Quantitative Analysis of the Interaction between the Fluorescent Probe Eosin and the Na^+/K^+-ATPase Studied through Rb^+ Occlusion†

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ABSTRACT: We report a study on the effect of the fluorescent probe eosin on some of the reactions involved in the conformational transitions that lead to the occlusion of the K^+-congener Rb^+ in the Na^+/K^+-ATPase. Eosin decreases the equilibrium levels of occluded Rb^+, this effect being fully attributable to a decrease in the apparent affinity of the enzyme for Rb^+ since the capacity for occlusion remains independent of eosin concentration. The results can be quantitatively described by a model that assumes that two molecules of eosin are able to bind to the Na^+/K^+-ATPase, both to the Rb^+-free and to the Rb^+-occluded enzyme regardless of the degree of cation occlusion. Concerning the effect on the affinity for Rb^+ occlusion, transient state experiments show that eosin reduces the initial velocity of occlusion, and that, like ATP, it increases the velocity of deocclusion of Rb^+. Interactions between eosin and ATP on Rb^+-release experiments seem to indicate that eosin binds to the low-affinity site of ATP from which it exerts effects that are similar to those of the nucleotide.

In the plasma membrane of eukaryote cells, the Na^+/K^+-ATPase hydrolyzes ATP in a process that is coupled to the efflux of three Na^+ and the influx of two K^+ (*1*). This reaction involves conformational changes of the enzyme, which at least imply the transition between a state with high affinity for Na^+, E_1, and another with high affinity for K^+, E_2 (*2*). E_1 can be phosphorylated by ATP forming E_1P, which may undergo a transition that leads it to E_2P. Dephosphorylation of E_2P yields E_2, which regenerates E_1 after a new transition. Some of the states of the enzyme sequester the transported cation, reducing its ability to exchange with the medium; this process is known as *occlusion*, and cations in this situation are called *occluded cations*. It is generally considered that occlusion takes place while the cations are *traveling* across the membrane during active transport. Present experimental evidence indicates that Na^+ becomes occluded in E_1P and K^+ becomes occluded in E_2 (*2*). Occlusion of K^+ and its congeners can occur under two different experimental conditions: (i) via the so-called "physiological" route, in media with Na^+, Mg^{2+}, and ATP during the hydrolysis of the nucleotide, concomitantly with K^+-promoted dephosphorylation of E_2P, or (ii) after mixing the enzyme with K^+ or its congeners, with no formation of phosphoenzyme, via the "direct" route.

One of the procedures to detect conformational changes in the Na^+/K^+-ATPase uses the fluorescence probe eosin-Y (eosin) (*3*, *4*). As this probe binds noncovalently, it is convenient to obtaining labeled enzyme without irreversible modifications. As shown by Skou and Esmann (*3*, *5*, *6*), in media with Na^+ eosin fluorescence increases because the probe binds to the enzyme ($K_d = 0.25-0.5\ \mu M$), probably at the high-affinity site for ATP, whereas in media with K^+ fluorescence is low. These properties make eosin a useful probe of the E_1 form of the enzyme.

In our laboratory, we have developed a method that accurately measures the states of the enzyme holding occluded cations and applied it to the measurement of occlusion of the K^+ congener Rb^+ (*7*). According to the current views on occlusion, this should test the E_2 form of the enzyme.

Our procedures make it possible to perform parallel experiments in which both eosin and Rb^+ are present in the media as to follow changes in E_1 (via fluorescence) and in E_2 (via Rb^+ occlusion). When performing these experiments, it is important to be aware that eosin binds to the enzyme and by itself could affect the reactions involved in the $E_1 \leftrightharpoons E_2$ equilibrium. Therefore, to obtain reliable results it is mandatory to know the effect of eosin on these reactions.

The binding of eosin to the high-affinity site in E_1 was studied in detail by fluorescence techniques (*6*, *8*). These studies also show evidence for low-affinity binding of eosin in a high $[K^+]$ medium. However, this interaction cannot directly be monitored from fluorescence experiments due to self-quenching of the probe at concentrations higher than about 1 μM (*9*).

The core of this paper consists of a quantitative study of the effects of eosin on the equilibrium between free and occluded Rb^+ as well as on the kinetics of occlusion and release of this cation from the occluded state. From the results obtained in equilibrium assays, we propose a minimal model for the interactions between the probe and the enzyme where two eosin molecules are able to bind both to the Rb^+-free

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and to the Rb^+-containing enzyme. The Rb^+ occlusion experiments presented in this paper extend the studies of the interactions between eosin and the enzyme at the low-affinity site for ATP and support the idea of eosin acting as an ATP analogue.

EXPERIMENTAL PROCEDURES

Enzyme. Na^+/K^+-ATPase was partially purified from pig kidney according to Jensen et al. (*10*). The specific activity at the time of preparation was 23−25 (μmol of Pi) min^{-1} (mg of protein)$^{-1}$ measured under optimal conditions (150 mM NaCl, 20 mM KCl, 3 mM ATP, and 4 mM $MgCl_2$ in 25 mM imidazole-HCl, pH 7.4 at 37 °C). This corresponds to an ADP-binding site concentration of 2.4−2.7 nmol (mg of protein)$^{-1}$.

Materials. [^{86}Rb]RbCl ($^{86}Rb^+$) was obtained from Perkin-Elmer NEN Life Sciences (USA). The fluorescent probe eosin (eosin-Y, free acid) and ATP were from Sigma Chemical Co (USA). ATP was freed of Na^+ by passing a solution of ATP through a column containing a cation-exchange resin (Bio Rad AG MP-50). Contaminant [Na^+] in the eluate, measured by flame photometry, was less than 0.05% of the [ATP] on a mol-to-mol basis. All other reagents were of analytical grade.

Reaction Conditions. Incubations were performed at 25 °C in media containing 25 mM imidazol-HCl (pH 7.4 at 25 °C) and 0.25 mM EDTA. The concentrations of other components varied according to the experiments and are indicated under Results. Free Mg^{2+} was taken as equal to [$MgCl_2$] minus [EDTA].

Determination of Occluded Rb^+. This was performed according to Rossi et al. (*7*). Briefly, reactions were carried out in a rapid-mixing apparatus (SFM4 from Bio-Logic, France) connected to a chamber that contained a Millipore filter through which an ice-cold solution of 30 mM KCl and 20 mM imidazole-HCl (pH 7.4 at 0 °C) was flowing at a rate of 40 mL/s. As the reaction mixture is injected into the chamber, quenching occurs due to a sudden drop in temperature and in ligand concentrations and the enzyme is retained and washed in the Millipore filter. The filter is then removed, dried, and counted for radioactivity. Blanks were estimated from the amount of $^{86}Rb^+$ retained by the filters when the enzyme was omitted.

Since occlusion occurred in media lacking Na^+ and ATP, occluded Rb^+ was formed via the "direct route" in both equilibrium and transient experiments.

Equilibrium occlusion of Rb^+ was attained by incubating enzyme (30 μg/mL) between 10 to 100 min (*11*) with different Rb^+ and eosin concentrations.

Time Courses of Formation and Breakdown of Occluded Rb^+. The time course of formation of occluded Rb^+ was measured after mixing the enzyme suspension (150 μg of protein/mL) in the rapid mixing apparatus with an equal volume of a medium having 200 μM $^{86}Rb^+$ and different concentrations of eosin. To measure the time course of breakdown of occluded Rb^+, one volume of an enzyme suspension (560 μg of protein/mL) equilibrated in a medium with 100 μM $^{86}Rb^+$ was mixed in the rapid mixing apparatus with 19 vol of a solution with 100 μM of unlabeled Rb^+ as to cause a 20-fold decrease in the specific activity of $^{86}Rb^+$. Reaction media also contained eosin, ATP, and/or $MgCl_2$ at the concentrations indicated under Results.

Treatment of the Data. The equations were fitted to the experimental data by a nonlinear regression procedure based on the Gauss−Newton algorithm using commercial software (Excel 7.0 for Windows and Sigma-Plot 2.0 for Windows).

Model Selection. Regression procedures permitted us to define the goodness of fit of a given equation to the experimental results and to choose among different models by using the Second-Order Akaike Information Criterion (see ref *12*), $AIC_C = N \ln(SS/N) + 2KN/(N - K - 1)$, with $N =$ number of data, $K =$ number of parameters of the fitted function plus 1, and SS = sum of weighted squared errors of residuals. Unitary weights were considered in all cases. The best equation was chosen as that which gave the lower value of AIC_C. To compare the goodness of fit between two different models, 1 and 2, we used the evidence ratio ($= \exp(1/2\ \Delta AIC_C)$, with $\Delta AIC_C = AIC_{C(\text{model 1})} - AIC_{C(\text{model 2})} > 0$), which indicates how many times more probable model 2 is, with the lower AIC_C value, than model 1 is to be the true one.

The quantitative analysis of Rb^+ occlusion and eosin binding and the calculation of the equilibrium constants were performed according to a procedure based on that described in ref *13*.

RESULTS

The Effects of Eosin on the Equilibrium Distribution between Occluded and Free Rb^+. We measured the amount of occluded Rb^+ (Rb_{occ}) after incubation of Na^+/K^+-ATPase with Rb^+ and eosin for a sufficient length of time (more than 10 min) as to reach equilibrium. The results plotted as Rb_{occ} vs [eosin] for different fixed concentrations of Rb^+ are given in Figure 1A. It can be seen that Rb_{occ} decreased asymptotically to a level that depended on [Rb^+] as the concentration of eosin increased. Note that the value of Rb_{occ} of about 4.4 nmol (mg of protein)$^{-1}$ obtained at zero eosin and at high [Rb^+] is consistent with a maximum of two Rb^+ occluded per enzyme unit (see Experimental Procedures). Figure 1B shows that, as the concentration of Rb^+ increased, the $K_{0.5}$ for eosin increased, indicating a decrease in apparent affinity for eosin with Rb^+.

Analysis by nonlinear regression was employed to work out a minimal model that could describe these results. The complete procedure, which is detailed in a previous paper (*13*), requires first establishing the stoichiometric coefficients that measure the maximal number of occluded Rb^+ and of bound eosin to the enzyme. Since up to two Rb^+ are known to be occluded in the enzyme, we focused our analysis to determine the maximal stoichiometry of eosin binding. This seems to be particularly important since it has been reported that, depending on the conditions, one or two eosin molecules can bind per phosphorylation site (*3*, *14*). Assuming that a single eosin binds per ATPase (a model that would correspond to the first two rows of the model in Figure 3), Rb_{occ} will follow eq 1, which was fitted to the curves in Figure 1 for each [Rb^+] tested

$$Rb_{occ} = \frac{A_0 K_{0.5} + A_\infty [eo]}{K_{0.5} + [eo]} \qquad (1)$$

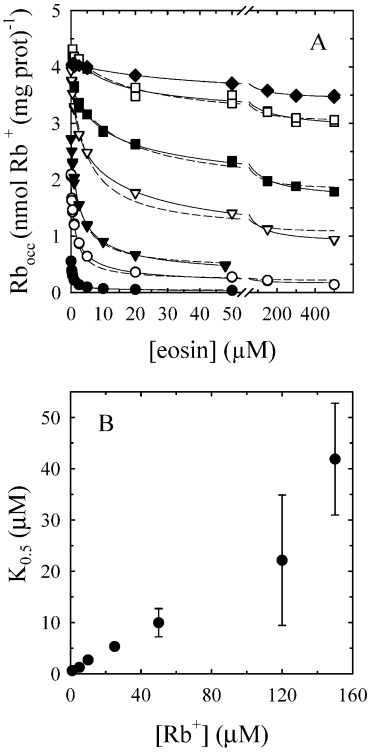

FIGURE 1: The effects of eosin on the equilibrium distribution between free and occluded Rb^+. The equilibrium level of Rb_{occ} as a function of [eosin] in media containing 1 (●), 5 (○), 10 (▼), 24 (▽), 49.5 (■), 120 (□), and 150 (◆) μM Rb^+, in media containing from 0 to 500 μM eosin (panel A). Dashed and continuous lines are plots of eq 1 or eq 2, respectively, for the best fitting values of their parameters. Panel B shows the effect of Rb^+ on $K_{0.5}$ for eosin (± 1 SE). Values of $K_{0.5}$ were calculated for each [Rb^+] from the best fitting curves (continuous lines in panel A) as the concentration of eosin that gives a value of Rb_{occ} equal to $1/2(A_0 + A_2)$.

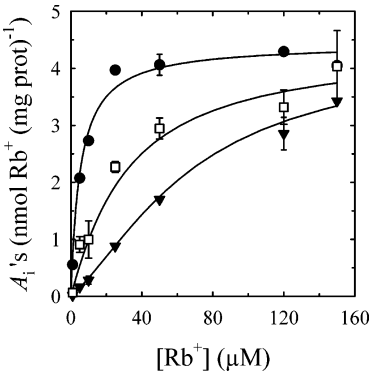

FIGURE 2: The best fitting values of A_0 (●), A_1 (□), and A_2 (▼) in eq 2 as a function of the concentration of Rb^+. These were obtained by fitting eq 2 to the results in Figure 1 for each [Rb^+] tested. The continuous lines are the predicted values of A_o, A_1, and A_2 according to their meaning in Table 2 using values of E_T and $K_{i,j}$'s given in Table 3. Vertical bars are ± 1 SE.

In this equation, A_0 and A_∞ are the values of Rb_{occ} when the concentration of eosin (eo) is zero or infinity, respectively, and $K_{0.5}$ is an apparent dissociation constant for eosin. As shown by the dashed lines in Figure 1, except at 150 μM Rb^+ eq 1 fitted the results with some degree of bias. A small but consistent improvement in the fit, accompanied by a decrease in the bias, was obtained when two eosin molecules were considered to bind per ATPase (continuous lines in Figure 1). This case corresponds to the model in Figure 3. In these circumstances, since free and occluded

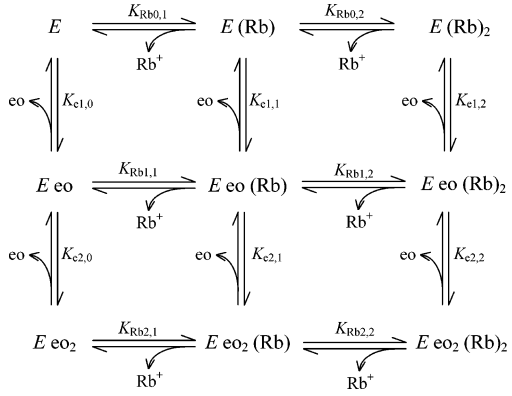

FIGURE 3: A minimal model for the equilibria between enzyme, Rb^+, and eosin during direct occlusion of Rb^+ in the Na^+/K^+-ATPase. Parentheses denote an occluded Rb^+, and eo and *E* denote eosin and Na^+/K^+-ATPase, respectively.

Table 1: Comparison of the Fit to the Results in Figure 1 Considering the Binding of One (eq 1) or Two (eq 2) Molecules of Eosin to the Na^+/K^+-ATPase[a]

[Rb^+] (μM)	eq 1		eq 2		
	SS	AIC_C	SS	AIC_C	evidence ratio[b]
1	0.000825	−168.7	0.000459	−170.7	2.70
5	0.0572	−106.5	0.00820	−137.5	5.48×10^6
10	0.0229	−140.7	0.00939	−153.1	486.6
24	0.202	−81.18	0.00857	−136.6	1.10×10^{12}
49.5	0.0682	−75.70	0.0133	−92.20	3825
120	0.0681	−89.31	0.0522	−85.53	0.151
150	0.00633	−122.9	0.00631	−113.9	0.0110

[a] SS and AIC_C were obtained as described under Experimental Procedures. [b] In all cases, the evidence ratio was calculated as $\exp[1/2(AIC_{C(eq\ 1)} - AIC_{C(eq\ 2)})]$.

Rb^+ are in equilibrium, Rb_{occ} will obey eq 2

$$Rb_{occ} = \frac{A_0K_1K_2 + A_1K_2[eo] + A_2[eo]^2}{K_1K_2 + K_2[eo] + [eo]^2} \qquad (2)$$

where A_0, A_1, and A_2 represent now Rb_{occ} with none, one, or two molecules of eosin (eo) bound, respectively, and K_1 and K_2 are apparent dissociation constants for eosin. It can be seen that Rb_{occ} will go from A_0 to A_2 as the concentration of eosin goes from zero to infinity.

A quantitative estimate of the improvement of fit obtained using eq 2 as compared with eq 1 is given in Table 1 which makes clear that, except for the two highest [Rb^+] tested, each $Rb_{occ} = f([eosin])$ curve gave values of AIC_C that were considerably lower when this equation instead of eq 1 was fitted. The relative improvement of the goodness of fit using eq 2 relative to that using eq 1 is also shown by the evidence ratio values given in the last column of Table 1. We also tried an equation that considered the binding of three eosin molecules per ATPase. This only slightly improved the fitting but gave values of AIC_C that, in general, were higher than those obtained using eq 2 (data not shown). This statistical evidence favored the selection of eq 2 as the one that provides the best description for the curves in Figure 1.

Once the maximal stoichiometry of 2 for eosin binding was established, we investigated which enzyme states containing bound eosin and bound or occluded Rb^+ were present. As a first approach, we plotted the best-fitting values of A_0, A_1, and A_2 in eq 2 as a function of [Rb^+] (Figure 2).

Table 2: Meaning of Coefficients A_0, A_1, A_2, K_1, and K_2 of eq 2 in Terms of the Equilibrium Constants of the Scheme in Figure 3[a]

parameters	meaning
A_o	$E_T\,(K_{Rb0,2}[Rb^+] + 2[Rb^+]^2)/(K_{Rb0,1}K_{Rb0,2} + [Rb^+]K_{Rb0,2} + [Rb^+]^2)$
A_1	$E_T\,(K_{Rb1,2}[Rb^+] + 2[Rb^+]^2)/(K_{Rb1,1}K_{Rb1,2} + [Rb^+]K_{Rb1,2} + [Rb^+]^2)$
A_2	$E_T\,(K_{Rb2,2}[Rb^+] + 2[Rb^+]^2)/(K_{Rb2,1}K_{Rb2,2} + [Rb^+]K_{Rb2,2} + [Rb^+]^2)$
K_1	$(K_{Rb1,1}K_{Rb1,2}K_{e1,0} + K_{Rb1,2}K_{e1,1}[Rb^+] + K_{e1,2}[Rb^+]^2)/(K_{Rb1,1}K_{Rb1,2} + K_{Rb1,2}[Rb^+] + [Rb^+]^2)$
K_2	$(K_{Rb2,1}K_{Rb2,2}K_{e2,0} + K_{Rb2,2}K_{e2,1}[Rb^+] + K_{e2,2}[Rb^+]^2)/(K_{Rb2,1}K_{Rb2,2} + K_{Rb2,2}[Rb^+] + [Rb^+]^2)$

[a] E_T represents the total amount of enzyme expressed in nmol (mg of protein)$^{-1}$.

In agreement with results obtained under similar conditions (*8*, *11*, *15*), $A_0 = f([Rb^+])$ can be adequately described by a single hyperbola with a value of $K_{0.5}$ of 5.67 ± 0.88 μM. In contrast with the hyperbolic shape of the curve for A_0, that for A_2 is a sigmoid function of $[Rb^+]$. It is clear in Figure 2 that A_0, A_1, and A_2 tend to the same value when $[Rb^+]$ tends to infinity, indicating that, when eosin is bound, the maximal occlusion capacity of the enzyme is not affected. In other words, in a model in which a maximum of two eosin molecules are able to bind to the enzyme, the states holding one or two eosin and two Rb^+ occluded must exist.

A further regression analysis of the whole set of results, based on the procedure described by González-Lebrero et al. (*13*), led us to postulate the minimal model shown in Figure 3. As it was to be expected from our previous evidence, in the absence of eosin the amount of enzyme species with bound, but not occluded, Rb^+ is negligible (*13*). According to our analysis, this is also true in the presence of any amount of eosin.

The model in Figure 3 can be expressed in terms of eq 2 taking into account the definitions for the parameters, which are now the functions of $[Rb^+]$ given in Table 2. From these equations, it can be seen that the model in Figure 3 adequately predicts that A_0, A_1, and A_2 (i.e., the states with none, one, or two eosin molecules bound) tend to the same value ($2E_T$) as the concentration of Rb^+ tends to infinity. Correspondingly, the model predicts that K_1 and K_2 will vary from $K_{e1,0}$ and $K_{e2,0}$, to $K_{e1,2}$ and $K_{e2,2}$.

After the parameters in eq 2 were replaced by the definitions in Table 2, the resulting function was fitted to the whole set of data in Figure 1, thus obtaining the values of equilibrium constants and total amount of enzyme (E_T) shown in Table 3. As in previous papers, the constraint $K_{Rb0,2} = 4K_{Rb0,1}$ was fixed to keep the hyperbolic shape of $Rb_{occ} = f([Rb^+])$ observed in the absence of ligands other than Rb^+ (see A_0 in Figure 2).

To test if the model of Figure 3 was minimal, i.e., if no subset of states was able to describe equally well the results, equations derived by systematically omitting enzyme states from the model in Figure 3, in different combinations, were also fitted to the data. We found that the best fitting was obtained when all possible states with occluded Rb^+ and bound eosin were taken into account (SS = 1.30, $AIC_C = -585$). For instance, when omitting the states with two bound eosins, Eeo_2, $Eeo_2(Rb)$, and $Eeo_2(Rb_2)$, the values for SS and AIC_C where 1.66 and -560, respectively. The evidence ratio calculated from these AIC_C values, which equals 2.68×10^5, strongly favors the two-site model. Also, when only Eeo_2 was omitted (SS = 1.65, $AIC_C = -555$), the values of the equilibrium constants for the dissociation of eosin from $Eeo_2(Rb)$ and $Eeo_2(Rb_2)$ became so large (higher than 10^{10} μM) that the amount of these states were made negligibly small.

Table 3: Best Fitting Values of the Equilibrium Constants of the Scheme in Figure 3[a]

constant	best fitting value (μM $\pm$ SE)
$K_{Rb0,1}$	2.66 ± 0.28
$K_{Rb0,2}$	10.7 ± 1.1
$K_{Rb1,1}$	19.3 ± 4.7
$K_{Rb1,2}$	52 ± 12
$K_{Rb2,1}$	83 ± 15
$K_{Rb2,2}$	55.4 ± 8.7
$K_{e1,0}$	0.248 ± 0.031
$K_{e1,1}$	1.80 ± 0.59
$K_{e1,2}$	8.8 ± 4.3
$K_{e2,0}$	18 ± 12
$K_{e2,1}$	76 ± 48
$K_{e2,2}$	82 ± 26

[a] $K_{Rb1,1}$, $K_{Rb1,2}$, $K_{Rb2,1}$, and $K_{Rb2,2}$ were calculated using the thermodynamic equivalence of pathways (see ref *13*) and propagating the error of the estimations of the fitted constants. E_T was 2.218 ± 0.021 nmol (mg of protein)$^{-1}$.

Hence, the presence of relatively large error values for some of the equilibrium constants in Table 3 does not justify the exclusion of the involved states from the model.

From the values of equilibrium constants in Table 3 it can be seen that, as the number of eosin bound to the enzyme "i" increases, $K_{Rbi,1}$ and $K_{Rbi,2}$ increase in such a way that the ratio $K_{Rbi,2}/K_{Rbi,1}$ decreases. This indicates that eosin decreases the affinity for Rb^+, but it promotes an increase in the positive interaction for the occlusion of the second Rb^+, explaining why A_i versus $[Rb^+]$ becomes progressively more sigmoid and shifted to the right at higher eosin concentrations. Besides, as the number of Rb^+ occluded in the enzyme "j" increases, $K_{e1,j}$ and $K_{e2,j}$ increase and the ratio $K_{e2,j}/K_{e1,j}$ decreases, indicating a drop both in the affinity for eosin and in the difference between the affinities for the two eosins (this is reflected in Figure 1 and Table 1, where the better fit given by eq 2 with respect to that given by eq 1 tends to disappear at high $[Rb^+]$). Note that the value of the equilibrium constant for the dissociation of eosin from the Eeo complex, $K_{e1,0}$, is very close to that of 0.25−0.45 μM reported by Skou and Esmann (*3*) from experiments measuring equilibrium binding of eosin in media containing 20 or 150 mM Na^+.

Since the decrease caused by eosin in Rb_{occ} observed in equilibrium conditions could be due to a slowing of Rb^+ occlusion or to an acceleration of Rb^+ deocclusion, we investigated the effect of the probe in transient experiments.

Effects of Eosin on the Time Course of Rb^+ Occlusion. To evaluate the effect of eosin on Rb^+ occlusion, Na^+/K^+-ATPase was incubated during periods ranging from 0.037 to 150 s in media containing 100 μM Rb^+ and from 0 to 600 μM eosin. In control experiments (not shown), we measured the time course of $^{86}Rb^+$ occlusion in media in

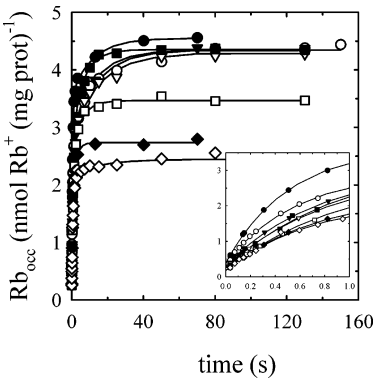

FIGURE 4: Effects of eosin on the time course of Rb^+ occlusion. Reaction media contained 100 μM Rb^+ and 0 (●), 0.1 (○), 0.4 (▼), 2 (▽), 5 (■), 50 (□), 250 (◆), or 600 (◇) μM eosin. The inset shows the initial 1 s of the incubation times. The continuous lines are plots of the sum of two increasing exponential functions of time plus a constant term.

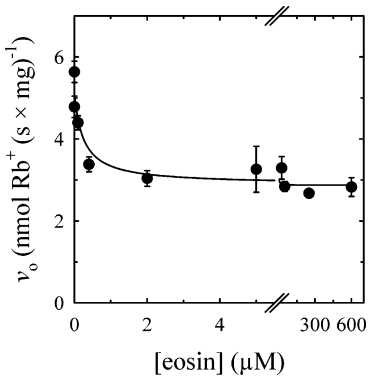

FIGURE 5: Initial rate of Rb^+ occlusion (v_o) as a function of [eosin]. v_o was obtained as described in the main text for each curve shown in Figure 4, and vertical bars indicate 1 ± SE. The continuous line is the plot of eq 3 setting the values of $K_{e1,0}$ and $K_{e2,0}$ as those given in Table 3 and adjusting those of v_{oi}. The best fitting values obtained for v_{o0}, v_{o1}, and v_{o2} were (± 1 SE): 5.10 ± 0.22, 2.92 ± 0.25, and 2.87 ± 0.25 nmol of Rb^+ (mg of protein)$^{-1}$ s^{-1}, respectively.

which eosin had either been equilibrated with the enzyme before adding $^{86}Rb^+$ or added together with $^{86}Rb^+$. No differences were detected between the time courses of $^{86}Rb^+$ occlusion under both conditions. This strongly suggests that eosin reaches equilibrium with the enzyme before Rb^+ occlusion. On the basis of these results, we considered it unnecessary to incubate the eosin with enzyme prior to addition of $^{86}Rb^+$. This has the advantage of avoiding the risk of the inactivation by light of the Na^+/K^+-ATPase in media with eosin reported by Esmann (*6*).

The results of Rb^+ occlusion experiments are shown in Figure 4 as plots of Rb_{occ} vs incubation time for each of the eosin concentrations tested. It can be seen that Rb_{occ} increased with time reaching a constant value that corresponds to the attainment of equilibrium, this value being lower at higher concentrations of eosin (cf. Figure 1). Best fit to each of the curves in Figure 4 was achieved by the sum of two increasing exponential functions of time plus a constant term that probably represents a component that is so fast that it reaches equilibrium before the shortest measured time. The plot of the initial part of the time courses (up to 1 s, inset to Figure 4) shows that the initial rate of occlusion also decreased with eosin concentration.

We estimated the values of the initial rate of occlusion (v_o) for each curve using the mathematical device of fitting the points measured at $t \leq 0.8$ s by the second-order polynomial: $a + v_o t + ct^2$. The first derivative of this function at $t = 0$ will be equal to v_o. The dependence of v_o on eosin concentration obtained in the experiments in Figure 4 is given in Figure 5. It can be seen that the initial rate of occlusion drops with eosin concentration along a curve that asymptotically decreases to a nonzero value. If this response were consistent with the scheme in Figure 3 and eosin were in rapid equilibrium with the enzyme, v_o should be described by eq 3.

$$v_o = \frac{v_{o0}K_{e1,0}K_{e2,0} + v_{o1}K_{e2,0}[eo] + v_{o2}[eo]^2}{K_{e1,0}K_{e2,0} + K_{e2,0}[eo] + [eo]^2} \quad (3)$$

This equation considers that the occlusion of Rb^+, at time tending to zero, takes place into *E*, *E*eo, and *E*eo$_2$ of the scheme in Figure 3 and that v_o is a linear combination of the occlusion velocities for each state (i.e., v_{o0}, v_{o1}, and v_{o2}). Equation 3 was fitted to the data in Figure 5, setting the values for $K_{ei,0}$ as those shown in Table 3. The continuous line in Figure 5 is the plot of eq 3 for the values of v_{oi} given in the legend of the figure. It is apparent that the model in Figure 3 and the values of the $K_{ei,0}$ in Table 3 are able to provide an adequate prediction of v_o as a function of eosin concentration. Figure 5 also shows that there is a significant change in v_o for eosin concentrations between 0 and 0.5 μM, 0.2−0.5 μM being the concentrations of the probe usually employed in fluorescence experiments (*5*, *8*, *16*).

Using the values of E_T and of v_o at 100 μM Rb^+, the calculated value of k_{on}[1] for Rb^+ occlusion in the absence of eosin is 0.0245 s^{-1} μM^{-1}, which is very similar to that already reported by us (*11*).

Effects of Eosin on the Time Course of Release of Occluded Rb$^+$. To look at this effect, Na^+/K^+-ATPase equilibrated with 100 μM $^{86}Rb^+$, which yields about 90% full occupation, was mixed with enough of media with eosin and the same concentration of unlabeled Rb^+ as to produce a 20-fold isotopic dilution of the cation. Eosin concentration in the deocclusion media varied from 0 to 950 μM. As for the case of occlusion assays, we obtained evidence from deocclusion experiments that eosin equilibrates very rapidly with the ATPase. Thus, there was no need to preincubate the enzyme containing occluded $^{86}Rb^+$ with the probe, which was added together with the unlabeled Rb^+.

Results of these experiments are given in Figure 6 as plots of Rb_{occ} vs time after isotopic dilution for each eosin concentrations tested. It can be seen that eosin induces a significant increase in the rate of deocclusion. In most cases, the breakdown of Rb_{occ} was described by the sum of two decreasing exponential functions of time plus a time-independent term, but at the higher concentrations of eosin tested a better fit to the results was obtained by a single-exponential function plus a time-independent term. Note that, as the time courses were not corrected for the amount of occluded $^{86}Rb^+$ remaining at infinite time after the 20-fold isotopic dilution, the time-independent term must include this value.

[1] Abbreviations: k_{on}, bimolecular binding rate constant; k_{off}, dissociation rate constant.

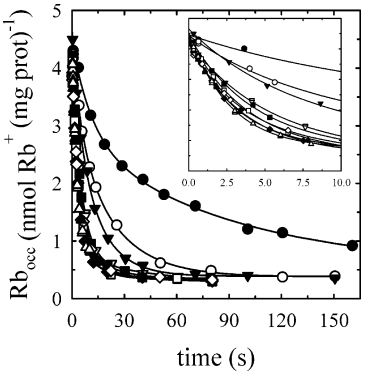

FIGURE 6: Effects of eosin on the time course of release of occluded Rb^+. Occluded $^{86}Rb^+$ remaining after a 20-fold dilution of the specific activity of $^{86}Rb^+$ was plotted as a function of time. During deocclusion, the concentration of eosin was either 0 (●), 5 (○), 10 (▼), 36 (▽), 47 (■), 118 (□), 238 (◆), 475 (◇), 750 (▲), and 950 (△) μM. Inset shows a detail of the first 10 s. The continuous lines are the plot of the sum of two decreasing exponential functions of time plus a time-independent term.

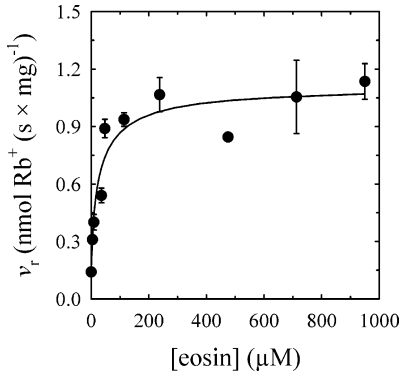

FIGURE 7: Initial rate of release of occluded Rb^+ (v_r) as a function of [eosin]. v_r was calculated as described in the main text for each curve shown in Figure 6, verticals bars indicate 1 ± SE. The best fitting values obtained for v_{r0}, v_{r1}, and v_{r2} were (± 1 SE): 0.12 ± 0.10, 0.64 ± 0.13, and 1.1 ± 0.07 nmol of Rb^+ (mg of protein)$^{-1}$ s^{-1}, respectively.

Following an analogous procedure as that used when measuring the initial rate of occlusion, we calculated the initial rate of Rb^+ release (v_r) fitting the first 5−7 points of the time courses to the second-order polynomial: $a - v_r t + ct^2$. Figure 7 shows that as [eosin] tended to infinity, v_r increased tending to a constant value that is almost an order of magnitude higher than that obtained in the absence of the probe. According to the scheme in Figure 3, as eosin rapidly equilibrates with the states of the enzyme containing two Rb^+, results should be described by

$$v_r = \frac{v_{r0}K_{e1,2}K_{e2,2} + v_{r1}K_{e2,2}[eo] + v_{r2}[eo]^2}{K_{e1,2}K_{e2,2} + K_{e2,2}[eo] + [eo]^2} \qquad (4)$$

This equation considers v_r as the linear combination of v_{r0}, v_{r1}, and v_{r2}, which respectively correspond to the initial rates of Rb^+ release from $E(Rb)_2$, $Eeo(Rb)_2$, and $Eeo_2(Rb)_2$, whose relative distribution in equilibrium will be determined by the concentration of eosin. The continuous line in Figure 7 is a plot of eq 4 for the best fitting values of v_{r0}, v_{r1}, and v_{r2} (see legend to Figure 7), using the values of the equilibrium constants of Table 3.

Equation 4 indicates that v_r will go from v_{r0} in the absence of the probe to v_{r2} when two molecules of eosin are bound to the Rb^+-occluded enzyme (right-hand side of the scheme in Figure 3) following a curve whose shape is consistent with the model obtained in equilibrium conditions. Figure 7 shows that eosin increases v_r 9-fold (see legend to the figure), with an affinity that is much lower than that for the effect of eosin on the initial rate of Rb^+ occlusion (cf. Figure 5 and the values for $K_{ei,2}$ and $K_{ei,0}$ in Table 3). The value of the rate coefficient for deocclusion (k_{off}) in the absence of eosin calculated from v_{r0} and the ordinate value of Rb_{occ} is 0.062 s^{-1}, in very good agreement with published results (*11*, *17*).

Table 4: Effects of Eosin and ATP on the Initial Rate of Release of Occluded Rb^+ (v_r)[a]

[eosin] mM	[ATP] mM	[$MgCl_2$] mM	v_r nmol of Rb^+ (mg of protein)$^{-1}$ s^{-1}
0.1	none	1.25	4.73 ± 0.27
none	0.1	1.25	13.44 ± 0.98
1	0.1	1.25	4.85 ± 3.04
0.1	none	1.90	4.40 ± 0.82
none	0.1	1.90	11.88 ± 1.92
1	0.1	1.90	4.68 ± 0.40
0.1	none	none	0.94 ± 0.04
none	0.1	none	1.25 ± 0.13
1	0.1	none	1.63 ± 0.17

[a] v_r (values ± 1 SE) were obtained as indicated in the main text. Reaction media contained 100 μM Rb^+ and eosin, ATP, or $MgCl_2$ as indicated.

Interaction between ATP and Eosin at the Low-Affinity Site for ATP. It is known that ATP stimulates with low affinity the rate of deocclusion of K^+ and its congeners (*17*−*19*), and that eosin competes with ATP for the high-affinity site in the enzyme (*3*, *6*). In Figure 7, we have shown that eosin, like ATP, also increases the rate of Rb^+ release. To investigate if this effect of eosin is exerted by the binding of the probe to the low-affinity site for ATP, we performed experiments measuring the initial rate of Rb^+ release (v_r) seeking a possible blockage by eosin of the ATP-stimulated deocclusion. This would become apparent if ATP were significantly more effective than eosin in promoting deocclusion of Rb^+, providing that both ligands competed for the same site.

Rb^+-release experiments were performed in media with ATP, eosin, and 1.25 mM $MgCl_2$ (1 mM free Mg^{2+}). Mg^{2+} was added to enhance the effect of ATP on the deocclusion rate (*17*), expecting to increase the differences between the rates of eosin and ATP-stimulated deocclusion, although it is a known fact that "per se" this cation only slightly changes the rate of deocclusion of Rb^+ (*18*). For instance, when we measured this rate in media with 7 μM Rb^+, addition of 1 mM $MgCl_2$ (0.7 mM free Mg^{2+}) just increased the rate coefficient from 0.081 ± 0.009 to 0.186 ± 0.016 s^{-1}.

Values of v_r in Table 4 show that, when Mg^{2+} is present, 100 μM ATP is 2.5−3-fold more effective than 100 μM eosin to accelerate Rb^+ deocclusion. When ATP and excess eosin (1 mM) were added together in the deocclusion medium, the effect of ATP disappeared and v_r reached a value similar to that obtained with 100 μM eosin. This behavior strongly suggests that both ligands compete for the site(s) from which they stimulate deocclusion. Since similar results were obtained in the presence of 1.9 mM $MgCl_2$, the blocking effect of eosin seems not to be due to changes in the availability of Mg^{2+} in the assay medium. Additionally,

1.25 mM $MgCl_2$ were sufficient to activate the effects of both ATP and eosin on the rate of Rb^+ release.

Table 4 also shows that, in the absence of Mg^{2+}, simultaneous addition of both ATP and eosin produces only a small difference as compared to that observed for the separate addition of ATP or eosin. This is probably related to the fact that Mg^{2+} activates both the ATP- and the eosin-promoted deocclusion of Rb^+, but while the former increased about 10-fold, the latter increased only 5-fold under these conditions.

DISCUSSION

The kinetic study of the effects of eosin, Rb^+, and ATP on the Na^+/K^+-ATPase reported in this paper provided important information about the interaction of eosin on the E_2 conformer of the ATPase. One of the main findings of our experiments is that the effects of eosin are remarkably similar to those of ATP not only on the E_1 but also on the E_2 conformer of the pump.

From the results of equilibrium experiments, in which occluded Rb^+ was measured in media with different concentrations of eosin and Rb^+, we found that like ATP (*13*, *20*) eosin is unable to fully displace occluded Rb^+, indicating that the enzyme can hold simultaneously occluded Rb^+ and eosin.

Despite the fact that eosin does not affect the capacity for Rb^+ occlusion, which remains constant at two Rb^+ ions per phosphorylation site, the binding of eosin induces significant changes in the affinity for Rb^+ of the Na^+/K^+-ATPase. The change observed in Figure 2 in the shape of $Rb_{occ} = f([Rb^+])$ curves, from hyperbolic in the absence of eosin (A_0) to sigmoid in the presence of this ligand (A_2), reveals an alteration in the interaction between the affinities of the sites from which Rb^+ occlusion takes place. In relation to this, the ratio between the macroscopic occlusion constants ($K_{Rbi,2}/K_{Rbi,1}$ in the scheme in Figure 3) are no longer 4 as it happens in the absence of eosin.

Thermodynamic considerations indicate that, if eosin decreases the affinity for Rb^+, Rb^+ will do the same with the affinity for eosin. This is manifest both for the binding of one or two molecules of eosin (compare $K_{e1,0}$ vs $K_{e1,2}$ and $K_{e2,0}$ vs $K_{e2,2}$).

The effects of ATP on Rb^+ occlusion reported by us in a previous paper (*13*) are remarkably analogous to the above-mentioned effects for the probe. Like eosin, ATP changes from hyperbolic to sigmoid the shape of $Rb_{occ} = f([Rb^+])$ curves and concomitantly Rb^+ decreases the affinity of the pump for the nucleotide.

As it is proposed in the model of Figure 3, our analysis suggests that up to two eosin molecules would be able to bind both to the Rb^+-free and to the Rb^+-saturated enzyme. Since this proposal does not come from a direct measurement of the binding of eosin to the enzyme, the alternative explanation of heterogeneity in the Na^+/K^+-ATPase preparation should be considered. If that were the case, however, this feature should be expected to appear not only as a response to eosin but also to other ligands, a fact that we did not observe (*13*). Therefore, the possibility of heterogeneity of the preparation seems to be improbable.

Our evidence for a possible binding of two eosin per enzyme unit should be compared with the results obtained by Skou and Esmann, who, measuring binding in equilibrium, showed that in media containing from 2 to 150 mM Na^+ between one and two high-affinity bound eosin were detected per ^{32}P-labeling site (*3*, *14*). The same authors also found two high-affinity eosin-binding sites in media with 5 mM Mg^{2+}.

Despite the fact that eosin is a fluorescent sensitive reporter for nucleotide binding, it must be stressed that the possible existence of two sites for eosin does not necessarily imply the existence of two nucleotide sites proposed by others (*21*−*23*).

The constant $K_{e1,0}$ for the dissociation of eosin from the state having only one eosin bound (*E*eo), obtained by fitting the equations of the minimal model proposed in this work to the experimental data, was 0.25 ± 0.03 μM. This is coincident with the K_d of 0.25 μM for the dissociation of eosin from the enzyme in media with 20 mM Na^+ (probably in E_1) measured by binding assays (*3*). Therefore, it is clear that in media with 25 mM imidazol and without added cations, the affinity of eosin for the free enzyme is identical to that obtained for E_1. We have observed that the fluorescence signal in 25 mM imidazol is very similar to that obtained in media with Na^+ (data not shown). It would seem therefore that imidazol seems to mimic the ability of Na^+ in displacing the ATPase toward its E_1 conformer, in agreement with findings by Esmann and Fedosova (personal communication).

Particularly interesting is the fact that the affinity for eosin to the free enzyme is very close to that previously obtained for ATP at the high-affinity site (*3*, *13*, *24*).

The decrease in the equilibrium levels of Rb_{occ} in media with limiting concentrations of Rb^+ could be caused by effects on the velocity of Rb^+ occlusion and/or of deocclusion. Transient experiments in this paper indicate that both factors contribute to the process.

Inspection of Figure 5 shows that the formation of *E*eo suffices to decrease the rate of Rb^+ occlusion, whereas the binding of the second eosin lacks any effect. This can be seen in the fact that the value of v_{o1} is one-half of that of v_{o0} and very similar to that of v_{o2}. The good fit of eq 3 to the values of v_o vs [eosin] plotted in Figure 5, setting the values of $K_{i,j}$ to those obtained from the equilibrium experiments, provides additional evidence for the agreement between the proposed model and the experimental results.

Attention must be paid to the fact that most fluorescence experiments using eosin were performed with concentrations of the probe between 0.2 and 0.5 μM (*5*, *8*, *16*, *25*), which are coincident with those that produce almost a complete decrease in the rate of Rb^+ occlusion.

The increase in the rate of Rb^+ deocclusion produced by eosin agrees with unpublished results by Glynn and coworkers (*25*). The initial rate of Rb^+ release is increased about nine-fold by the probe, the affinity for this effect being much lower than that exhibited for the decrease in the initial rate of Rb^+ occlusion (cf. Figure 5 and Figure 7). This difference in affinities parallels that observed for the equilibrium binding of eosin to the free and the Rb^+-containing enzyme.

Additionally, from the results in Figure 6 we observed that the higher the eosin concentration, the more the shape of the curves of Rb^+ deocclusion was shifted from biexponential to single-exponential decreasing functions of time.

Since both this change in shape and the accelerating effect on Rb^+ deocclusion are features that eosin shares with ATP (*17*, *19*), it is likely that the probe and the nucleotide bind to the same site in E_2. This possibility received confirmation in the experiments of this paper showing that eosin seems to compete with ATP for accelerating Rb^+ deocclusion.

As it was observed by Forbush for ATP (*17*), we found that Mg^{2+} enhances the ability of eosin and ATP to promote Rb^+ deocclusion. Under our experimental conditions, ATP was up to 2−3 times more effective than eosin to stimulate deocclusion, but if enough eosin was present, the effect of ATP was annulled and the initial deocclusion rate dropped to that obtainable by eosin alone. Probably, the simplest explanation for these results would be that eosin occupies the sites for ATP but it accelerates deocclusion to a lesser degree than the nucleotide. The use of 1.25 or 1.9 mM $MgCl_2$ produced no significant differences on the results, indicating that eosin does not act by lowering the free Mg^{2+} required for enhancing the ATP-stimulated deocclusion.

The interaction of eosin with the E_1 conformer of the Na^+/K^+−ATPase has been extensively studied measuring the fluorescence changes of eosin. Because of the low affinity for the binding of the probe to E_2, florescence studies could not be applied in the case of this conformer. In this paper, we show how this hindrance can be overcome. Our results show that eosin does not only bind to the high-affinity site in E_1 but also shares with ATP the low-affinity site for the nucleotide in E_2 where it induces qualitatively similar effects. These results widen the effectiveness of eosin as a tool to study the interaction of ATP with the enzyme since they make possible the interpretation of experiments in which both ligands are present. Additionally, the present study allows us to define the conditions for using eosin to measure the rate of the conformational change which occurs during Rb^+ occlusion.

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