Sequential Potassium Binding at the Extracellular Side of the Na,K-Pump

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Abstract. Ion binding at the extracellular face of the Na,K-ATPase is electrogenic and can be monitored by the styryl dye RH 421 in membrane fragments containing a high density of the Na,K-pumps. The fluorescent probe is noncovalently bound to the membrane and responds to changes of the local electric field generated by binding or release of cations inside the protein. Due to the fact that K^+ binding from the extracellular side is an electrogenic reaction, it is possible to detect the amount of ions bound to the pump as function of the aqueous concentration. The results are in contradiction to a second order reaction, i.e., a simultaneous binding of two K^+ ions. A mathematical model is presented to discuss the nature of the two step binding process. On the basis of this model the data allow a quantitative distinction between binding of the first and the second K^+ ion. The temperature dependence of ion binding has been investigated. At low temperatures the apparent dissociation constants differ significantly. In the temperature range above 20°C the resulting apparent dissociation constants for both K^+ ions merge and have values between 0.2 and 0.3 mM, which is consistent with previous experiments. The activation energy for the half saturating concentration of K^+ is 22 kJ/mol. Additional analysis of the titration curve of K^+ binding to the state $P - E_2$ by the Hill equation yields a Hill coefficient, n_{Hill} , of 1.33, which is in agreement with previously published data.

Key words: Na,K-ATPase — Potassium binding — Electrogenic transport — Cation binding site — Sequential binding — Activation energy

Introduction

The reaction cycle of the Na,K-pump consists of a sequence of ion binding and release reactions and confor-

mational changes, by which cations are transported across the membrane against their electrochemical potential gradient. In conformational state E_1 , the ion binding sites are facing the cytoplasm and the protein binds three Na^+ ions. After the enzyme is phosphorylated by ATP and a spontaneous conformational transition to E_2 occurs, the ions are transferred to the extracellular side and released into the aqueous phase. Under physiological conditions, the pump binds subsequently two K^+ ions from the extracellular medium and occludes them after dephosphorylation (Glynn, 1985; Läuger, 1991). The complete reaction cycle is shown in Fig. 1 in a modified version of the Post-Albers cycle (Läuger, 1991). Binding and release of K^+ ions to state $P - E_2$ occurs in an ordered sequence, as has been shown by a rapid filtration technique of Na,K-ATPase preincubated with $^{86}Rb^+$ (Forbush, 1987). Binding of K^+ is associated with charge translocation from the aqueous phase into the membrane. This electrogenic process produces a change of the electrical field inside the membrane dielectric (Apell 1989). Changes of the electric field can be detected by a fluorescence change of the noncovalently bound dye probe RH 421 (Bühler, Stürmer, Apell & Läuger, 1991).

The simplest reaction scheme of the binding of two cations, M , to the phosphorylated enzyme in conformation E_2 would be

where M holds for monovalent cations like the physiological relevant K^+ or its congeners, Rb^+ , Cs^+ , NH_4^+ , Na^+ , Li^+ . If linearity can be assumed between the change of fluorescence intensity and the number of bound K^+ ions, the fluorescence change $\Delta F(c)$, of the probing dye can be described in terms of the K^+ concentration of the solution:

$$\Delta F(c) = \Delta F_{\infty} \frac{c^n}{K + c^n} \quad (2)$$

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where ΔF_∞ is the saturation value of the fluorescence change of $c \rightarrow \infty$. $c \equiv [M^+]$ is the concentration of the bound cation species, K is the apparent dissociation constant and n the Hill coefficient. If two cations are bound simultaneously as indicated in Eq. (1), the condition $n = 2$ will hold. If sequential binding of ions occurs, a more detailed reaction scheme will describe this process:

To resolve the two reaction steps, a necessary condition is that they have properties which produce detectable differences. Those differences may be based on kinetic, spectroscopic or electric properties of the protein. Eq. (3) represents the simplest sequential model. A more complicated model could include for example a structural rearrangement of the sites between the two binding steps.

Different approaches were discussed in the literature to analyze the potassium binding mechanism and to determine corresponding kinetic parameters. However, due to the lack of a directly observable parameter representing the state of K⁺ binding, only subsequent actions of the Na,K-ATPase were detected. Transmembrane fluxes of ⁴²K⁺ in red blood cells, which depend on the total reaction cycle, were measured as function of the extracellular K⁺ concentration (Sachs & Welt, 1967) and the results predicted a cooperative two-state model. Different approaches were detection of K⁺ – K⁺ exchange (Karlisch & Stein, 1982), of *p*-nitrophenyl-phosphatase activity (Gache et al., 1976) or activation of Na⁺ efflux (Sachs, 1980). The most direct experiments so far are Forbush's studies of Rb⁺ occlusion. He could determine the existence of a sequentially ordered fast and slowly accessible site by competition of radiolabeled Rb⁺ and unlabeled K⁺. His findings from kinetic experiments could be explained consistently by sequential binding in a "flickering-gate model" (Forbush, 1987, 1988).

In this paper, results from equilibrium titration experiments are presented, in which the occupation of the ion binding sites in phosphorylated states of E₂ was measured by fluorescence changes of the dye RH 421. This styryl dye detects changes of the local electric field induced by K⁺ binding within the Na,K-ATPase in its E₂ conformation. The simplest mathematical model of sequential binding, which described the experimental findings, is presented and applied. The deduced kinetic properties of the extracellularly presented ion binding sites are discussed.

Materials and Methods

Na,K-ATPase was prepared from the outer medulla of rabbit kidneys using procedure C of Jørgensen (Jørgensen 1974). The specific ATPase activity was measured by the pyruvate kinase/lactate dehydro-

genase assay (Schwartz et al., 1971) and the protein concentration determined by the Lowry method (Lowry et al., 1951), using bovine serum albumin as a standard. For most preparations the specific activity was in the range between 1900 and 2000 μmol P_i per hr and mg protein at 37°C.

The fluorescence styryl dye RH 421 was obtained from Molecular probes (Eugene, Oregon) and added from an ethanolic stock solution of 250 μM to obtain a final concentration of 250 nM.

NaCl and KCl were used in Suprapur quality (Merck, Darmstadt), all other reagents were of analytical grade. Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase and Na₂-ATP (disodium salt, Sonderqualität) were obtained from Boehringer, Mannheim. The solution of inorganic phosphate, P_i, was obtained by adding 50 mM *ortho*-H₃PO₄ (Riedel-de Haen, Hannover) to a buffer containing 200 mM imidazole. The pH was then adjusted to 7.2 by addition of HCl. 10 μl of this solution were added to 1 ml probe volume to get a final P_i concentration of 500 μM. In control experiments was shown that addition of 2 mM imidazole to the buffer had no significant effect in the experiments.

Fluorescence measurements were carried out with a Perkin Elmer LS50B luminescence spectrometer. The thermostated cell holder was equipped with a magnetic stirrer. The excitation wavelength was set to 580 nm (slit width 15 nm) and the emission wavelength to 650 nm (slit width 15 nm). Under these conditions the phosphorylation of the Na,K-ATPase in the presence of 20 mM Na⁺ yielded a relative RH 421 fluorescence changes up to 100%. RH 421 is an amphiphilic electrochromic styryl dye. It is thought to insert into the lipid domains of Na,K-ATPase membrane fragments. It does not respond to conformational transitions but detects changes of electric field strength inside the membrane dielectric (Bühler et al., 1991). Such changes in electric field strength are caused by binding or release of ions and/or by movement of charge inside the membrane. The fluorescence levels allow the discrimination between differently charged states of the enzyme. The relative fluorescence change is assumed to be a linear function of the change of the electric field (Grinvald et al., 1982; Bühler et al., 1991). Although there is general agreement that RH styryl dyes respond to changes of the electric field in the membrane, the mechanism is not explained completely so far (Grinvald et al., 1982; Bühler et al., 1991; Clarke 1992). While Bühler and colleagues (Bühler et al., 1991) attributed the main effect to an electrochromic mechanism, Clarke and colleagues (Clarke, Schrimpf & Schöneich, 1992) presented evidence for additional voltage-sensitive aggregation of dye molecules. According to Clarke and colleagues the differences may be explained by higher dye concentrations they used compared to Bühler and colleagues (Clarke et al., 1992).

Results

The Post-Albers scheme (Fig. 1) describes states of the Na,K-ATPase having different numbers of cations bound or occluded. In a suspension of membrane fragments containing a high number of pumps, the resulting charge density within the membrane after addition of K⁺ ions to the P – E₂ state is measured by the electrochromic dye RH 421 (Bühler et al., 1991). Na,K-ATPase membrane fragments were suspended in 1 ml buffer containing (in mM): 30 imidazole, 1 EDTA and 5 MgCl₂, pH 7.2, to a final protein concentration of 9 μg/ml. The suspension was thermostated at the desired temperature and equilibrated with 250 nM RH 421. After the fluorescence intensity became stable, successively 20 mM NaCl and 500

Fig. 1. Post-Albers scheme of the pump cycle of the Na,K-ATPase, adapted from Lauger (1991). E₁ and E₂ are conformations of the enzyme with binding sites facing the cytoplasm and the extracellular medium, respectively. In the "occluded" states (Na₃)E₁-P, E₂(K₂) and ATP · E₂(K₂) the bound ions are unable to exchange with the aqueous phase. P-E₂(K₂) and P-E₂(Na₂) indicate states, which may occlude ions upon binding of cardiotonic steroids. Dashes indicate covalent bonds and dots indicate noncovalent bonds. When the enzyme is phosphorylated by ATP in state E₁, 3 Na⁺ ions become occluded (Na₃E₁ · ATP → (Na₃)E₁-P). The spontaneous transition to conformation E₂ leads to the release of the Na⁺ ions to the extracellular side. The subsequent binding of potassium initiates enzyme dephosphorylation and occlusion of K⁺ ions. Low-affinity binding of ATP shifts the conformation to state E₁ from which K⁺ is released to the cytoplasm.

μM Na₂-ATP were added and the fluorescence signal was monitored until the signal became stationary. In the presence of 20 mM Na⁺ the pumps can be assumed to be essentially in the state P-E₂ after phosphorylation (Lauger, 1991). Subsequently K⁺ was added in different aliquots of 5 or 10 μl to titrate K⁺ binding in 15 steps to a saturating concentration, in which no further fluorescence decrease beyond the small dilution effect (<1% per step) was observed. The fluorescence response to Na⁺, ATP and K⁺ addition is shown in Fig. 2A. According to the properties of RH 421 in the given experimental conditions, the fluorescence decrease indicated an increase of positive charge within the membrane/protein dielectric (Buhler et al., 1991). As a control an experiment is included, in which instead of K⁺ only buffer containing 20 mM NaCl was added. This trace represents the dilution and drift effects which had to be taken into account when the titration experiments were analyzed. The drift within each titration level was negligible, even upon addition of low concentrations of K⁺, when |ΔF/F₀| < 1%. The temperature was 20°C. The fluorescence decrease caused by the titration with K⁺ has been taken as a measure for the binding of K⁺ to the states of P-E₂. The data points in Fig. 2B represent the relative fluorescence change, ΔF/F₀, versus the K⁺ concentration. The reference fluorescence intensity, F₀, is the intensity level before addition of K⁺ (cf. Fig. 2A). The data plotted in Fig. 2B were corrected for the dilution of RH 421 and K⁺ according to the increasing aqueous volume during the titration according to the control trace. Similar experiments were performed in the temperature range between

Fig. 2. Relative fluorescence changes of RH 421 labeled Na,K-ATPase membranes. (A) Open membrane fragments (9 μl protein/ml) were equilibrated with 0.25 μM RH 421 in the presence of (in mM): 30 imidazole, 1 EDTA and 5 MgCl₂, pH 7.2. The temperature was 20°C. The fluorescence was excited at 580 nm (slit width 15 nm) and recorded at 650 nm (slit width 15 nm). After having obtained a stable fluorescence level 20 mM NaCl and 0.5 mM Na₂-ATP were added successively to reach essentially state P-E₂. Subsequently buffer containing KCl was added in various amounts to titrate to the binding sites or K⁺ free (bold trace). To demonstrate the dilution effects and the (negligible) fluorescence drift a control experiment is included, in which K⁺ free buffer was added (light trace). (B) The relative fluorescence changes, corrected for dilution effects, is plotted as function of the K⁺ concentration in the solution. Equation (4) is fitted to the data points (solid line). Their error bars are smaller than the size of the points. A Hill function with a Hill coefficient, n_{Hill}, set to 1 (dotted) and 2 (broken line) do not fit the data. A fit of the Hill function resulted with a variable n_{Hill} = 1.33 which was not significantly different from the drawn continuous line (not shown).

10°C and 35°C in intervals of 5°C. Each experimental series was repeated at least four times. The experimental uncertainty of the data point was within the size of the symbol.

Control experiments were performed to demonstrate that the applied concentration of ATP was high enough to make sure that hydrolysis during the titration experiment did not reduce the concentration in a way that bind-

Table. Analysis of K⁺ binding after enzyme phosphorylation

	F_{∞}	K_1/mM	K_2/mM	$c_{1/2}/\text{mM}$
10°C, 20 μM ATP	-0.519 ± 0.019	0.176 ± 0.099	0.034 ± 0.012	0.072 ± 0.006
10°C, 500 μM ATP	-0.537 ± 0.019	0.230 ± 0.068	0.090 ± 0.023	0.140 ± 0.007
10°C, 500 μM P _i	-0.409 ± 0.011	0.290 ± 0.047	0.224 ± 0.022	0.255 ± 0.010
15°C, 20 μM ATP	-0.515 ± 0.005	0.157 ± 0.023	0.028 ± 0.004	0.065 ± 0.002
15°C, 500 μM ATP	-0.519 ± 0.005	0.198 ± 0.043	0.121 ± 0.014	0.153 ± 0.008
20°C, 500 μM ATP	-0.606 ± 0.017	0.169 ± 0.031	0.220 ± 0.022	0.192 ± 0.009
20°C, 500 μM P _i	-0.496 ± 0.018	0.316 ± 0.017	0.260 ± 0.030	0.286 ± 0.013

Enzyme phosphorylation by 20 μM ATP or 500 μM inorganic phosphate, P_i. Experiments have been performed as described in Fig. 2 with exception of the phosphorylation step, in which ATP concentration has been varied or ATP has been replaced by P_i.

ing to the low affinity binding site was significantly altered. When ATP was added in a concentration of 500 μM and no K⁺ was added, the fluorescence level corresponding to state P – E₂ was maintained for times longer than at least 1 hr. The estimated ADP (and P_i) concentration at the end of a titration experiment was in the order of 50 μM , a concentration without significant inhibitory effect to the pump (Apell et al., 1986).

In the presence of Na⁺, K⁺ and ATP the Na-pump runs through the physiological transport cycle (Fig. 1). K⁺ ions are occluded and released to the intracellular side after conformational transition of the pump from E₁ to E₂. Na⁺ binding, phosphorylation and sodium release complete the cycle back to the state P – E₂. In the absence of K⁺ the mainly populated state of the pump will be P – E₂ (Heyse et al., 1994). Addition of increasing amounts of K⁺ will not only populate the states P – E₂ · K and P – E₂ · K₂, but also other states around the cycle, mainly the states before the two rate limiting steps, ATP – E₂ · (K₂) and (Na₃) · E₁ · P. Since the fluorescence intensity of these states will contribute to the signal, control experiments had to be performed to determine the contributions of subsequent partial reactions to the obtained apparent K⁺ binding affinities, which were evaluated by the presented experiments. The reaction step, which follows K⁺ binding is the dephosphorylation, P – E₂ · K₂ → E₂ · (K₂). In forward direction it is a spontaneous reaction, which cannot be controlled by substrate conditions. However, in the presence of inorganic phosphate, P_i, the kinetic equilibrium can be shifted towards state P – E₂ · K₂. The subsequent conformational transition, E₂ · (K₂) → K₂ · E₁, can be accelerated by high ATP concentrations, K₂ · E₁ → K₂ · E₁ · ATP → ATP · E₁ · K₂ → ATP · E₁ (Glynn, 1985). These functional properties of the Post-Albers cycle were used in additional experiments.

Experiments as shown in Fig. 2 were repeated in the presence of only 20 μM ATP, which is a concentration in the range of the half-saturating concentration of the low-affinity ATP binding in state E₂ · (K₂). All other conditions were the same as described above. Under this con-

dition the reaction E₂ · (K₂) → K₂ · E₁ is the main rate limiting reaction step of the cycle with 1 s⁻¹ (Glynn, 1985). Due to its low concentration, in these experiments ATP consumption played a major role. To control ATP consumption the following experiments were performed. After addition of K⁺ in a concentration close to c_{1/2} the fluorescence level was monitored. Due to the lack of ATP the fluorescence intensity returned to the initial level before addition of ATP after a certain period of time. These controls revealed that the available time was temperature dependent. Evaluable titration experiments could be performed in the presence of 20 μM ATP therefore only at 10°C and 15°C (*data not shown*).

A third series of experiments made use of the observation that the Na,K-ATPase can be phosphorylated by P_i in the absence of Na⁺ (Glynn, 1985). In a buffer containing (in mM): 30 imidazole, 1 EDTA and 5 MgCl₂ membrane fragments with a final concentration of 9 $\mu\text{g}/\text{ml}$ were equilibrated with 250 nM RH 421. Experiments were performed at 10°C, 20°C and 30°C. After a stable fluorescence intensity was reached 10 μl of a 50 mM solution of P_i was added. The fluorescence signal increased by approximately 50%. Thereafter aliquots of KCl were added to titrate the binding sites. Since no Na⁺ and ATP were present in these experiments, the protein states in the upper half of the Post-Albers cycle could not be populated and contribute to the fluorescence signal. For comparison with ATP phosphorylation the results of the analysis of the experiments taken at 20°C are included in the Table.

THEORETICAL ANALYSIS

Sequential binding of 2 K⁺ ions to the extracellular binding sites of the protein according to Eq. (3) could be described by a mathematical model as explained in the Appendix. The assignment of fluorescence levels for the states P – E₂, P – E₂ · K, and P – E₂ · K₂ allowed the description of the relative fluorescence change of K⁺ binding as function of equilibrium dissociation constants, K₁ and K₂.

$$\frac{\Delta F}{F_0} = F_\infty \frac{\frac{\alpha c}{K_1} + \frac{c^2}{K_1 K_2}}{1 + \frac{c}{K_1} + \frac{c^2}{K_1 K_2}} \quad (4)$$

where F_∞ is the saturation value of the fluorescence change, $c \equiv [M^+]$ the concentration of the bound monovalent cation species, K_1 the apparent dissociation constant of the partial reaction $P - E_2 \leftrightarrow P - E_2 \cdot M$ and K_2 that of $P - E_2 \cdot M \leftrightarrow P - E_2 \cdot M_2$. $\alpha = (f_1 - f_0)/(f_2 - f_0)$ is the ratio of the fluorescence levels of $P - E_2 \cdot M$ (F_1) and $P - E_2 \cdot M$ (F_2). With parameters α , K_1 and K_2 the half-saturating concentration $c_{1/2}$ can be calculated:

$$c_{1/2} = K_2 \left((0.5 - \alpha) + \sqrt{\frac{K_1}{K_2} + \alpha^2 - \alpha + 0.25} \right) \quad (5)$$

$c_{1/2}$ is defined by the condition $F(c_{1/2}) = F_\infty/2$. For $\alpha = 0.5$ Eq. (5) is reduced to

$$c_{1/2} = K_2 \sqrt{\frac{K_1}{K_2}} = \sqrt{K_1 K_2} \quad (6)$$

The solid line in Fig. 2B shows a calculation of $\Delta F/F_0$ according to Eq. (4), fitted to the experimental data for $\alpha = 0.5$ and K_1 and K_2 optimized by a Marquardt fitting routine. The fitting program converged to the same set of parameters independent from the starting value, which were set in a range between 0.001 and 10 mM. The fit of Eq. (4) was compared to the Hill plot with coefficients $N_{\text{Hill}} = 1$ (dotted line) and $n_{\text{Hill}} = 2$ (broken line). When the Hill coefficient was used as a fitting parameter in a least-square fit, n_{Hill} was determined to be 1.33. The corresponding curve is not presented in Fig. 2B, because it is almost identical to the solid curve of the fit of Eq. (4).

The temperature dependence of the parameters $c_{1/2}$, K_1 and K_2 is shown in Fig. 3A and B. The values of $c_{1/2}$ have been obtained from the data, K_1 and K_2 were determined by fitting Eq. (4) to the data. A comparison of the experimentally obtained values of $c_{1/2}$ and of Eq. (6) gave agreement within the experimental errors. At each temperature four titration experiments were carried out and Eq. (4) was fitted to the data sets. Except of the temperature, the experimental conditions were the identical to those described for the experiments of Fig. 2. The data points in Fig. 3 represent the mean value of the respective parameters, the error bars indicate the standard deviation, σ_{n-1} . Fig. 3A shows the temperature dependence of the half saturating concentration, $c_{1/2}$, of the relative fluorescence change after K⁺ addition to the enzyme in state $P - E_2$. The activation energy of E_a of $c_{1/2}$ was evaluated as 22 kJ/mol by fitting the Arrhenius equation, Eq. (7), to the data (continuous line in Fig. 3A).

$$c_{1/2}(T) = A_e^{-E_a/RT} \quad (7)$$

Fig. 3B shows the temperature dependence of the parameters K_1 and K_2 .

The analysis of the K⁺ binding experiments to state $P - E_2$ after phosphorylation by 20 μM ATP at 10°C and 15°C and after phosphorylation by 500 μM P_i at 20°C was performed using Eq. (4) accordingly. The results are presented in the Table.

Discussion

In this paper we described experiments that allowed the measurement of K⁺ binding to the Na,K-ATPase in its E_2 conformation. As has been discussed in the literature, the affinity of the two-ion-binding sites is significantly higher for K⁺ than for Na⁺ in conformation E_2 (Glynn, 1985; Forbush, 1987; Forbush, 1988; Glynn & Richards, 1982). The sites are not necessarily equivalent and may be distinguished by the kinetics of ion release. Forbush carried out detailed studies of the kinetics of occlusion and phosphate-induced deocclusion of ⁸⁶Rb and was able to discriminate between a fast and a slowly accessible site (Forbush, 1988). According to his “flickering-gate model,” release of ⁸⁶Rb from the slowly-exchanging binding site could be blocked by K⁺ with an affinity of approximately 0.1 mM. With this preposition sequential binding can be modeled and formalized as presented. In Eq. (4), the equilibrium dissociation constant of the first and the second cation, K_1 and K_2 respectively, are distinguished. The concentration dependence of the relative fluorescence change, $\Delta F/F_0$, as measured in this study depended only on both dissociation constants and the ratio, α , of the contribution of states with one and two ions bound (Eq. A7). These parameters could be obtained by fitting Eq. (4) to the experimental signals.

In opposition to previously published studies, we did not monitor effects following K⁺ binding from the extracellular side, e.g., enzymatic activity or transmembrane ion transport. Direct detection of K⁺ binding is possible by the fact that it is an electrogenic process step (Läuger, 1991; Stürmer et al., 1991; Rakowski et al., 1991). Ion binding to the Na,K-ATPase in its $P - E_2$ state is proposed to consist of two steps: a migration of the ions from the surface through a narrow access channel or “ion well” to the site and a subsequent binding reaction (Sagar & Rakowski 1994). The latter step may be slower than first.

In this study, we used Na,K-ATPase-containing membrane fragments labeled with the fluorescence dye RH 421, which detects mainly the movement of charges from the aqueous phase into the membrane dielectric and vice versa (Bühler et al., 1991; Läuger, 1991). The applied method is precise enough to discriminate between

different numbers of cations bound to the extracellularly presented sites (Fig. 2B). It is less sensitive to charge movements inside the plain of adsorbed dye molecules in the membrane, as has been derived in the appendix of Bühler et al. (1991). If the dielectric length of the access channel between aqueous phase and binding sites is in the order of the distance of the adsorption plane of the dye from the membrane surface (or longer), then both ions will contribute with the same fluorescence change to the RH 421 signal. This would produce a strong constraint to the parameter α , which then has to be close to 0.5. Experimental indications which support this constraint were given by Heyse et al. (1994). They studied extracellular Na⁺ binding to the P – E₂ state of the Na,K-ATPase by RH 421 experiments and found that the first two Na⁺ ions, which bind to extracellularly presented sites, produced the same contribution to the fluorescence signal. The ions migrate over a dielectric distance of 20% of the total membrane thickness (Heyse et al., 1994). Since it is generally accepted that two of the Na⁺ and K⁺ ions bind to the same protein moiety, we fixed α to a value of 0.5. All states with two ions bound and all E₁ states except (Na₃)E₁ and (Na₃)E₁ – P exhibit the same level of fluorescence (Stürmer et al., 1991; Heyse et al., 1994). In addition we performed fits of Eq. (4) to the data, in which α could vary to obtain better fits with lower values of chi-square. However, the improvements were marginal and the values of K_1 and K_2 varied less than 10% (with a few exceptions).

To analyze the titration curves in the proposed way, two underlying assumptions had to be verified. The first assumption was that an equilibrium distribution between the three states P – E₂, P – E₂K and P – E₂K₂ exists. This is valid provided all steps associated with extracellular K⁺ binding are rapid compared to the reactions, which border the two states, and from which they are emptied: P – E₂ → E₁, P – E₂ → P – E₂Na and P – E₂K₂ → E₂(K₂). According to Heyse et al. (1994), who presented a consistent set of rate constants of all involved reactions, the first two pathways fulfill the condition. The forward reaction, P – E₂K₂ → E₂(K₂) + P_i, has a rate comparable to ion binding and has to be included in the discussion of the contributing reactions as can be seen from the P_i dependence (Table). However, the next following reaction steps, E₂(K₂) → ATPE₂(K₂) → K₂E₁ATP, are sufficiently slow (Heyse et al., 1994). The second assumption is that no significant redistribution of pump intermediates occurs between the pool of K⁺-binding states and other states during addition of increasing concentration of K⁺. At low temperatures a redistribution has to be expected only between states P – E₂K₂ and E₂(K₂), as was shown by the presence of inorganic phosphate, which drives this reaction backwards (Table). Since the specific fluorescence levels of both states is the same, they may be pooled and treated as one state after binding of two ions. The subsequent reaction

(ATP-binding and/or conformation change) is again sufficiently slow (Heyse et al., 1994). At temperatures above 20°C detectable effects were found as discussed below.

Titration of the K⁺ binding sites have been performed in the presence of saturating concentrations of ATP in the temperature range between 10°C and 35°C as shown exemplarily in Fig. 2A. The subsequent analysis is presented in Fig. 2B. The data can be fitted neither by a function, which describes ion binding without any cooperativity between both K⁺ ions ($n_{\text{Hill}} = 1$), nor by simultaneous binding of both ions, as represented by a Hill function with $n_{\text{Hill}} = 2$. Using the Hill coefficient as a phenomenological parameter to find an optimum fit, we obtained $n_{\text{Hill}} = 1.33$ ($T = 20^\circ\text{C}$). The corresponding curve is not included in Fig. 3B, since it was similar to the solid line, which represents the fit of Eq. (4). The phenomenological quantity n_{Hill} does not explain any kinetical or mechanistic properties of binding, it should be taken only as an indicator of some kind of complex process.

The temperature dependence of experimentally determined values of $c_{1/2}$ and of the apparent affinities, K_1 and K_2 are presented in Fig. 3, as obtained by fitting Eq. (4) to the data. As a test of consistency, the experimentally and theoretically obtained values of $c_{1/2}$ agreed within the error bars in Fig. 3A for all temperatures. The temperature dependence of the half saturating K⁺ concentration obeyed the Arrhenius equation (Eq. 7) with an activation energy, E_A , of 22 kJ/mol. The activation energy under turnover conditions was determined to be 90–120 kJ/mol (Marcus et al., 1986), which is significantly larger. The large discrepancy between the activation energy of the rate-limiting reactions under turnover conditions, (Na₃)E₁ – P → P – E₂(Na₂) and ATPE₂(K₂) → K₂E₁ATP, and the K⁺-binding steps was an additional test that the observed effects were not controlled by subsequent steps. K_1 and K_2 differed not significantly at temperatures above 20°C, but they diverged for lower temperatures (Fig. 3B). The error bars represent the standard deviation of different series of experiments, σ_{n-1} .

To explain the lower values of K_2 compared to K_1 in the temperature range between 10°C and 15°C and their agreement above 20°C, one has to compare the reaction scheme of Eq. (4) with the simple model of the complete cycle in Fig. 4. After two K⁺ ions are bound, the pump has the chance to run through the subsequent reaction cycle, which includes a whole series of reaction steps: occlusion of K⁺, the conformational transition to E₁ and ion release, and only after Na⁺ binding, phosphorylation by ATP another conformational change to E₂, the release of Na⁺ brings the pump back to the reaction scheme of Eq. (3). At low temperatures the conformational change ATP · E₂(K₂) → ATP · K₂E₁ is so slow that the pathway through the cycle was negligible and the hardly revers-

Fig. 3. (A) Half saturating K⁺-concentration, $c_{1/2}$, and (B) apparent dissociation constants, K_1 and K_2 for the first and second K⁺ binding site as a function of temperature. The numbers have been obtained as best fits of Eq. (4) to experiments as presented in Fig. 2. Each data point represents the mean value of four independent experiments. The error bars indicate the standard deviation, σ_{n-1} , for each group of four experiments.

ible dephosphorylation reaction $P - E_2(K_2) \rightarrow E_2(K_2)$ produced an apparent higher affinity for the second binding site. At higher temperatures the state $E_2(K_2)$ was "short circuited" into state $P - E_2$ and prevented an accumulation in state $E_2(K_2)$.

Control experiments in the presence of 20 μ M ATP were performed, in which the transition $E_2(K_2) \rightarrow K_2E_1$ was rate limiting with a rate constant of $\leq 1 \text{ s}^{-1}$ in the temperature range below 20°C. Above this temperature no experiments were possible due to artifacts produced by ATP consumption. Since there was virtually no inorganic phosphate present, the back reaction $E_2(K_2) \rightarrow P - E_2(K_2)$ could not occur, and even low K⁺ concentrations lead to a significant population of state $E_2(K_2)$. This effect should produce an apparently higher affinity for K⁺ as was shown (Table). If experiments were performed in the presence of a high concentration of P_i (500 μ M) the back reaction $E_2(K_2) \rightarrow P - E_2(K_2)$ was sup-

Fig. 4. Simplified Post-Albers cycle with expanded reaction steps of extracellular K⁺ binding. After sequential binding of two K⁺ ions to binding site within the membrane dielectric, the enzyme is spontaneously dephosphorylated. The reactions from state $E_2(K_2)$ to $P - E_2$ are dependent only on ATP and Na⁺ ions which are kept at constant values in an experimental series.

ported and an apparently lower affinity could be observed. The half saturating K⁺ concentration, $c_{1/2}$, increased from 0.19 mM to 0.29 mM (Table). These observations lead to the suggestion that the obtained apparent K⁺ binding affinities approach the true affinities at high temperatures.

The data presented in this contribution exclude the simultaneous binding of the two cations to the extracellularly presented sites, but are consistent with the notion of two extracellular ion binding sites with equal affinity (Läuger & Apell, 1986). Since Forbush proved an ordered release of the potassium congener Rb⁺ (Forbush, 1987; Forbush, 1988), an independent access to two separated binding pockets can be excluded in addition. An explanation of sequential binding under this constraint can be proposed by the "flickering gate model" (Forbush, 1987; Läuger, 1991), in which the first K⁺ ion binds with the affinity K_1 to the first site at the bottom of an ion well, jumps back and forth between the first and second binding site while an occlusion gate flickers between open and close inside the ion well. The second site is not directly accessible from the well. If the gate is open and the first ion bound is placed second, inner binding site, then a second ion can be bound to the first site, directly accessible from the well, with the same affinity as the first ion, $K_2 = K_1$. When both binding sites are occupied, subsequently the dephosphorylation and (permanent) occlusion will occur. In this model different apparent affinities may be observed by influence of subsequent reaction steps, e.g., the rephosphorylation step of the pump in the presence of P_i. In nonequilibrium experiments the rate constants of the flickering process and the jumping of the ion between both sites may easily produce differing apparent binding affinities.

On the basis of the fact that the RH 421 method discriminates only between states with no, one or two ions in the binding sites, but does not between the open or closed flickering gate and between one ion in the fast or slowly accessible site, Forbush's model with eight

states is reduced to the three-state model according to Eq. (3), in which only the ion-binding steps are under experimental control. With respect to this simplification all presented data and the mathematical modeling (Eq. 3) are in agreement with the model suggested by Forbush (1988).

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Appendix

The relative fluorescence change, $\Delta F/F_0$, of RH 421-labeled Na,K-ATPase fragments at different cation concentrations $[M]$ at the extracellular face can be described by Eq. (4) with the parameters y_{∞} , α , K_1 and K_2 . The assumed reaction scheme is:

We denote the fraction of pump molecules in state A by $x[A]$ and introduce the following variables:

$$x_0 = x[P - E_2], x_1 = x[P - E_2 \cdot M], x_2 = x[P - E_2 \cdot M_2], x_0 + x_1 + x_2 = 1 \quad (A2)$$

The equilibrium dissociation constants of the two partial reactions are defined by

$$K_1 = \frac{cx_0}{x_1}, K_2 = \frac{cx_1}{x_2} \text{ with } c \equiv [M] \quad (A3)$$

When f_0, f_1, f_2 are the specific fluorescence levels of the states $P - E_2$, $P - E_2 \cdot M$, and $P - E_2 \cdot M_2$, respectively, the total fluorescence, F , is calculated by

$$F = x_0 f_0 + x_1 f_1 + x_2 f_2 \quad (A4)$$

and the relative fluorescence change, $\Delta F/F_0$ is introduced by

$$\frac{\Delta F}{F_0} = \frac{F - F_0}{F_0} \quad (A5)$$

Under the assumption that the initial state of the protein is the state with no ions bound, $x[E_2P] = 1$, the initial fluorescence intensity will be $F_0 = f_0$. This allows the expansion of Eq. (A5)

$$\frac{\Delta F}{F_0} = \frac{F - f_0}{f_0} = x_0 + x_1 \frac{f_1}{f_0} + x_2 \frac{f_2}{f_0} - 1 \quad (A6)$$

Substituting according to Eq. (A3) $x_1 = \frac{cx_0}{K_1}$, $x_2 = \frac{cx_1}{K_2} = \frac{c^2 x_0}{K_1 K_2}$

and defining $\alpha \equiv \frac{f_1 - f_0}{f_2 - f_0}$ (A7)

the relative fluorescence change can be expressed as

$$\frac{\Delta F}{F_0} = x_0 + x_0 \frac{\alpha c f_2}{K_1 f_0} + x_0 \frac{c^2}{K_1 K_2 f_0} - 1. \quad (\text{A8})$$

Further substitution of $x_0 = \frac{1}{1 + \frac{c}{K_1} + \frac{c^2}{K_1 K_2}}$

and introduction of $F_\infty = \frac{f_2 - f_0}{f_0}$ as maximum fluorescence change

for the condition, when all enzyme is in state E₂(K₂), finally yields in Eq. (4) of the text

$$\frac{\Delta F}{F_0} = F_\infty \frac{\frac{\alpha c}{K_1} + \frac{c^2}{K_1 K_2}}{1 + \frac{c}{K_1} + \frac{c^2}{K_1 K_2}}.$$