Influence of Na^+ on Conformational States in Membrane-Bound Renal Na,K-ATPase†

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ABSTRACT: Conformational states of the Na,K-ATPase and rates of transition between these are studied using the fluorescent dye eosin as a marker for the Na^+ form (E_1) and occlusion of $^{86}Rb^+$ as a marker for the K^+ form (E_2). The aim of the present paper is to propose that the E_1 form of the Na,K-ATPase can be liganded with a number of Rb^+ and Na^+ ions and that only some of these E_1 forms bind eosin and thus probably nucleotides with high affinity. Experiments are performed with Na,K-ATPase isolated from pig kidney. Binding of eosin occurs only when Na^+ is present at millimolar concentrations, and the observed rate of binding is slow when Rb^+ is present. The rate of eosin binding after a sudden increase in the Na^+ concentration is about the same as the rate of deocclusion of Rb^+, suggesting that eosin monitors the rate of the E_2 to E_1 transition. Titrations of eosin fluorescence with Na^+ indicate that binding of more than one Na^+ occurs when high-affinity eosin binding takes place. With 0.05 mM RbCl and 4 mM NaCl present, the Na,K-ATPase is a mixture of at least two enzyme species which do not bind eosin with high affinity. One species is the E_2 form with Rb^+ occluded, and transition of this form to E_1 gives rise to a small observed rate constant for eosin binding when the Na^+ concentration is suddenly increased to about 25 mM. The other enzyme species—about 25% of total enzyme—has a very high observed rate constant of eosin binding when the Na^+ concentration is suddenly increased, and the observed rate constant is proportional to the eosin concentration. The interpretation of the results is that these two enzyme species represent E_1 forms of the enzyme with fewer Na^+ bound than those required for high-affinity eosin binding and that there is a rapid equilibrium between the E_1 forms with Na^+ and/or Rb^+ bound. A consequence of this is that the Na,K-ATPase may exist in several kinetically distinguishable E_1 states, only some of which are liganded in such a way—presumably with two or three Na^+ bound—as to allow for high-affinity binding of eosin or the nucleotides ADP and ATP.

The Na,K-ATPase (E.C. 3.6.1.37) transports Na^+ out of and K^+ into most eukaryotic cells at the expense of ATP [Skou, 1957; Post et al., 1960; Dunham & Glynn, 1961; recently reviewed by Glynn (1992)]. A property of this enzyme is the ability to occlude Na^+ and K^+ during the reaction cycle. The occluded ions exchange only very slowly with ions in the bulk phase. The deocclusion of K^+ from the enzyme seems to be the rate-limiting step in the reaction cycle under physiological conditions, and in this context interest in the properties of the occluded conformation is particularly due to the accelerating effects of Na^+ and ATP on the rate of release of the occluded ions (Post et al., 1972; Glynn & Richards, 1982; Forbush, 1987; Glynn & Karlish, 1990; Glynn, 1992; Rossi & Nørby, 1993).

Occlusion of K^+ occurs during hydrolysis of ATP ("the physiological route"), but there is also a spontaneous occlusion of K^+ in the absence of ATP ("the direct route"). This occlusion reaction is observed at very low K^+ concentrations with an apparent K_D in the 10–20 μM range (Glynn & Richards, 1982; Forbush, 1987; Esmann, 1985; Shani et al., 1987), and it is abolished by a high Na^+ concentration. The major conformations of the Na,K-ATPase [recently rewieved by Robinson and Pratap (1993)] are related to these properties, with the E_2 conformation (the "K^+ form") having two (or more) K^+ occluded (Glynn & Richards, 1982; Glynn & Karlish, 1990; Hasenauer et al., 1993; Rossi & Nørby, 1993) and a slow rate of transition to the E_1 conformation (the "Na^+ form"). An indicator for the E_1 conformation has been the ability to bind the nucleotides ADP and ATP (and the fluorescent dye eosin) with high affinity (Nørby & Jensen, 1971; Hegyvary & Post, 1971; Skou & Esmann, 1981).

The present investigation is concerned with binding of Na^+ and Rb^+ (a K^+ congener) to the E_1 and E_2 forms of the Na,K-ATPase and is aimed at determining the influence of the cations on the equilibrium and transition rates between these two forms. The effect of Na^+ on Rb^+ occlusion is studied under equilibrium conditions and in transient experiments, and under the same experimental conditions the rate of appearance of the enzyme form which binds eosin with high affinity is studied. The principal experimental findings are interpreted in a model where some of the enzyme molecules are neither in the E_2 form with Rb^+ occluded nor in an E_1 form with eosin bound.

The results thus suggest that the E_1 form of the Na,K-ATPase consists of several rapidly equilibrating enzyme species, liganded with Na^+ and/or K^+, and that not all these species bind eosin and nucleotides with high affinity. This implies that a more specific nomenclature is required for describing properties of enzyme conformations rather than just the E_1 and E_2 notation, as also suggested by Jencks (1989), Glynn and Karlish (1990), and Pratap and Robinson (1993).

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MATERIALS AND METHODS

Preparation of Pig Kidney Enzyme. Na,K-ATPase was isolated in the membrane-bound form from pig kidney outer medulla by the method of Jørgensen (1974) followed by selective extraction of the plasma membranes with SDS. The enzyme was stored at −20 °C in 250 mM sucrose, 12.9 mM imidazole, and 0.625 mM EDTA at pH 7.5. The specific ouabain-inhibitable Na,K-ATPase activity was 1200–1500 μmol (mg of protein)$^{-1}$ h^{-1} at 37 °C. Na,K-ATPase activity, phosphorylation capacity, and protein content were determined as previously described (Esmann, 1988). In some experiments the enzyme was resuspended in 10 mM NaCl and washed repeatedly by centrifugation in 20 mM histidine (pH 7.0) in order to remove Na^+ and traces of K^+.

Measurement of Rb^+ Occlusion Using the Cation-Exchange Procedure. The method used is essentially as described by Glynn and Richards (1982). The carboxylic resin Bio-Rad Bio-Rex 70 is equilibrated in 10 mM histidine (pH 7.5) and 1 mM CDTA. Na,K-ATPase (0.4–0.8 mg/mL) in 10 mM histidine (pH 7.5), 0.5 μM eosin, and 1 mM CDTA is incubated with $^{86}Rb^+$ in the presence of 0–50 mM NaCl for 60 min at 20 °C in the dark, allowing for the slow equilibration of Rb^+ with the enzyme. About 300 μL of reaction mixture is forced through the 1-mL column at 20 °C, with the speed of the piston being adjusted to allow the enzyme suspension to be in contact with the resin for about 0.9 s (this is calculated from the flow rate and the volume of the liquid phase in the resin). The amount of $^{86}Rb^+$ emerging from the column is determined from γ-radiation. Forcing the enzyme through the column at half the speed given above did not change the amount of radioactivity emerging, in agreement with the very slow spontanous deocclusion rate (see later). Determination of the amount of protein in the effluent from the resin revealed that 76 ± 7% (SD, n = 8) of the protein was recovered in the effluent. Protein determination was not carried out on all effluents, and it was also observed that the accuracy of the data was not improved significantly by determination of protein. Analysis of the data was performed using the program GraphPAD Inplot, version 3.00 (GraphPAD Software, San Diego, CA).

Stopped-Flow Fluorimetry. Measurements of changes in eosin fluorescence were determined using an SFM-2 stopped-flow apparatus (Biologic, Claix, France). Excitation was at 530 nm, and emission was measured with a photomultiplier equipped with a cutoff filter at 550 nm. Data were collected with an A/D converter interfaced to a HP 9820 microcomputer. The signal-to-noise ratio was increased by digitally adding 5–7 tracings. Nonlinear least-squares calculations of exponential decays were performed using a program kindly provided by Robert Clegg, Göttingen.

Samples for the experiments shown in Figures 5 and 6 were prepared in the following way: Both stopped-flow syringes contained 10 mM histidine (pH 7.5), 1 mM CDTA, and eosin as indicated. In addition, one syringe contained 0.1 mg/mL Na,K-ATPase protein, 0.05 or 0.1 mM RbCl, and 0–12 mM NaCl as indicated in Figure 5. The other syringe contained no protein, 0.05 or 0.1 mM RbCl, and 50 mM NaCl (Figure 5) or between 50 and 900 mM NaCl (Figure 6). The volume delivered from each syringe was 150 μL per shot, and the flow time was chosen to be 200 ms. This instrumental setting gives a dead time of about 2.1 ms as determined by the procedures supplied by Biologics (France). With this dead time and a rate-constant of, for example, 80 s^{-1}, the determined fluorescence amplitude is equal to 100% × exp(−80 × 0.0021) = 85% of the total fluorescence change.

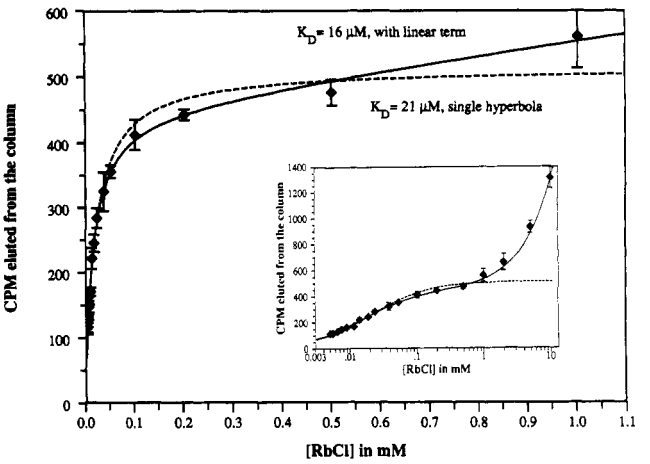


FIGURE 1: Occlusion of Rb^+ by pig kidney Na,K-ATPase. Here is shown the amount of Rb^+ carried through a cation-exchange resin at 20 °C by 0.4 mg/mL Na,K-ATPase in 10 mM histidine (pH 7.5), 1 mM CDTA, and 0.5 μM eosin at the Rb^+ concentrations indicated. The sample is in contact with the resin for 0.9 s. Each point represents the average of 3 or 4 experiments (with SD indicated). The curves are obtained by nonlinear least-squares analysis. The dotted line is drawn according to a simple binding isotherm of the form $Y = Y_{max}/(1 + K_D/[Rb^+])$, with K_D = 21 μM. The solid line is drawn according to the same equation with the addition of a term which is linear in $[Rb^+]$: $Y = Y_{max}/(1 + K_D/[Rb^+]) + K_{non}[Rb^+]$. Here K_D is 16 μM, K_{non} = 107 cpm mM^{-1}, and Y_{max} = 452 cpm, equivalent to about 1.8 Rb^+ occluded per phosphorylation site with a measured value of 2.2 nmol of phosphorylation sites per milligram of protein for this preparation. The inset shows these and additional data in a plot with a logarithmic [RbCl] axis.

Materials. $^{86}Rb^+$ was obtained from Risø National Laboratories, Roskilde, Denmark.

RESULTS

The aim of the present experiments is to study the effect of Na^+ on Rb^+ occlusion under equilibrium conditions and in transient experiments, and under the same experimental conditions to monitor the rate of appearance of the enzyme form which binds eosin with high affinity. First the equilibrium properties of the Na^+/Rb^+ antagonism are investigated, and second the rates of deocclusion and transition between the different enzyme species are determined.

Equilibrium Occlusion Experiments. Figure 1 shows the amount of Rb^+ occluded as a function of the total Rb^+ concentration at 20 °C in the presence of 0.5 μM eosin (pH 7.5). A large component with high affinity (K_D = 16 ± 2.4 μM, n = 3) is observed in addition to a component which appears to depend linearly on the RbCl concentration up to at least 10 mM RbCl. The dashed line in Figure 1 gives the concentration dependence of the amount of occluded Rb^+ in terms of a single hyperbolic binding isotherm, and the solid line shows the dependence when a linear term is added to the binding equation (see caption to Figure 1). There is no significant deviation either from the hyperbolic term or from the composite equation at low RbCl concentration (see inset in Figure 1), consistent with earlier observations at 20 °C (Shani et al., 1987). The dissociation constant K_D = 16 μM is in the same range as previously found (Shani et al., 1987; Hasenauer et al., 1993) and is not affected by the presence of 0.5 μM eosin (not shown). In some cases a higher K_D was found, but this could be attributed to a K^+ contamination of the preparations.

The stoichiometry of high-affinity Rb^+ occlusion is consistent with two Rb^+ occluded per phosphorylation site (with 2.2 nmol of phosphorylation sites per milligram protein). The

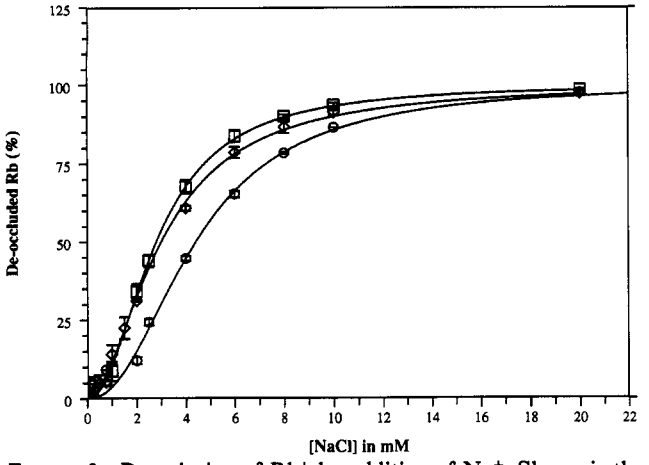


FIGURE 2: Deocclusion of Rb^+ by addition of Na^+. Shown is the decrease in the amount of Rb^+ carried through the cation-exchange resin when the protein is allowed to be in contact with the resin for 0.9 s at 20 °C as a function of the [Na^+] in the equilibrated sample. In order to ease comparison with Figure 3, the data are given as the loss of Rb^+ occlusion as a function of Na^+. Na,K-ATPase at a protein concentration of 0.2 mg/mL in 10 mM histidine (pH 7.5), 1 mM CDTA, 0.5 μM eosin, and 0.05 mM RbCl (□, ◇) or 0.1 mM RbCl (○) is titrated with NaCl up to 55 mM. Each point represents the average of 3 or 4 experiments (±SD). The amount of radioactivity carried through the column was about 500 cpm in the absence of NaCl (0% deoccluded). The solid lines are drawn according to the Hill equation: $Y = Y_{max}[Na^+]^n/(K_{0.5}^n + [Na^+]^n)$, where Y is the percentage of deoccluded Rb^+. The Hill coefficient n and the [Na^+] giving half-maximal response ($K_{0.5}$) were determined from a nonlinear least-squares analysis. For [RbCl] = 0.05 mM n was found to be 2.1 (□) and 1.9 (◇) with $K_{0.5}$ = 2.8 and 3.0 mM, respectively. With 0.1 mM RbCl, n was 2.2 and $K_{0.5}$ was 4.4 mM.

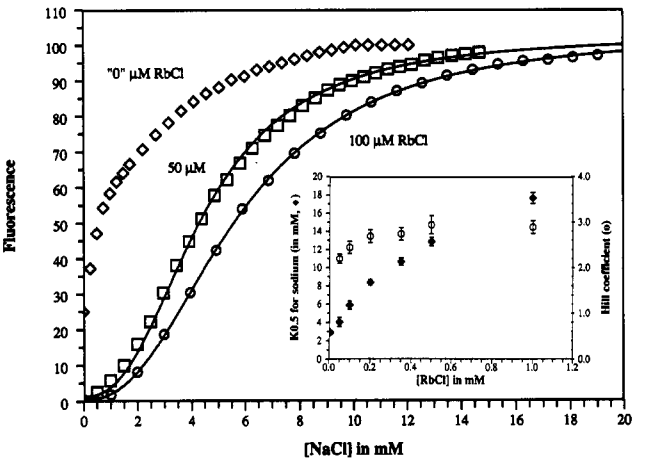


FIGURE 3: Titration of eosin fluorescence with Na^+ and Rb^+. Pig kidney Na,K-ATPase at a protein concentration of 0.1 mg/mL in 10 mM histidine (pH 7.5), 1 mM CDTA, 0.5 μM eosin, and 0, 0.05, or 0.10 mM RbCl is titrated with NaCl. The equilibrium fluorescence of eosin is given as the percentage of the fluorescence at saturating NaCl concentration, with the zero level being the fluorescence of eosin in the presence of 0.1 mM ADP (where all eosin is displaced from the binding sites by ADP). The solid lines are drawn according to the Hill equation: $F = F_{max}[Na^+]^n/(K_{0.5}^n + [Na^+]^n)$. Average values (±SD) from 2–4 experiments for the Hill coefficient n and the [Na^+] giving half-maximal response ($K_{0.5}$) were determined from a nonlinear least-squares analysis and are shown in the inset for [RbCl] from 0 to 1.0 mM.

increase in the amount of occluded Rb^+ at concentrations exceeding 0.2 mM was almost linear up to 10 mM RbCl (see inset in Figure 1; note the log scale). Whether this represents nonspecific binding of Rb^+ (which is not trapped during passage of the cation resin) or, as discussed by Hasenauer et al. (1993), additional Rb^+ being occluded (acting with a low affinity) cannot be determined from these data. At 10 mM RbCl a stoichiometry of at least 6 Rb^+ per phosphorylation site is obtained, which is far more than the two transported Rb^+.

NaCl displaces occluded Rb^+. In Figure 2 is shown the decrease in the amount of occluded Rb^+ as a function of the NaCl concentration with RbCl concentrations of 0.05 (□, ◇) or 0.1 mM (○). The titration curves are fitted adequately by the Hill equation (see caption to Figure 2), giving estimates for the Hill coefficient n of about 2 (□, ◇) and 2.2 (○), respectively, and the Na^+ concentrations giving half-maximal response ($K_{0.5}$) are about 2.9 and 4.4 mM, respectively. Oligomycin increases the apparent affinity for Na^+ considerably (Esmann, 1992), and in agreement with this much less Na^+ was required to displace occluded Rb^+ in the presence of 10 μg/mL oligomycin (data not shown).

Equilibrium Fluorescence Experiments. The competition between Rb^+ and Na^+ can also be seen from the effect of Rb^+ on the Na^+-dependent binding of eosin, monitored from the fluorescence increase when enzyme in the presence of eosin is titrated with NaCl (Figure 3) [eosin bound to the Na,K-ATPase in the E_1 form has a 3–5-fold higher fluorescence yield than unbound eosin (Skou & Esmann, 1981)]. In the absence of added RbCl a fraction of the eosin seems to be bound in the absence of added NaCl, presumably due to the known Na-like effects of most buffers (Skou & Esmann, 1980) (0% fluorescence is the level of fluorescence when bound eosin is displaced by 0.1 mM ADP, which acts as a competitor for eosin). Interpretation of the Na^+ titration curve in the absence of added RbCl is therefore difficult. In the presence of 0.05 or 0.1 mM RbCl the titration curves are markedly sigmoid (Figure 3). The titration curves in this case are fitted adequately by the Hill equation (see caption to Figure 3), giving estimates for the Hill coefficient n and the [Na^+] giving half-maximal response ($K_{0.5}$). The inset in Figure 3 shows the dependence of $K_{0.5}$ for Na^+ and n on the concentration of Rb^+ in the range 0.01–1 mM. $K_{0.5}$ for Na^+ is 2.9 mM at 10 μM Rb^+, increasing to about 17 mM at 1 mM Rb^+. The Hill coefficient increases from about 2.2 at low Rb^+ concentrations to about 3 at 1.0 mM Rb^+. Note the similarity of the Hill parameters for the titrations in Figures 2 and 3.

Transient Experiments. In preliminary experiments the rate of occlusion of Rb^+ was studied in order to define conditions where equilibrium is obtained between occluded and nonoccluded forms of the enzyme. The amount of occluded Rb^+ approached equilibrium in an exponential fashion with a half-time of about 4 min at 6 °C (corresponding to an observed rate constant of about 0.18 min^{-1}) when Rb^+ was mixed with Na,K-ATPase in the absence of Na^+. After 40 min of incubation there was still a slow increase in the amount of Rb^+ occluded, in agreement with recent observations by Hasenauer et al. (1993). The rate constant describing the occlusion reaction was found to decrease with an increase in the Rb^+ concentration from 0.01 to 0.2 mM [not shown, but in agreement with the results of Hasenauer et al. (1993)]. At 20 °C the half-time was less than 1 min when 0.05 or 0.1 mM RbCl was added to the enzyme (not shown), and there was no difference in the amount of Rb^+ occluded between 30 and 60 min. A mixture of NaCl, RbCl, and Na,K-ATPase was therefore considered to be in equilibrium after 60 min at 20 °C. At 20 °C it was also observed that the rate constant describing the occlusion reaction decreased with increasing Rb^+ concentrations (tested at 0.01, 0.2, and 0.5 mM RbCl; data not shown).

The rate of deocclusion of Rb^+, induced by addition of NaCl, was followed at 10 or 20 °C in order to determine the

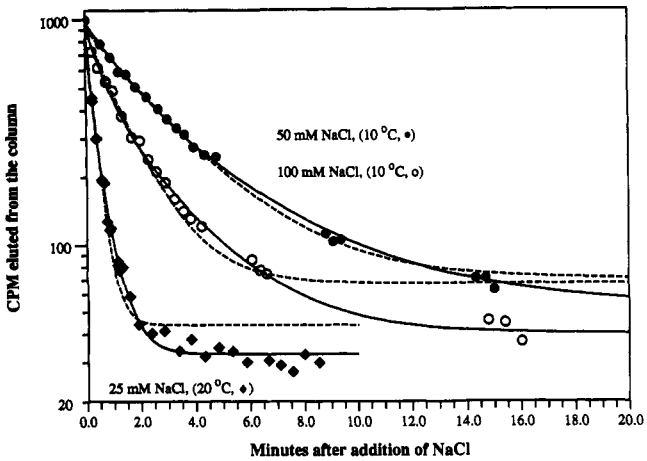


FIGURE 4: Time dependence of the deocclusion of Rb^+ studied by addition of Na^+. Shown is the effect of addition of 25–100 mM Na^+ on the time course of the amount of occluded Rb^+. First occlusion of Rb^+ is allowed to take place in the absence of Na^+ with incubation at 10 (○, ●) or 20 °C (◆) for 60 min in a medium with a protein concentration of 0.2 mg/mL, 10 mM histidine (pH 7.5), 1 mM CDTA, 0.5 μM eosin, and 0.1 mM RbCl. NaCl is added as a concentrated solution at time zero, and after the indicated times, the suspension is forced through the cation-exchange resin and the amount of Rb^+ carried through is determined. The dashed lines represent single-exponential curve fits of the data with rate constants of 0.35 and 0.72 min^{-1} (upper and middle tracings, respectively). The solid lines represent biexponential curve fits, assuming a model with two equally sized populations of deoccluding Rb^+, with pairs of rate constants of 0.22 and 0.59 min^{-1} (upper curve), 0.4 and 1.5 min^{-1} (middle curve), and 1.9 and 5.9 min^{-1} (lower curve) and with errors in the range 4–8%.

rate constant for deocclusion in the presence of Na^+. Figure 4 shows the time dependence of the residual amount of Rb^+ occluded after addition of NaCl. The data shown in Figure 4 are fitted by single exponentials (dashed lines) and also by a double-exponential decay, where the amplitudes of the two exponentials are fixed to be equal (solid lines in Figure 4; a model with two equal amplitudes is chosen from the assumption that two Rb^+ are occluded per phosphorylation site (Glynn & Richards, 1982; Rossi & Nørby, 1993)]. Clearly there is an accelerating effect of an increase in the NaCl concentration from 50 to 100 mM for the experiments done at 10 °C, and the data are fitted better by the double-exponential curves. At 20 °C the deocclusion reaction is not fitted satisfactorily by a single exponential (rate constant, 3.2 min^{-1}), but seems to be adequately described by the double-exponential decay with rate constants of about 1.9 and 5.9 min^{-1} with 25 mM NaCl (note that with rate constants this close there are a number of combinations of amplitudes and rate constants that will give visually almost identical fits, so the parameters are poorly determined).

The rate of deocclusion of Rb^+ may also be followed using the stopped-flow fluorescence technique. This is shown in Figure 5, where the fluorescence is given as a function of time on a short (panel A) or long time scale (panel B) when enzyme in the presence of eosin, 0.05 mM RbCl, and between 0 and 12 mM NaCl is mixed with NaCl to give a final NaCl concentration of 25–31 mM.

When NaCl is absent in the enzyme-containing syringe, there is no detectable rapid fluorescence increase but rather a slow fluorescence increase, characterized by a half-time of about 15 s (an observed rate constant of about 2.8 min^{-1} in a single-exponential curve fit; panel B). This rate constant of 2.8 min^{-1} is close to the 3.2 min^{-1} found for the single-exponential fit of deocclusion of Rb^+ under the same conditions (lower data set in Figure 4). Deocclusion of Rb^+ thus follows the fluorescence increase closely with time, in agreement with earlier observations (Glynn et al., 1987). The present data do not allow for a further kinetic analysis of the coupling between the deocclusion reaction and the fluorescence increase.

Interestingly, there is a marked effect of low concentrations of NaCl (0.2–12 mM) in the enzyme-containing syringe: the fluorescence signal becomes clearly biphasic with a very rapid component (Figure 5, panel A), the relative size of which increases with the NaCl concentration. The total fluorescence change, as expected, is decreased when the NaCl concentration is increased from 0 to 12 mM in the enzyme-containing syringe (see below) since some of the enzyme is titrated to the eosin-binding form (cf. Figure 3). In addition to the rapid component, there is a slow component ascribed to the residual enzyme with occluded Rb^+ (Figure 5, panel B; note that the transients shown in Figure 5, panels A and B, are amplified to different extents; this is discussed below).

An interpretation of this result is that the mixture of enzyme species in the enzyme-containing syringe before mixing consists of both some enzyme in the occluded state [$E_2(Rb_2)$] and some enzyme which has no eosin bound, the latter reacting rapidly with Na^+ and eosin when the Na^+ concentration is increased to 25–31 mM. This can also be deduced from the equilibrium experiments shown in Figures 2 and 3. With 0.05 mM RbCl the concentration of Na^+ required for displacing 50% of the occluded Rb^+ is about 2.9 mM (Figure 2), whereas about 4.1 mM Na^+ is required to give a 50% increase in eosin fluorescence (Figure 3). Therefore, at some intermediate Na^+ concentration (for example, 4 mM) a fraction of the enzyme molecules has neither Rb^+ occluded nor eosin bound.

The nature of the rapid fluorescence increase was investigated with respect to the influence of NaCl and eosin. In Figure 5 (panel A), the rapid component is described by a rate constant of about 60 s^{-1} at 0.5 μM eosin, and the rate constant seemed independent of the Na^+ concentration in the enzyme-containing cuvette (see inset in Figure 5A). Experiments such as those shown in Figure 5A with 2 mM NaCl were performed at eosin concentrations ranging from 0.1 to 1 μM, and it was found that the observed rate constant describing the rapid fluorescence increase depends linearly on the eosin concentration (Figure 5C). Analysis of such a relationship suggests that (Gutfreund, 1969)

$$k_{obs} = k_{off} + k_{on}[\text{eosin}] \quad (1)$$

with a dissociation rate constant, k_{off}, of about 17 s^{-1} and a binding rate constant, k_{on}, of about 64 μM^{-1} s^{-1}, giving an equilibrium dissociation constant K_D ($=k_{off}/k_{on}$) for eosin of about 0.26 μM. These values are all consistent with an interpretation where the rapid phase of the fluorescence increase is attributable to the binding of eosin to an enzyme form which appears very rapidly when the Na^+ concentration is increased, for example, from 2 to 26 mM.

The amplitudes of the rapid and slow fluorescence changes (Figure 5, panels A and B) are shown in Figure 5, panel D, as a function of the Na^+ concentration in the enzyme-containing syringe. A close relationship between the amount of Rb^+ occluded and the amount of slow fluorescence increase is observed (compare Figures 2 and 5D). The half-maximal effect of Na^+ is about 3 mM for both the decrease in Rb^+ occlusion and the amplitude of the slow fluorescence change at 0.05 mM RbCl, and the half-maximal effect of Na^+ is about 4.4 mM at 0.1 mM RbCl (deocclusion data shown in Figure 2; fluorescence data not shown). The slow fluorescence increase can thus be attributed to the deocclusion reaction,

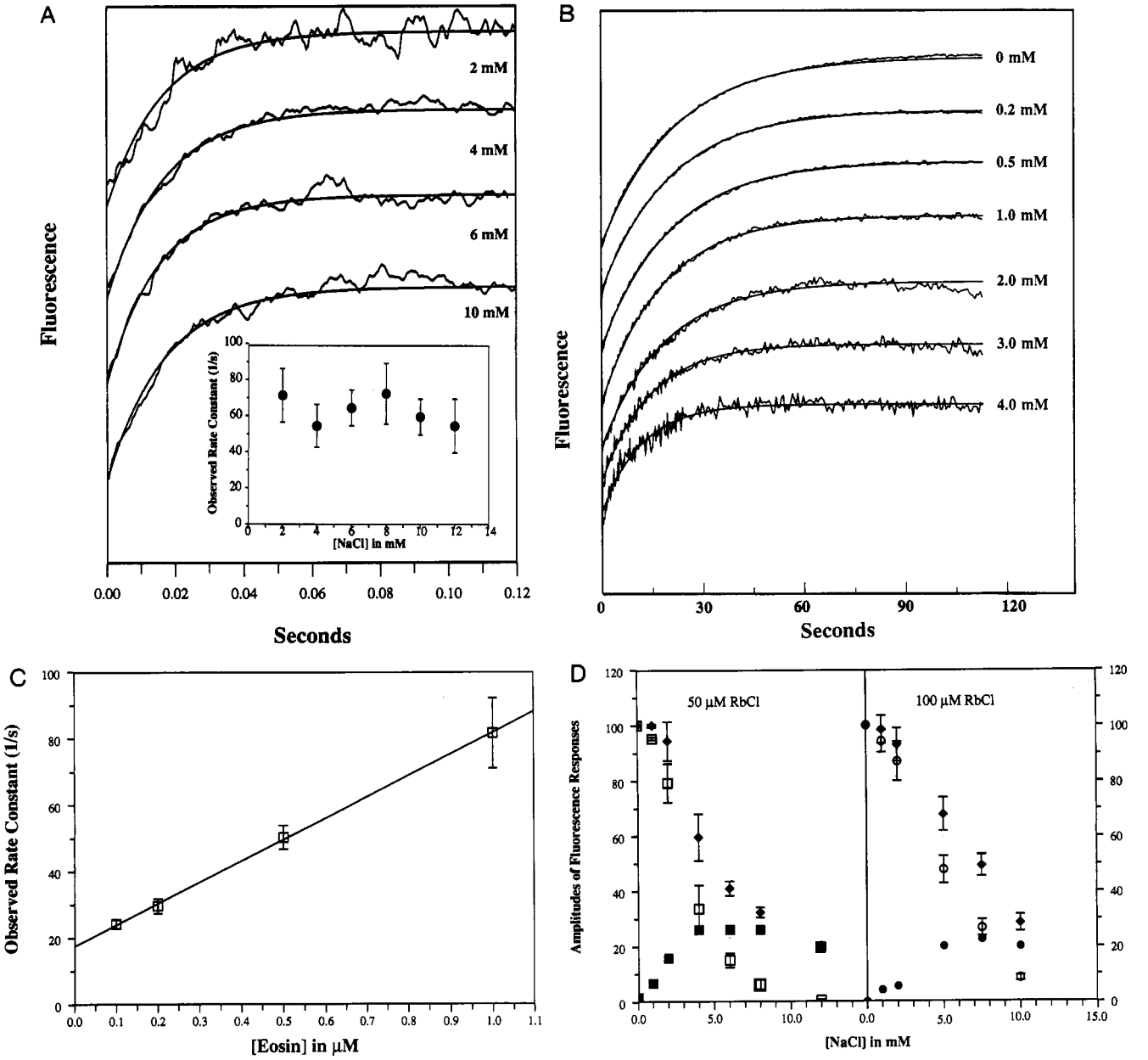


FIGURE 5: Effect of low concentrations of Na^+ on the rate of binding of eosin. Na,K-ATPase at a protein concentration of 0.1 mg/mL in a buffer containing 10 mM histidine (pH 7.5), 1 mM CDTA, 0.5 μM eosin, 0.05 mM RbCl, and between 0 and 12 mM NaCl, as indicated, was mixed in the stopped-flow apparatus with an equal volume of buffer containing 10 mM histidine (pH 7.5), 1 mM CDTA, 0.5 μM eosin, 0.05 mM RbCl, and 50 mM NaCl. The temperature was 20 °C. Panel A shows the initial rapid fluorescence increase upon mixing, and [NaCl] was (from top to bottom) 2, 4, 6, or 10 mM, respectively, in the enzyme-containing cuvette. The transients shown are each the average of 5–7 tracings, and 240 data points were collected during the 120-ms sampling period. The inset shows the average observed rate constant obtained from 5–7 *single* tracings (±SD) as a function of the [NaCl] in the enzyme-containing cuvette. Panel B shows the additional slower phase (due to the data collection procedure, the rapid phase is not seen here). The transients in panel B were fitted by a single exponential of the form $F(t) = F(\infty) - Fe^{-k_{obs}t}$, with the values for k_{obs} ranging from 2.4 to 3.6 min^{-1}. Note that the tracings have been normalized to about the same amplitude and displaced vertically to ease comparison; the real amplitudes are given in panel D. Panel C shows the dependence of the observed rate constant for the rapid phase (panel A) on the eosin concentration when 0.1 mg/mL Na,K-ATPase in a buffer containing 10 mM histidine (pH 7.5), 1 mM CDTA, 0.05 mM RbCl, eosin, and 2 mM NaCl was mixed with an equal volume of buffer containing 10 mM histidine (pH 7.5), 1 mM CDTA, 0.05 mM RbCl, eosin, and 50 mM NaCl. The eosin concentration was the same in the two syringes and was varied between 0.1 and 1 μM. Error bars indicate the SD of 6 *single* tracings. The straight line has a slope of 64 μM^{-1} s^{-1} and an intercept of 17 s^{-1}, determined from linear regression. Panel D shows an analysis of the dependence of the amplitudes of the fluorescence responses such as those shown in panels A and B on the NaCl concentration in the enzyme-containing syringe when the Rb^+ concentration is 50 (left) or 100 μM (right). In the absence of NaCl, the total amplitude is set to 100% (◆; ±SD) and is the sum of the amplitudes of the slow (□, ○; ±SD) and rapid phases (■, ●).

since both the amount of occluded Rb^+ and the amount of slowly converting enzyme decrease in parallel with Na^+ and the rates of deocclusion and of slow fluorescence increase are about the same. The interpretation is that a conformational change ($E_2 \rightarrow E_1$) is taking place followed by a very rapid equilibration of E_1 forms with Na^+ which subsequently leads to eosin binding.

The increase in deocclusion rate constant with the NaCl concentration (Figure 4) can also be observed with the fluorescence technique. The observed rate constant describing the slow fluorescence change also increases with the NaCl concentration (Figure 6), as expected from the effect of NaCl on the rate of deocclusion of Rb^+ (Figure 4, circles). There seems to be no saturation of the observed rate constant in the

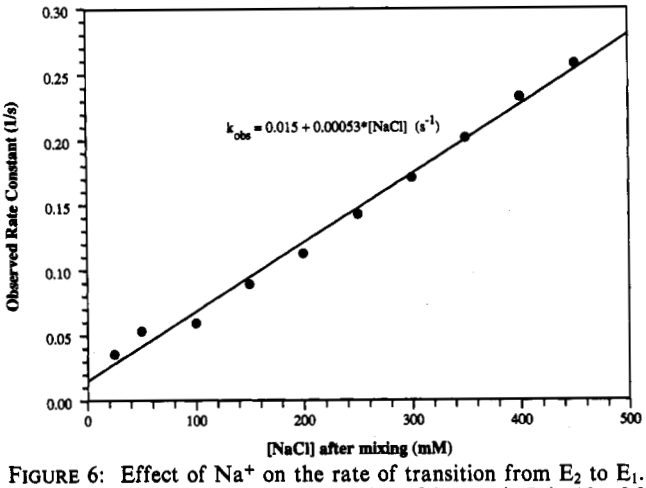


FIGURE 6: Effect of Na^+ on the rate of transition from E_2 to E_1. Na,K-ATPase at a protein concentration of 0.1 mg/mL in 10 mM histidine (pH 7.5), 1 mM CDTA, 0.5 μM eosin, and 0.05 mM RbCl was mixed with an equal volume of buffer containing 10 mM histidine (pH 7.5), 1 mM CDTA, 0.5 μM eosin, 0.05 mM RbCl, and between 50 and 900 mM NaCl. The temperature was 20 °C. The transients were fitted by a single exponential of the form $F(t) = F(\infty) - Fe^{-k_{obs}t}$, and k_{obs} is given as a function of the NaCl concentration after mixing.

range tested (final NaCl concentration up to 450 mM), but rather a linear dependence as indicated in Figure 6. Similar effects have been observed by Forbush for a number of cations on the rate constant describing deocclusion of Rb^+ [Figure 9 in Forbush (1987)]. It has previously been observed (Skou & Esmann, 1983) that both Na^+ and K^+ accelerate the $E_2 \rightarrow E_1$ conversion. If Na^+ is acting by binding to the $E_2(Rb_2)$ form—the resulting complex having a higher rate of transition to E_1—then the affinity for Na^+ must be very low since no saturation is seen up to 450 mM (Figure 6). However, the detailed kinetic implications of this effect remain obscure at the moment.

DISCUSSION

The new information gained from the experiments reported here is the proposed rapid equilibrium between a number of E_1 forms of the enzyme with Na^+ and/or Rb^+ bound. It is observed that at appropriate combinations of Na^+, Rb^+, and eosin some enzyme molecules have neither Rb^+ occluded nor eosin bound and that these molecules are transferred to the eosin-binding state very rapidly when the Na^+ concentration is increased (Figure 5). The experiments also suggest that the previous interpretation of similar experiments on shark enzyme in terms of E_2 forms of the enzyme with no cations occluded and a relatively rapid transition to E_1 (Esmann & Skou, 1983) should be reevaluated. In addition, the results reported here also confirm previously reported properties of the occlusion/deocclusion reactions, notably the strictly hyperbolic saturation curve for Rb^+ occlusion (Shani et al., 1987), the Na^+/Rb^+ antagonism, and the closeness of the deocclusion rate for Rb^+ and the slow fluorescence change associated with the conformational transition (Glynn et al., 1987; Steinberg & Karlish, 1989). Rate constants for the fluorescence changes reported here are also in agreement with recent detailed studies on fluorescein isothiocyanate modified enzyme (Faller et al., 1991; Smirnova & Faller, 1993).

Rb^+-Dependence of the Occlusion Reaction. The hyperbolic component of the curve showing the relationship between the Rb^+ concentration and the amount of occluded Rb^+ (Figure 1) indicates a binding of about 2 Rb^+ per phosphorylation site with a K_D of about 16 μM. In addition to this apparent high-affinity binding, there is a component which increases linearly with the Rb^+ concentration (up to 10 mM). Previously this has been considered to be Rb^+ trapped within the preparation in a nonspecific manner (Esmann, 1992). Support for this interpretation also comes from the finding that detergent-solubilized active Na,K-ATPase has a 10–30-fold lower amount of nonspecific Na^+ or Rb^+ bound (Esmann, 1985, 1992). Tl^+ binding and occlusion experiments have similarly been interpreted in terms of both a specific component (with 2 Tl^+ occluded or bound per ADP binding site) and a nonspecific component which in binding experiments was quite large (Jensen & Nørby, 1989). Recently Hasenauer et al. (1993) has questioned this type of interpretation and suggested the presence of additional allosteric Rb^+ occlusion sites. The present experiments are performed at 20 rather than 4 °C (Hasenauer et al., 1993), which could be an important difference.

Deocclusion of Rb^+ by NaCl. Deocclusion of Rb^+ upon addition of NaCl followed a time course which could be interpreted as a sum of two exponential terms of equal amplitude (Figure 4). The time resolution using the manual technique is such that the first time point is about 13 s after addition of NaCl, so a detailed analysis of the initial phase is not possible with this technique at 20 °C due to the high rate constant governing deocclusion. At 10 °C, on the other hand, the accelerating effect of increasing the NaCl concentration from 50 to 100 mM could be clearly seen through the whole time course. This effect is also observed in the fluorescence experiments (Figure 6). The slow fluorescence changes shown in Figure 5B are fitted adequately by single exponentials, but a better fit could be obtained if two exponentials were used in order to get exact duplication of the deocclusion time course (not shown).

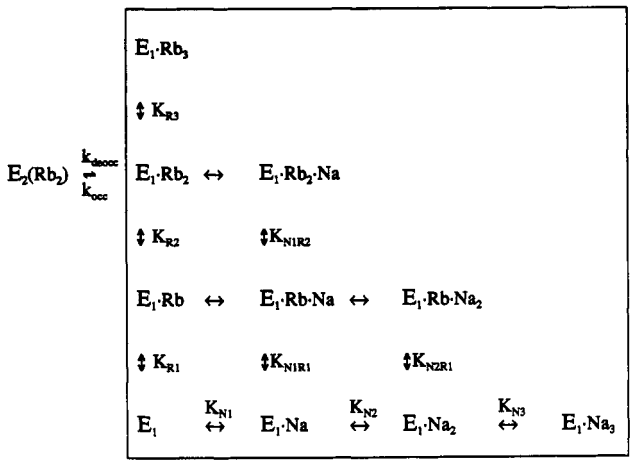
The Na^+ concentration required for displacement of half of the occluded Rb^+ is about 2.9 mM with 0.05 mM Rb^+ and about 4.4 mM with 0.1 mM Rb^+ (Figure 2). From Figure 3 it is seen that the Na^+ concentrations required to get half-maximal fluorescence of eosin under the same experimental conditions are about 4.1 and 5.8 mM, respectively. This discrepancy also points to the presence of some enzyme species that have no Rb^+ occluded but, on the other hand, that either do not have enough Na^+ bound to give a high affinity for eosin (and high fluorescence) or still have some Rb^+ bound (but not occluded), this species having low affinity for eosin. The transient experiments shown in Figure 5 with two components with markedly different rate constants support this notion.

A Model for the Interactions between Rb^+, Na^+, and Eosin. The data presented here can be qualitatively evaluated in terms of the model depicted in Scheme 1. Here all the E_1 species within the box are assumed to be in rapid equilibrium (defined as having rate constants too large to be detected with the stopped-flow method).

The individual rate constants for eosin binding (k_{on} = 64 $\mu M^{-1}\,s^{-1}$) and dissociation (k_{off} = 17 s^{-1}) are determined from Figure 5C, and the deocclusion rate constant (k_{deocc}, about 0.05 s^{-1}) is determined from the measurements shown in Figure 4. For simplicity, the deocclusions of the two Rb^+ are lumped together in a single process here [for a discussion of this, see Forbush (1987) and Glynn and Karlish (1990)]. The value for the occlusion rate constant (k_{occ}) depends on the assumed dissociation constants for Rb^+ for the E_1 form. k_{occ} has been estimated from fluorescein iodoacetamide labeled enzyme to be about 150 s^{-1} (Steinberg & Karlish, 1989).

The dissociation constants governing Na^+ and Rb^+ binding to E_1 (a total of nine independent constants; Scheme 1) could in principle be determined by model fitting of the data reported

Scheme 1



[a] The scheme shows the enzyme species present when Na,K-ATPase is mixed with Rb^+ and Na^+, assuming that only two Rb^+ can be occluded and that there are three cation sites on the E_1 form, which may be occupied by Rb^+ and/or Na^+. The equilibrium constants are defined, for example, as $K_{N2R1} = [Rb^+]\cdot[E_1\cdot Na_2]/[E_1\cdot Rb\cdot Na_2]$, and the rate constants governing occlusion and deocclusion define $K_c = k_{occ}/k_{deocc}$. Some dissociation constants are not indicated, since these, due to microscopic reversibility, can be expressed as ratios of those indicated.

in Figures 1–5. There are some limitations on these constants. As discussed by Shani et al. (1987), the strictly hyperbolic saturation curve for the high-affinity binding (Figure 1) is incompatible with binding of two Rb^+ in a sequential fashion to E_1 followed by the occlusion reaction unless widely different values for the dissociation constants for the first and second Rb^+ are assumed: the dissociation constant for the first Rb^+, K_{R1}, must be less than the smallest Rb^+ concentration employed in the binding experiments, and the dissociation constant for the second Rb^+, K_{R2}, must be in the 10 mM range (Shani et al., 1987). Recently Smirnova and Faller (1993) in a detailed transient kinetic study have found a sigmoidal dependence of the rate of transition from E_1 to E_2 with FITC-modified Na,K-ATPase. This is in disagreement with observations on the concentration dependence of equilibrium occlusion [see Jørgensen and Petersen (1982) and Shani et al. (1987)]. This discrepancy remains to be elucidated.

The data in Figures 2, 3, and 5 are evaluated using the Hill equation (see captions to these figures). The dependence of the amount of eosin-binding species or the amount of Rb^+ occluded on the Na^+ concentration can be evaluated in terms of the dissociation and rate constants describing Scheme 1 using equilibrium ligand binding analysis (Gutfreund, 1969; Segel, 1975). The sigmoidal behavior of both the fluorescence titration curves and the deocclusion measurements suggests binding of more than one Na^+ to the enzyme. This would, in a quantitative analysis of Scheme 1, lead to higher order terms in $[Na^+]$, which in the limit of high cooperativity can be approximated by the Hill equation (Segel, 1975). The sigmoid decrease in "slow" fluorescence amplitude (Figure 5, panel D) attributed to disappearence of $E_2(Rb_2)$ can also be qualitatively described by the model.

The model shown in Scheme 1 does not, however, encompass all the experimental observations reported here (and elsewhere). For example, in both an occlusion and a deocclusion experiment an increase in the Na^+ concentration should decrease the observed rate constant due to the displacement of the E_1 forms away from $E_1\cdot Rb_2$ [for a discussion, see, for example, Faller et al. (1991) and Smirnova and Faller (1993)]. Figures 4 and 6 show the opposite behavior, namely, an increase in the observed rate constants for deocclusion and slow fluorescence change. These effects must be due to the binding of Na^+ to sites other than those shown in Scheme 1.

In a model such as the one depicted in Scheme 1, the observed rate constant for Rb^+ occlusion at a very low Rb^+ concentration should be about the same as the deocclusion rate constant, k_{deocc}, since the observed rate constant for occlusion is the sum of k_{deocc} and k_{occ} times the fraction of E_1 species in the $E_1\cdot Rb_2$ state, this fraction being very small at low Rb^+ concentrations:

$$k_{obs} = k_{deocc} + k_{occ}[E_1\cdot Rb_2]/[\text{sum of other } E_1 \text{ forms}]$$

With increasing $[Rb^+]$, k_{obs} should increase due to displacement of E_1 forms toward $E_1\cdot Rb_2$, which is the opposite of the observed behavior (Hasenauer et al., 1993; this paper).

Interestingly, in the absence of added Rb^+ or Na^+ there is a large fraction—about 40% of the fluorescence change—which binds eosin rapidly when exposed to 25 mM NaCl (not shown, but the experiments are performed analogously to those shown in Figure 5, omitting RbCl in the enzyme-containing syringe). The remaining 60% of the fluorescence change is slow, indicative of a deocclusion reaction. With 1 mM NaCl in the enzyme-containing syringe there is no observable slow phase upon an increase in the Na^+ concentration to 25.5 mM, and the observed rate constant for the remaining rapid phase depends on the eosin concentration in the same manner as shown in Figure 5C. This suggests either that there is a contamination of the enzyme or buffer solutions by K^+ (or a congener, but K^+ seems most probable) or that the enzyme in the absence of added cations displays an equilibrium between, on the one hand, E_1 forms (which bind Na^+ rapidly and thus give rise to a rapid eosin binding) and, on the other, an "occluded-like" form (with no cation trapped) that has a low rate of transition to E_1. At present neither of these two possibilities can be ruled out, but since the observed rate constant for the slow fluorescence increase is the same at that seen when 20 μM K^+ is added, it seems most probable that we have a small K^+ contamination in the preparations. It should be noted that cations other than Na^+ influence the equilibrium between E_2 and E_1. Protonated Tris or histidine can also induce a conformation of the enzyme which binds eosin with high affinity, but the affinities for these cations are markedly lower than the affinity for Na^+ (Skou & Esmann, 1980).

In conclusion, the experiments reported here suggest the presence of a number of E_1 forms of the Na,K-ATPase in rapid equilibrium, only one (or a few) of which binds eosin and thus nucleotide with high affinity. The antagonism between Rb^+ (K^+) and Na^+ for nucleotide binding is thus exerted not only through a displacement between different conformations of the enzyme (E_1 and E_2) with different affinities for nucleotide but also through the binding of Rb^+ and/or Na^+ to species of the E_1 conformation, and presumably only those species liganded with (two or) three Na^+ and no Rb^+ bind eosin and nucleotides (Jensen et al., 1984) with high affinity.

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