fixed stoichiometry (cf. Equation 9).

After inspection of duplicate errors in large samples of data, we used statistical weights \(1/s^2\) for \(v_1\) and for \(Rb_{\text{occ}}\). When values of parameters were nonsignificantly different from 0 (i.e., whose mean ± S.E. interval included 0), their corresponding terms were eliminated from the equation, giving rise to equations with less number of parameters. To evaluate the significance of eliminating parameters and choose among different equations, Akaike Information Criterion (AIC = \(N \times \ln(S) + 2 \times P\), with \(N = \) number of data, \(P = \) number of parameters, and \(S = \) sum of weighted square errors of residuals) (22) was applied. The best fitting equation was considered the one that showed the lowest AIC value.

Results

The present paper investigates the role of occluded \(^{86}\text{Rb}^+\) (acting as a congener of \(^{39}\text{K}^+\)) in the reaction mechanism of \(\text{Na}^+/\text{K}^+\)-ATPase. Our fast quenching procedure with a time resolution of a few milliseconds has allowed us to determine quantitatively the concentration of occluded \(^{86}\text{Rb}^+\) (\(\text{Rb}_{\text{occ}}\)) formed via the physiological route, that is by the \(^{86}\text{Rb}^+\)-activated dephosphorylation of the phosphoenzyme \(E_p\). We have used the procedure to measure the steady-state level of \(\text{Rb}_{\text{occ}}\) and the rate of deocclusion of \(^{86}\text{Rb}^+\) at 25 °C. We compared these measurements with estimations of steady-state \(\text{Na}^+/\text{K}^+\)-ATPase activity carried out in parallel experiments under exactly the same reaction conditions. To complete the information, we also measured the \(\text{Na}^+/\text{K}^+\)-ATPase activity in steady state. In all the experiments, [NaCl] was 150 mM, [free Mg\(^{2+}\)] was 0.5 mM, and the pH was kept at 7.4 with 20 mM imidazole HCl. The concentrations of \(^{86}\text{Rb}^+\) and ATP depended on each experiment and are given in the legend of each figure.

The results were analyzed using the scheme in Fig. 1, which shows in more detail that part of the Albers-Post model involved in the formation and breakdown of the intermediates of the \(\text{Na}^+/\text{K}^+\)-ATPase that are supposed to hold occluded \(^{86}\text{Rb}^+\). As it is clear from the inspection of Fig. 1, the amount of enzyme containing occluded \(^{86}\text{Rb}^+\) (\(E_{\text{occ}}\) and \(E_{\text{occ}}\text{ATP}\)) is a function of the concentrations of \(^{86}\text{Rb}^+\) and ATP. The effect of ATP is 2-fold since the nucleotide not only provides the phosphate of the phosphoenzyme intermediates, but it also accelerates deocclusion by binding to \(E_{\text{occ}}\). Table I shows the equations generated by the analytical solution of the scheme in Fig. 1, for the particular conditions of our experiments, and the meaning of each of its coefficients in terms of rate and/or equilibrium constants. By using the equations for \([E_{\text{occ}}]\) and \(v_1\) in Table I, the overall ratio \(v_1/[E_{\text{occ}}]\) will be as indicated in Equations 20 and 21:

\[
v_1 = \frac{k_dK_{ATP} + k_{d1}[ATP]}{[E_{\text{occ}}]} \tag{Eq. 20}
\]

\[
f([Rb^+]) = \frac{k_d[Rb^+]^2}{k_dK_{ATP}K_{B} + k_{d1}[ATP][Rb^+] + k_{d2}[Rb^+]} \tag{Eq. 21}
\]

It is obvious that for saturating \([Rb^+]\), i.e., \([Rb^+] \to \infty, f([Rb^+]) = 1\), and Equation 20 is then identical with the general Equation 8. At subsaturating \([Rb^+]\), \(f([Rb^+]) < 1\), signifying that \(v_1\) either >0 under these conditions.

The Steady-state Concentration of Occluded \(^{86}\text{Rb}^+\) (\(\text{Rb}_{\text{occ}}\))—\(\text{Rb}_{\text{occ}}\) was measured during steady-state hydrolysis in three series of experiments. First, it was determined within a broad range of ATP (10–960 \(\mu\)M) and \(^{86}\text{Rb}^+\) (0.1–2.5 mM) concentrations with the results shown in Fig. 3, A and B. Second, the same measurements were focused in the low ATP concentration range (1–10 \(\mu\)M, Fig. 4) to cover in more detail this region. These experiments were performed in media with 0.5–20 mM \(^{86}\text{Rb}^+\) and allowed us to obtain reliable, extrapolated values for \(\text{Rb}_{\text{occ}}\) when [ATP] \(\to \infty\). The results of these experiments are shown in Fig. 5.
Table II, column 3 or 4, respectively. All the equilibrium constants and the maximal amount of $R_{bocc}$, $R_{bocc(max)}$, were fitted by nonlinear regression.

As shown in Fig. 3A for concentrations of ATP above 10 $\mu$M, $R_{bocc}$ increased with $[\text{Rb}^+]$ tending to saturation along sigmoidal curves. The dotted and continuous curves are solutions for the scheme in Fig. 1 with the parameters given in Table II, columns 3 and 4, respectively, adjusting only the values of $R_{bocc(max)}$. The lower curve belongs to 0.5 mM $\text{Rb}^+$, the upper to 20 mM $\text{Rb}^+$.

The rate constant between the two sets of parameters lies mainly in the value of $k_1$, which is taken as equal to $k_{3o}$ for drawing the dotted line and larger than $k_{3o}$ for drawing the continuous lines (the reason for checking both alternatives will be explained below).

When plotted as a function of [ATP] (Fig. 3B), it becomes apparent that $R_{bocc}$ decreases as [ATP] rises tending to constant positive values (see Fig. 5 for similar but more extended data with another enzyme preparation). The fact that $R_{bocc}$ does not tend to 0 as [ATP] → ∞ indicates that the rate of deocclusion does not increase indefinitely with [ATP] but rather that it reaches a value comparable to that of at least one of the other elementary steps in the reaction sequence.

For concentrations of ATP between 1 and 10 $\mu$M and of $\text{Rb}^+$ between 0.5 to 20 mM, $R_{bocc}$ remained approximately constant at a value that is close to that of maximal occlusion (Fig. 4). This is expected from the properties of the model in Fig. 1 because, under these conditions (see Table II), the rate of occlusion is much higher than the rate of deocclusion. The agreement between the model and the experiments is shown by the good fit to the data points of the dotted and continuous lines in Fig. 4, which were calculated using the constants in Table II only adjusting the value for $R_{bocc(max)}$.

In the media with the concentration of NaCl (150 mM) used in our experiments, no occlusion of $\text{Rb}^+$ is detectable in the absence of ATP, so that no $R_{bocc}$ is formed by the direct route described by Glynn and Richards (Ref. 4 and see under “Experimental Procedures†). Therefore, all our curves of $R_{bocc} = \beta([\text{ATP}])$ should start at 0 when [ATP] is 0, pass through a maximum, and finally decrease toward constant values as the acceleration of deocclusion by high [ATP] reaches saturation. The fact that the rising phase of the $R_{bocc}$ versus [ATP] curves is not noticeable in Figs. 3B and 4 indicates that, even at the lowest concentration of ATP used (1 $\mu$M), the velocity of formation of the $\text{Rb}^+$-sensitive phosphoenzyme ($E_2P$) was not rate-limiting. Moreover, the fact that in Fig. 4 $R_{bocc}$ does almost not decrease with [ATP] indicates that for concentrations of the nucleotide up to 10 $\mu$M its accelerating effect on deocclusion was insufficient to affect significantly the steady-state level of $R_{bocc}$.
4, and 5 can be seen that the curves give an adequate description of the experimental results, indicating that models like those in Fig. 1 are able to predict the response of steady-state Rbocc to a wide range of ATP and Rb⁺ concentrations. The simulation in Fig. 3 was obtained assuming a fixed stoichiometry of occlusion. The numerical value of this stoichiometry was determined independently by measuring the ratio between the amount of occluded rubidium (Rbocc) and the maximum amount of occluded rubidium used in Table I to fit the results in Figs. 3–6.

The values for kdeocc measured by means of experiments like those in Fig. 7 in media with either 1, 5, or 10 μM ATP, are plotted as a function of [ATP] in Fig. 8A, together with the values of v(1/[Eocc]) calculated from the data in Figs. 6A and 4. Since [Eocc] was nearly constant and v was a linear function of [ATP] for each given Rb⁺ concentration, the plots of v(1/[Eocc]) against [ATP] are straight lines. Linearity with [ATP] is expected both for the ratio v(1/[Eocc]) and for kdeocc, since in these experiments [ATP] ≪ KATP (see Equations 6 and 20). Results in Fig. 8A show that when [Rb⁺] was less than 10 mM, the plots of v(1/[Eocc]) versus [ATP] have larger intercepts and perhaps slightly larger slopes than the plot of kdeocc, but that the differences tend to disappear at 10 and 20 mM Rb⁺. At these Rb⁺ concentrations the experimental points for v(1/[Eocc]) become very similar to those for kdeocc. The ordinate intercept of the continuous line that fits kdeocc is 0.132 ± 0.010 s⁻¹. This value corresponds to kdeocc, whereas the ordinate intercepts of the plots of v(1/[Eocc]) = f([ATP]) — curves correspond to kdeocc + vother/[Eocc] or kdeocc/1/[f([Rb⁺])] (see Equations 6 and 20, respectively).

Fig. 8B shows a plot of v(1/[Eocc]) vs. [ATP]₀, i.e., of the values of the ratio extrapolated to [ATP] = 0, against the concentration of Rb⁺. It can be seen that the ratio decreases as [Rb⁺] increases, from a value that is about 6 times higher than kdeocc at 0.5 mM Rb⁺ to values that are not significantly different from kdeocc at [Rb⁺] ≥ 10 mM. Thus, at very low [ATP]₀, only if [Rb⁺] is higher than 10 mM, vother = 0 and f([Rb⁺]) = 1 (Equations 6 and 20), and Eocc can be considered an obligatory intermediate for the ATP hydrolysis by Na⁺/[Rb⁺]-ATPase. The fact that v(1/[Eocc])/[ATP]₀ > kdeocc at [Rb⁺] < 10 mM is strong evidence for the existence of a route for ATP hydrolysis that does not pass through Eocc. As it will be shown in the following section, this feature is also apparent at high ATP concentrations.

We will turn now to the experiments performed at higher ATP concentrations. In these, the ATPase activity, v, and the
**Rb⁺ Occlusion during Na⁺/K⁺-ATPase Activity**

**FIG. 6.** ATPase activity (v₅) as a function of [ATP] (A) or [Rb⁺] (B). Media contained either 0.5 (●), 1 (○), 2.5 (▲), 5 (□), 10 (■), or 20 (▲) mM Rb⁺ (A) and either 1 (○), 2 (■), 4 (□), 6 (▲), 8 (○), or 10 (●) μM ATP (B). The continuous lines in A were calculated by weighted, linear regression and in B were traced by eye. Vertical bars are ± 1 S.D.

**FIG. 7.** Semilog plots of release of occluded Rb⁺ at μM [ATP]. 

$^{86}$Rb⁺ remaining occluded after a 20-fold dilution of the label is plotted as a function of time after dilution. Since dilution leaves 5% of the initial specific activity of the media, $^{86}$Rbocc, as a function of time after dilution. Since dilution leaves 5% of the initial specific activity of the media, $^{86}$Rbocc, as a function of time after dilution. $^{86}$Rbocc, was adjusted to the data to obtain the best fitting values of $k_{deocc}$, $k_{deocc}$, and $k_{deocc}$, were measured in media of identical composition containing from 100 to 2500 μM ATP and from 0.25 to 10 mM Rb⁺. The results are shown in Figs. 9 and 10.

The data for the rate of hydrolysis presented in Fig. 9 show the well known fact that Rb⁺ is a potent activator of the ATPase when [ATP] ≥ 100 μM. Activation is predicted by the model in Fig. 1 since, in the presence of enough ATP, acceleration by Rb⁺ of dephosphorylation is not limited by deocclusion, the next step in the cycle. The data were analyzed by nonlinear regression using Equation 19 under "Experimental Procedures," which predicts that the curves are hyperbolas with a positive intercept at the y axis. As shown in Fig. 9, there was a satisfactory correspondence between the curves and the data. The values of the coefficients corresponding to the denominator of Equation 19 are listed in Table III together with those obtained by a similar, independent analysis of the data for Rb⁺ in Fig. 5 using Equation 18. The comparison between the values of the two sets of coefficients allows us to test if the experimental results satisfy the theoretical prediction that both $v_5$ and $[E_{occl}]$ share the same denominator (see Equations 9 and 10 in Table I). The fact that both sets of values (and thus the denominators) are (approximately) the same satisfies the requirement for the two steady-state quantities as expressions of the functioning of the Na⁺/K⁺-ATPase and justifies us to

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**FIG. 8.** Effects of ATP (A) and Rb⁺ (B) on $v_5/[E_{occl}]$ and $k_{deocc}$. The effects of [ATP] on the values of $k_{deocc}$ ($\bullet$) measured in experiments as those in Fig. 7, and on the values of $v_5/[E_{occl}]$, calculated from the data in Fig. 6A and Fig. 4 for 0.5 (●), 1 (○), 2.5 (▲), 5 (□), 10 (■), or 20 (▲) mM Rb⁺ (A). All the values were fitted by weighted, linear regression. In the case of $k_{deocc}$ (continuous line) best-fitting values were as follows: slope = 0.119 ± 0.007 μM⁻¹s⁻¹ and intercept, $k_0 = 0.132 ± 0.010$ s⁻¹. Vertical bars are ± 1 S.D. A plot against [Rb⁺] of the ratio $v_5/[E_{occl}]$ at [ATP] = 0 obtained by extrapolating the lines in A to [ATP] = 0 (B). Vertical bars are ± 1 S.E. of the mean. The dotted lines and continuous lines are calculated using Equations 20 and 21 and the values for the rate constants given in Table I, columns 3 and 4, respectively. The horizontal band corresponds to the value of $k_0 ± 1$ S.E. from the plot in A.
calculate the ratio between $v_t$ and $[E_{\text{occ}}]^2$, where the denominator cancels out (see Table I, Equations 9–11, and below).

Fig. 10 shows the results of experiments in which the rate coefficient for deocclusion, $k_{\text{deocc}}$, was measured in media with the same ATP concentrations as in the experiments in Figs. 5 and 9 using the same procedure as that employed in the experiments with low ATP concentrations (Fig. 7). In additional experiments using 0.5 mM ATP, both during the occlusion and the deocclusion phases (not shown), we observed that $k_{\text{deocc}}$ did not change if $\text{Rb}^+$ concentration was varied from 0.5 to 25 mM in the media used for occlusion or for deocclusion or in both. This extends to high [ATP] the observations at low [ATP] about the absence of effect of $\text{Rb}^+$ on the rate of deocclusion. In the experiment in Fig. 10, like that in Fig. 7, the loss of $\text{Rb}^+$ followed single exponential functions of time, with rate coefficients that increased with the concentration of the nucleotide as expected from the model in Fig. 1.

In Fig. 11A, the ratio $v/[E_{\text{occ}}]^2$ calculated from the data in Figs. 9 and 5 and the directly measured value of $k_{\text{deocc}}$ obtained from Fig. 10 are plotted together against the concentration of ATP. Two features of the plots seem to be worth mentioning. First, the $v/[E_{\text{occ}}]^2$ versus [ATP] curves at low $[\text{Rb}^+]$ are positioned above those at higher $[\text{Rb}^+]$, and as $[\text{Rb}^+]$ increases, the curves become superimposable. This is comparable to what happens at micromolar [ATP] (Fig. 7) except that $k_{\text{deocc}}$ did not change if $\text{Rb}^+$ concentration was varied from 0.5 to 25 mM in the media used for occlusion or for deocclusion or in both. This extends to high [ATP] the observations at low [ATP] about the absence of effect of $\text{Rb}^+$ on the rate of deocclusion. In the experiment in Fig. 10, like that in Fig. 7, the loss of $\text{Rb}^+$ followed single exponential functions of time, with rate coefficients that increased with the concentration of the nucleotide as expected from the model in Fig. 1.

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Equation 22 gives the sum of the Na\(^+\)-ATPase activity and the activity when only one Rb\(^+\) is bound to E\(_{P}\). From the measured activity of the Na\(^+\)-ATPase activity, we have estimated \(k_{40} \approx 2.5 \text{ s}^{-1}\). In our initial simulations on the Rb\(_{occ}\) data (dotted curves in Figs. 3–5), we took arbitrarily \(k_{41} = k_{40}\) (column 3, Table II). If the equality between constants is kept when using Equations 20 and 21 to simulate the data of Figs. 8B and 11B, a very poor fit is obtained (as shown by the dotted curves in these figures) indicating that the calculated values of \(v_{\text{other}}/E_{\text{occ}}\) (Equations 5 and 7) and hence of \(v_\text{p}/[\text{Rb}\text{]}\) (Equation 21) are much smaller than the experimental values.

Obviously this difference can be diminished making \(k_{41} > k_{40}\). Since the estimate of \(k_{40}\) is reliable we explored the inequality by increasing the value of \(k_{41}\). We simulated the data in Figs. 8B and 11B using the constants in Table II, column 3, but searching for a value of \(k_{41}\) that would give good fit. This came out as 53 and 47 s\(^{-1}\), respectively. A few iterations, using the \(k_{41}\) values obtained from Figs. 8B and 11B and permitting \(K_{Rb_{2}}\) (which is closely connected to \(k_{41}\), see Equation 21) and \(E_{p}\) to be adjusted by the regression algorithm resulted in a new set of parameters that is listed in the 4th column of Table II. These were used to draw the full curves in Figs. 3–5. Hence as it will be analyzed in more detail under “Discussion,” a possible explanation for the data in Figs. 8B and 11B is that \(E_{p}\)Rb dephosphorylates with a rate constant \(\approx 50 \text{ s}^{-1}\) that is between that for \(E_{p}\) (\(\approx 2.5 \text{ s}^{-1}\)) and \(E_{p}\)Rb (\(\approx 250 \text{ s}^{-1}\)) without leading to the formation or Rb\(_{occ}\).

**DISCUSSION**

We have studied, during steady-state turnover of the enzyme, the dependence on [ATP] and on [Rb\(^+\)] of the amount of Rb\(^+\) occluded in the Na\(^+\)/K\(^+\)-ATPase as well as the rate coefficient for release of Rb\(^+\) from this state. In all cases Rb\(_{occ}\) was formed via the physiological route. We were thus able to get a previously unavailable, comprehensive set of measurements of Rb\(_{occ}\) and \(k_{\text{deocc}}\) over a very wide range of ATP and Rb\(^+\) concentrations.

We also performed parallel measurements of ATPase activity as a function of both [Rb\(^+\)] and [ATP] using the same enzyme preparation and in media with the same composition and temperature as those used to measure Rb\(_{occ}\).

Direct measurements of steady-state Rb\(_{occ}\) formed by the physiological route have been obtained by other workers who used either the passage of the incubation media through a cation exchange resin (4) or rapid filtration procedures (6). In neither case, however, were these studies performed in the range of concentrations of Rb\(^+\) and ATP or with the accuracy required for a quantitative treatment as that presented in this paper. Moreover, neither of these techniques are able to isolate Rb\(_{occ}\) in the millisecond time scale. In a few cases, Forbush (6) employed the filtration technique to measure occluded Rb\(^+\) in enzyme retained by filters, but in most of his experiments filtration was used to follow the time course of release of \(^{86}\)Rb into the filtrate. By using media in which both ATP and [Rb\(^+\)] were saturating, Forbush (6) concluded that \(E_{\text{occ}}\) is an obligatory intermediate of the overall ATPase reaction. Glynn and Richards (4) reported a sigmoidal response of Rb\(_{occ}\) to [Rb\(^+\)] in media with less than 10 \(\mu\text{M}\) [ATP] and showed that, as [ATP] increased, Rb\(_{occ}\) first increased and then fell. Although these results agree with those shown in this paper, a quantitative comparison is not possible because the method employed by Glynn and Richards (4) does not allow to accurately control the actual concentration of the nucleotide in the vicinity of the enzyme.

The **Kinetics of Rb\(^+\) Occlusion**—Comparison between simulations and the experimental data indicated that the response of the steady-state level of Rb\(_{occ}\) to [ATP] and [Rb\(^+\)] agreed with the predictions of reaction schemes like that shown in Fig. 1 in the following: (i) the stoichiometry of occlusion is constant; (ii) Rb\(^+\) binds to a conformer of the enzyme that is different from that which binds ATP; (iii) the release of occluded Rb\(^+\) is strongly accelerated by ATP binding at the conformer that occludes the cation; and (iv) the numerical values of the rate and equilibrium constants required to fit the model to the experimental data are close to those usually considered to govern the elementary steps of this model (for references, see Ref. 21).

The model also predicts adequately most of the properties of
the directly measured rate constants for deocclusion since (i) $k_{deocc}$ is independent of [Rb+] (1), (ii) $k_{deocc}$ increases with [ATP] along a rectangular hyperbola, and (iii) the values of $k_{deocc}$ attained for [ATP] = 0 or [ATP] → ∞ (0.13 and 27 s⁻¹, respectively) adequately fit the experimental results when they are included in the scheme in Fig. 1. A small discrepancy between theory and experiments is that the experimental value for the $K_{0.5}$ of the hyperbola relating $k_{deocc}$ to [ATP] is 194 ± 35 μM which is about one-half that obtained by the simulation of the scheme in Fig. 1.

The independence of $k_{deocc}$ with the concentration of Rb⁺ is consistent with results obtained by Glynn and Richards (4) and Forbush (6) who investigated the effects of the addition of various cations in the absence of ATP. Forbush (6) showed that in media with 4 mM ATP (but no Mg²⁺), there is a saturable increase of $k_{deocc}$ with the concentration of either K⁺ or Na⁺. Since the maximum value for $k_{deocc}$ for both cations is the same and is reached at about 150 mM Na⁺ or K⁺, the phenomenon would remain unobservable under our experimental conditions.

$E_{occt}$ as an Obligatory Intermediate for the Hydrolysis of ATP by Na⁺/K⁺-ATPase—Since the properties of the scheme in Fig. 1 that are satisfied by the experimental results on Rb₋ = f([ATP], [Rb⁺]) are those of the Albers-Post model of the Na⁺/K⁺-ATPase (here Na⁺/Rb⁺-ATPase), our results suggest that this model provides a satisfactory description of the steady-state kinetics of Rb⁺ occlusion. This, however, is not sufficient to prove that under all conditions $E_{occt}$ is an obligatory intermediate of the Na⁺/Rb⁺-ATPase reaction. To analyze this, we compared the response to ATP and Rb⁺ of the ratio $v_r/[E_{occt}]$ with that of $k_{deocc}$. At this point, it seems important to notice that the term $E_{occt} K_1 k_2 k_3$ of the numerator and the denominators of the steady-state equations for $v_r$ and $[E_{occt}]$ are canceled out when $v_r$ is divided into $[E_{occt}]$ (cf. Equations 9 and 10 in Table I). The cancellation of coefficients determines that $v_r/[E_{occt}]$ will only depend on the rate and equilibrium constants that participate directly in the turnover of the occluded species, being totally unaffected by changes in any of the other elementary steps that participate in the reaction of ATP hydrolysis.

The theoretical prediction of the cancellation of the denominator was actually fulfilled by our experiments, since the values of the denominators of the functions that best fitted $v_r$ and $[E_{occt}]$ were not significantly different (Table III), thus validating the use of the $v_r$ to $[E_{occt}]$ ratio in our results.

We have shown that for $E_{occt}$ to be an obligatory intermediate of the Na⁺/Rb⁺-ATPase reaction, the ratio $v_r/[E_{occt}]$ must be equal to $k_{deocc}$. In the particular case of our enzyme, in which the breakdown of $E_{occt}$ takes place through two different pathways controlled by the binding of ATP to a single class of sites in $E_{occt}$, two additional predictions can be made as follows: (i) at any constant [Rb⁺], $v_r/[E_{occt}]$ will increase with [ATP] along rectangular hyperbolas whose $K_{0.5}$ values are independent of [Rb⁺]; and (ii) the values of $v_r/[E_{occt}]$ at 0 and at infinity [ATP] will be decreasing functions of [Rb⁺] that will tend to $k_2$ and to $k_{occt}$, respectively, as the increase in [Rb⁺] shifts ATP hydrolysis away from Na⁺-ATPase toward Na⁺/Rb⁺-ATPase activity (see Fig. 1).

Our results showed that, in media in which [Rb⁺] was sufficiently high as to make negligible the contribution of the Na⁺-ATPase to the overall activity, $v_r/[E_{occt}]$ was much larger than $k_{deocc}$ and only approached or reached $k_{deocc}$ at Rb⁺ concentrations above 10 mM. At 1–10 μM ATP, there was a complete convergence between $v_r/[E_{occt}]$ and $k_{deocc}$. At nonlimiting [ATP], the convergence annulled about 90% of the difference between both parameters so that, albeit much reduced, $v_r/[E_{occt}]$ remained larger than $k_{deocc}$ at nonlimiting [Rb⁺]. It seems premature to consider this residual difference as the sign of a genuine phenomenon, since small systematic errors biasing the estimation of $v_r/[E_{occt}]$, and/or $k_{deocc}$ cannot yet be discarded. We have explored two possible sources of errors: one is the underestimation of $[E_{occt}]$ (which would artificially increase $v_r/[E_{occt}]$) and the other is an underestimation of $k_{deocc}$. Deviations in any of these quantities should increase with the concentration of ATP. Control experiments (17) demonstrated that the loss of Rb⁺ from the occluded state using our filtration technique will give at most a 10% loss at the highest (2500 μM) [ATP] tested. It is more difficult to conceive why the measurements of $k_{deocc}$ at high [ATP] should lead to underestimation of the real value, at least on the basis of a loss of occluded rubidium during the washing of the enzyme on the filter.

As already mentioned, we ruled out the possibility of a residual Na⁺-ATPase activity as the only cause for the discrepancies between $v_r/[E_{occt}]$ and $k_{deocc}$. This was based on the following quantitative evidence: (i) in the experiments performed at 1–10 μM ATP, $E_{occt}$ had reached saturation with respect to [Rb⁺] at all [ATP] and [Rb⁺] tested, so that Na⁺-ATPase activity should be practically zero; (ii) in the experiments performed at 100–2500 μM [ATP], the discrepancy between $v_r/[E_{occt}]$ and $k_{deocc}$ persisted after the maximal value of Na⁺-ATPase activity was subtracted from each of the values of $v_r$ before calculating $v_r/[E_{occt}]$.

An additional, more qualitative, discrepancy between our experiments and the prediction of the model in Fig. 1 is that, although our results are in agreement with this model in the sense that, for all [Rb⁺], $v_r/[E_{occt}] = f([ATP])$ were rectangular hyperbolas, the experimentally obtained values for $K_{0.5}$ decreased with [Rb⁺] instead of remaining constant, as should happen if no steps other than those shown in Fig. 1 governed the effect of ATP on $k_{deocc}$. As before, we cannot yet discard that small deviations in the measurement of $[E_{occt}]$ (now dependent on both [ATP] and [Rb⁺]) could explain this unexpected effect of Rb⁺.

Before analyzing if the model in Fig. 1 has to be modified or replaced to account for discrepancies between theory and experiment, it seems reasonable to see if these are caused by the restrictive assumptions of fixed stoichiometry of occlusion and irreversibility of deocclusion which we used to obtain numerical simulations of our model.

Let us, for the sake of the argument, disregard the abundant experimental evidence for fixed stoichiometry of occlusion and assume that the Na⁺/Rb⁺-ATPase activity also occurs through enzymes that occlude only one Rb⁺. In this case $E_{occt}$ would vary between Rb₋ and Rb₋/2, and if the species occluding one Rb⁺ had the same turnover than those occluding two Rb⁺, $v_r/[E_{occt}]$ would be at most twice as large as $k_{deocc}$ at sufficiently low [Rb⁺]. Since this is too small to account for the effects we observed in our experiments, the variable stoichiometry hypothesis would require the additional postulate that enzymes occluding one Rb⁺ have a higher turnover than those occluding two Rb⁺. An effect of this kind cannot be postulated, since it would have been detected as a decrease in the values of $k_{deocc}$ as high [Rb⁺] drove the putative state holding one occluded Rb⁺ to the state holding two occluded Rb⁺.

An additional assumption we used to solve the model in Fig. 1 is that the reactions of deocclusion ($E_{occt} → E_1$ and $E_{occt}ATP → E_2ATP$) are irreversible. If this were not the case, an increase in [Rb⁺] would decrease the net rate of hydrolysis ($v_r$) as the inverse reaction sets in, with the consequent decrease in $v_r/[E_{occt}]$. In the context of the model in Fig. 1, this kind of effect on $v_r$ would always yield values of $v_r/[E_{occt}]$ smaller than those of $k_{deocc}$. This is so because if deocclusion of Rb⁺ were reversible its net rate, and hence the overall Na⁺/K⁺-ATPase activity,
would decrease from $k_{\text{deocc}} \times [E_{\text{occ}}]$ toward 0 as $[\text{Rb}^+]$ goes from 0 to infinity. This is in sharp contradiction with our results that show that $v_{\text{f}}/[E_{\text{occ}}]$ is always higher than $k_{\text{deocc}}$. It must be stressed that in addition the assumption that the deocclusion reaction is irreversible under our experimental conditions rests on solid experimental basis. We have shown (“Experimental Procedures” in this paper and Ref. 17) that in media with 150 mM Na$^+$ and 0.5 mM Mg$^{2+}$, there is no occlusion via the direct route. Although there are no comparable studies in the literature, evidence from other authors favor the idea of irreversibility. Glynn and Richards (4) have shown that at 20 °C, 15 mM Na$^+$ prevents occlusion at 100 μM Rb$^+$ through the direct route. We (9) have earlier shown that at 20 °C, in media with 150 mM NaNO$_3$ and 100 μM Tl$^+$, there is no occlusion in the absence of ATP. In addition, Hasenauer et al. (26) in experiments performed at 4 °C, have shown that 10 mM Na$^+$ is sufficient to prevent occlusion by the direct route in media with 200 μM Rb$^+$.

The inability of the reversal of deocclusion to explain the properties of $v_{\text{f}}/[E_{\text{occ}}]$ agrees with previous studies of our laboratory on the mechanism of the inhibition of Na$^+$/K$^+$-ATPase by high [K$^+$] at low [ATP] (25). In these studies, reversibility of deocclusion was unable to account for the kinetics of inhibition.

It would seem, therefore, that it is reasonably well established that the differences we found between theory and experiment in the response to $[\text{Rb}^+]$ and to [ATP] of the ratio $v_{\text{f}}/[E_{\text{occ}}]$, rather than being attributable to the restrictions imposed in the simulations of the model in Fig. 1, represent a genuine property of the system. We have shown that the model in Fig. 1 describes adequately $k_{\text{deocc}} = f([\text{ATP}],[\text{Rb}^+])$ using currently accepted values for equilibrium and rate constants. Therefore, it seems reasonable to ascribe the above differences to the existence of a component of $v_{\text{f}}$ which is not accountable by the same set of values in Table II (column 3) that explain $k_{\text{deocc}} = f([\text{ATP}],[\text{Rb}^+])$. This component tends to disappear at a concentration of Rb$^+$ much higher than those needed to abolish Na$^+$/ATPase activity and can be expressed by defining a putative ouabain-sensitive ATPase activity, corresponding to the term $v_{\text{other}}$ defined in the Introduction. We can calculate the magnitude of $v_{\text{other}}$ from the difference between the total activity ($v_{\text{f}}$) and that of a reaction cycle in which $E_{\text{occ}}$ and $E_{\text{occ}}$ATP are obligatory intermediates, see Equation 23:

$$v_{\text{other}} = v_{\text{f}} - k_{\text{deocc}} \times [E_{\text{occ}}]$$

(Eq 23)

This putative ATPase activity has the following properties: (i) it is not coupled to the turnover of $E_{\text{occ}}$ and $E_{\text{occ}}$ATP; (ii) it displays a biphasic response to ATP concentration with high and low affinity components; and (iii) it tends to disappear at sufficiently high [Rb$^+$].

An indirect indication, different from evidence given in this paper for the existence of $v_{\text{other}}$, is provided in previous experiments by us in which, using preparations from pig (21) of from dog (25) kidney, we compared calculated and measured values of $V_{\text{max}}$ and $K_{\text{p}}$ of the high affinity component of the substrate curve of the Na$^+$/K$^+$-ATPase. Results showed that the experimental values of both parameters were from 5- to 10-fold larger than those predicted by models like that in Fig. 1. Using a value of $k_{\text{p}} = 0.2 s^{-1}$ which is similar to that measured in this paper (0.13 s$^{-1}$). At this time (21) we speculated that the reason for this could be that $k_{\text{deocc}}$ was much higher when measured in steady-state than in pre-steady-state conditions. The results in this paper make this hypothesis untenable and uphold the need of postulating a putative ATPase having the properties described above to explain our previous results.

Our conjecture of a putative extra ATPase activity does not intend to posit that a different enzyme is present in our preparations. It is rather an operational definition that may help to envision which modifications have to be introduced into a usually accepted model to account for the unexpected aspects of its behavior. With this in mind, it seems that the simplest modification would be to assume that when the enzyme binds a single K$^+$ (or Rb$^+$) ion its activity increases without the formation of occluded species. In terms of the model in Fig. 1, a likely hypothesis is that $k_{\text{deocc}} > k_{\text{occ}}$. If it were true, it would imply the existence of a new pathway for ATP hydrolysis, which would require, but not occlude, K$^+$ or its congeners. It is obvious that, if $k_{\text{deocc}} > k_{\text{occ}}$, for a certain intermediate range of Rb$^+$ concentrations ATPase activity will increase more than $[E_{\text{occ}}]$ leading to the inequality $v/[E_{\text{occ}}] > k_{\text{deocc}}$. When [Rb$^+$] is high enough all the reaction will take place through $E_{\text{occ}}$ and $v/[E_{\text{occ}}] = k_{\text{deocc}}$. Hence, the activity resulting from $k_{\text{deocc}} > k_{\text{occ}}$ would share with our experimental data the feature that the $v/[E_{\text{occ}}]$ ratio approaches, at sufficiently high [K$^+$], the classical behavior of the Na$^+$/K$^+$-ATPase. We have already shown the predictive ability of the hypothesis in the experiments shown under “Results.” In Figs. 3′–5, 8D, and 11B, we have shown the predictive ability of this hypothesis by plotting, together with the experimental data, two different simulations of the model in Fig. 1 as follows: in one $k_{\text{deocc}} = 2.5 s^{-1}$ and in the other $k_{\text{deocc}} = 20 \times k_{\text{occ}}$. The latter assumption did not modify substantially the fit to our results of Rb$^+$ (Figs. 3′–5), but it accounted almost completely for the behavior of $v/[E_{\text{occ}}]$ as a function of [Rb$^+$] (Figs. 8B and 11B).

Before relying on the predictive value of the assumption that $k_{\text{deocc}} > k_{\text{occ}}$, it must be stressed that the hypothesis does not account for the effects of [Rb$^+$] on the values of $K_{0.5}$ for ATP since, according to Equations 20 and 21 under “Results,” these should remain independent of [Rb$^+$] even when $k_{\text{deocc}} = 20 \times k_{\text{occ}}$. At this stage of our knowledge, to analyze in more detail these additional effects would be to take the speculation too far away from the experimental facts.

To the best of our knowledge, there is no direct experimental evidence for or against our modification of the scheme based on $k_{\text{deocc}} > k_{\text{occ}}$. However, the fact that this modification allows us to cancel at least in part the observed discrepancies between theory and experiment makes it still unnecessary to postulate that the Albers-Post model must be substantially modified to explain our results.

Further evidence to confirm the hypothesis could be obtained by measuring the rate of dephosphorylation and/or the rate of occlusion as a function of [Rb$^+$]. The rate of K$^+$ transport would be a diagnostic tool as well: if occlusion is taken as essential for cation pumping, no pumping of K$^+$ would take place through the pathway determined by the dephosphorylation of $E_{2PK}$. The existence of this pathway would decrease the apparent stoichiometry of cation pumping and reduce the efficiency of pumping because of the partial uncoupling between K$^+$ (and Na$^+$)-dependent ATPase and active transport.

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