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# Fluorescence study on cardiac glycoside binding to the Na,K-pump

## Ouabain binding is associated with movement of electrical charge

W. Stürmer and H.-J. Apell

*Department of Biology, University of Konstanz, D-7750 Konstanz, Germany*

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Recently we have presented evidence that the fluorescence probe RH 421 can be used to detect binding and release of ions at the extracellular face of the pump since these processes are associated with translocation of electrical charge [1]. Applying this method to experiments with cardiac glycosides we found that: (1) ouabain induced fluorescence changes of the electrochromic dye, RH 421, were caused by the change of charges bound to the enzyme; (2) independent of the sodium concentration, the final fluorescence amplitude indicated that approximately 2 $Na^+$ ions were bound to the pump; (3) the sodium release to the extracellular side involved two distinct electrogenic steps; (4) the kinetics of inhibition depended on the $Na^+$ concentration. Experiments with hydrophobic ions indicated that the kinetics of ouabain binding to the Na-ATPase is voltage dependent; and (5) the applied technique is a convenient tool to characterize binding of cardiac glycosides to the Na,K-pump.

Na,K-ATPase; Ion pump; Cardiac glycoside; Voltage-sensitive dye; Electrogenic transport

## 1. INTRODUCTION

Operation of the Na,K-pump involves a sequence of conformational transitions and ion binding and release reactions [2–4]. The Na,K-pump transports three Na ions out of and two K ions into the cell during a pumping cycle. According to the pumping scheme (Fig. 1), the enzyme can assume two principal conformations, $E_1$ and $E_2$, with ion binding sites facing the cytoplasm and the extracellular medium, respectively. In state $E_1$ the enzyme binds 3 $Na^+$ ions. When the protein is phosphorylated by ATP, the $Na^+$ ions become occluded. The spontaneous transition to state $E_2$-P induces the release of $Na^+$ to the extracellular side. After high-affinity binding and occlusion of $K^+$ ions, the enzyme is dephosphorylated. The pumping cycle is completed by an ATP facilitated transition back to state $E_1$ and release of $K^+$ to the cytoplasm.

Cardiac glycosides are specific inhibitors of the Na,K-pump. They act on the enzyme only from the extracellular face and bind preferentially to a P-$E_2$-state of the enzyme. Inhibition occurs with one molecule bound to an $\alpha\beta$-protomer [2,5–7]. Recently we have shown that the styryl dye RH 421 responds to changes of the local electric field in the membrane dielectric [8]. The results of these studies indicate, that binding and release of $Na^+$ and $K^+$ at the extracellular face of the pump is associated with translocation of electrical charge. An experiment illustrating this finding is shown in Fig. 2.

After additon of 100 $\mu$M ouabain (or analogue) to the phosphorylated enzyme fluorescence changes can be observed (Fig. 3a). The analysis of these signals allows an interpretation on a microscopic level. As we will show, valuable insight both in the mechanism of enzyme inhibition by cardiac glycosides and in $Na^+$-transport mechanism may be obtained.

## 2. MATERIALS AND METHODS

Sodium dodecylsulfate (SDS) was obtained from Pierce Chemical (Rockford, IL), sodium cholate from Merck (Darmstadt) and dioleoyllecithin from Avanti Polar Lipids (Birmingham, Alabama). Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, NADH and ATP (disodium salt, Sonderqualität) were from Boehringer (Mannheim). All cardiac glycosides were purchased from Sigma. RH 421 was obtained from Molecular Probes (Eugene, Oregon). The purity of the dyes was checked by thin-layer chromatography. Sodium tetraphenylborate ($TPB^-$), tetraphenylphosphonium chloride ($TPP^+$) and ethylenediamine tetraacetic acid (EDTA) were from Merck (Darmstadt). NaCl of Suprapur quality (Merck) was used. All other reagents were analytical grade.

Na,K-ATPase was prepared from the outer medulla of rabbit kidneys using procedure C of Jorgensen [9]. The specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay [10] and the protein concentration by the Lowry method [11], using bovine serum albumin as a standard. For most preparations the specific activity was in the range between 1500 and 2000 $\mu$mol $P_i$ per h and mg protein at 37°C.

Most steady-state fluorescence measurements were carried out with a Perkin-Elmer 650-40 fluorescence spectrophotometer. The thermostated cell holder was equipped with a magnetic stirrer. For experiments with RH 421, the excitation wavelength was set to 580 nm (slit width 10 nm) and the emission wavelength to 660 nm (slit width 10

*Correspondence address:* W. Stürmer, Department of Biology, University of Konstanz, D-7750 Konstanz, Germany. Fax: (49) (7531) 882966.

nm). For experiments with anthroylouabain the excitation wavelength was set to 365 nm, the emission to 510 nm (slit width 10 nm). For some experiments with the dye RH 421, we used a home made fluorescence spectrometer. The excitation and emission wavelength were selected with interference filters ($\lambda_{ex}$ = 580 nm, $\lambda_{em}$ = 660 nm, bandwidth 9 nm). Absorbance measurements were performed with a Perkin-Elmer Lambda 5 spectrophotometer. If not otherwise indicated, the experiments were carried out at 20°C.

## 3. RESULTS

The addition of cardiac glycosides to the phosphoenzyme leads to large fluorescence changes (Fig. 3a). Sign, amplitude and time course depended on $Na^+$ concentration. At low $Na^+$ (enzyme in state P-$E_2$) the fluorescence decreased, whereas at high $Na^+$ (enzyme in state $(Na_3)E_1$-P) a fluorescence increase could be observed. The final fluorescence was the same in all $Na^+$ concentrations. Similar results have been obtained with other analogues (digitoxigenin, strophanthidin, digoxin). Control experiments (data not shown) using the fluorescent ouabain analogue anthroylouabain (AO) [12] indicated, that for all $Na^+$ concentrations the amount of ouabain bound to the enzyme was the same. Therefore it is unlikely that cardiac glycosides directly affected the fluorescence changes. The ouabain induced fluorescence amplitudes are consistent with the assumption, that ouabain binds to the P-$E_2(Na_2)$ state in the pumping cycle when no $K^+$ is present. The states P-$E_2$, P-$E_2$(Na), P-$E_2(Na_2)$, and $(Na_3)E_1$-P are — on the time scale of ouabain binding — in equilibrium with each other. The addition of ouabain leads to a redistribution between these states, inducing the transport-inhibited P-$E_2(Na_2)$–ouabain complex. This state is obtained in low $Na^+$ buffers by binding of (up to 2) $Na^+$ ions within the protein dielectric, thus decreasing the dye fluorescence. At high sodium concentrations (up to one) $Na^+$ ion will be released upon ouabain addition, thus increasing the fluorescence. At about 500 mM $Na^+$, in the average 2 $Na^+$ ions seem to be buried in the protein dielectric, since no fluorescence changes can be observed after addition of ouabain. The $Na^+$ concentration dependence of ouabain-binding rates is shown in Fig. 3b. Due to the high ionic strength and low temperature (20°C) the rate constants were smaller than values reported in the literature [18]. A similar dependence was also found for anthroylouabain binding and Na-ATPase activity [13], which works with a stoichiometry of 3Na/2Na/1ATP [14,15]. All these experiments support the idea that ouabain binds to the P-$E_2(Na_2)$ state.

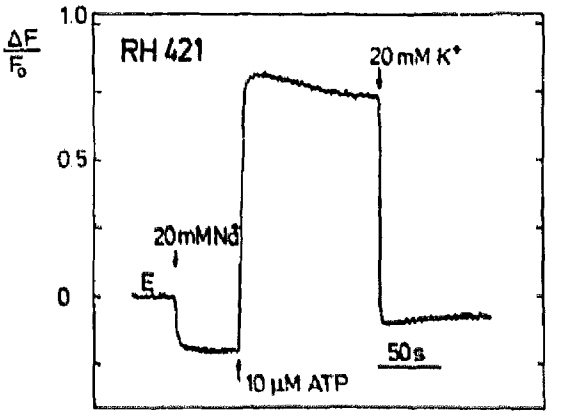


Fig. 2. Fluorescence changes of styryl dye RH 421 bound to Na,K-pump containing membrane fragments. The fragments contain approx. 5000 pumps/µm². About 9 µg protein/ml were equilibrated at 20°C with 200 nM RH 421 in the presence of 10 mM Mg, 25 mM histidine and 0.5 mM EDTA. The initial condition represents the fluorescence of the enzyme in absence of $Na^+$ and $K^+$ ions. Supplying 20 mM $Na^+$ leads into state $Na_3E_1$. The fluorescence decreased by approximately 25% indicating an import of positive charge into the protein dielectric [1]. Addition of ATP leads to phosphorylation, occlusion of $Na^+$, and transition to P-$E_2$. The corresponding release of 3 $Na^+$ ions induces a fluorescence increase of up to 100%. This amplitude of the fluorescence change $\Delta F/F_0$ depends on $Na^+$ concentration which is largest at low concentrations. It has been explained by a $Na^+$-induced shift of the distribution between states P-$E_2$, P-$E_2$(Na), P-$E_2(Na_2)$, and P-$E_1(Na_3)$ [1]. The subsequent addition of $K^+$ to the phosphorylated enzyme causes a transition from P-$E_2$ to $E_2(K_2)$ and is associated with a decrease in fluorescence, which reflects binding and occlusion of 2 $K^+$ ions.

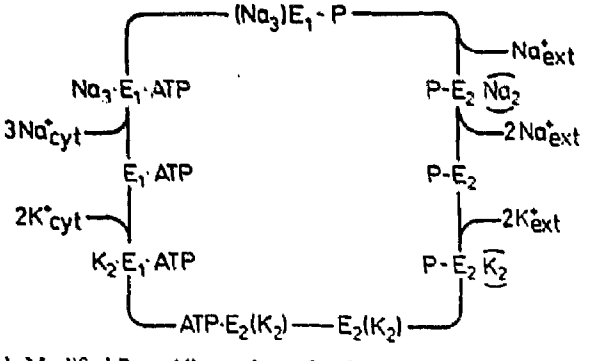


Fig. 1. Modified Post–Albers scheme for the pumping cycle of Na,K-pump. Brackets ( ) indicate occluded states (i.e. ions are unable to exchange with the aqueous phase). Horizontal brackets describe conditions in which ions are buried inside the protein dielectric.

From previous experiments [1,16] there is evidence that the apparent affinity of the extracellular ion binding sites can be changed by changing the electrostatic potential inside the membrane dielectric. Hydrophobic ions like tetraphenylphosphonium ($TPP^+$) or tetraphenylborate ($TPB^-$), which bind in lipid bilayers, change the electrostatic potential inside the dielectric when added to membranes, and modulate the extracellular binding affinity of the pump for $Na^+$ and $K^+$ [1]. If, as proposed, ouabain binds mainly to the P-$E_2(Na_2)$ state both the amplitude and the kinetics of the RH 421 binding signal should be affected by the addition of hydrophobic ions. The corresponding experiments (Fig. 4a,b) supported this assumption. In Fig. 4a the ATP and ouabain induced changes of the fluorescence signal are shown with 0 and 200 µM $TPP^+$. In the absence of $TPP^+$ a small fluorescence increase was observed. This

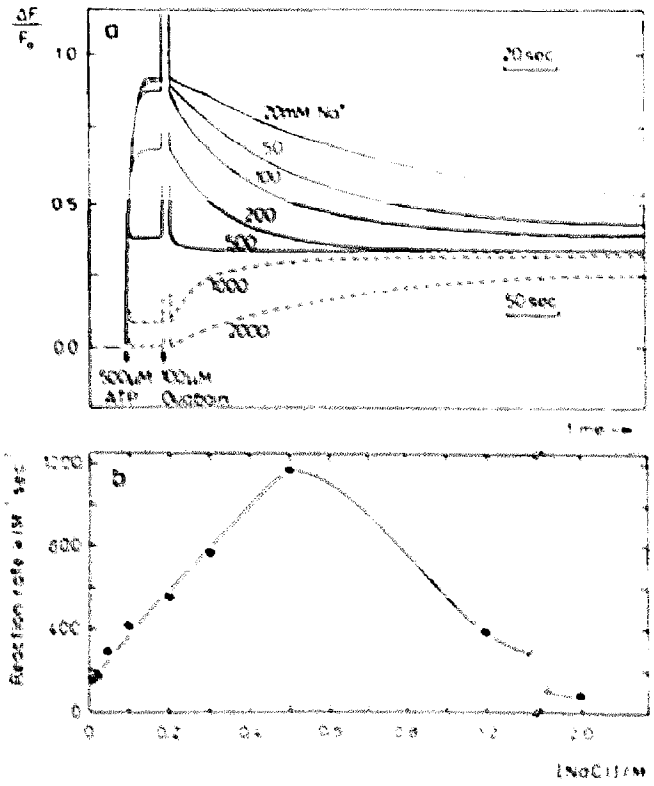


Fig. 3. Ouabain induced fluorescence changes of RH 421 labelled membrane fragments in a solution with 25 mM histidine, 0.5 mM EDTA, 10 mM Mg, and the indicated concentrations of NaCl ($T$ = 20°C). (a) Time course after phosphorylation by ATP. 100 µM ouabain was added to the buffer. Ionic strength has been maintained constant with choline chloride. (b) Na concentration dependent kinetics: the fluorescence changes as observed in part (a) can be fitted by a single exponential. The experimentally obtained values of the apparent rate constants $k$ are plotted as a function of the Na concentration. At the highest rate the fluorescence amplitude are minimal.

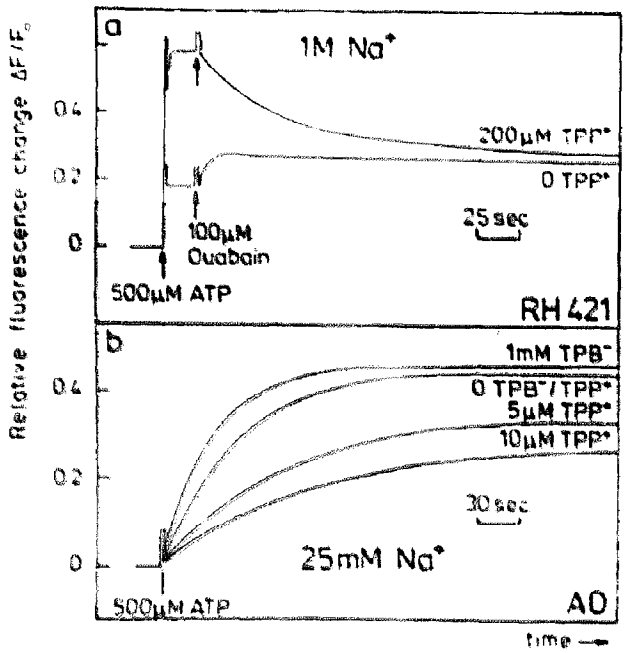


Fig. 4. Effect of hydrophobic ions on cardiac glycoside binding. a: Fluorescence change of RH 421 labelled membrane fragments in the presence and absence of 200 µM TPP+. Addition of ATP to a solution containing 1 M NaCl, 25 mM histidine, 10 mM Mg, and 0.5 mM EDTA, resulted in phosphorylation of the enzyme and in subsequent transition to $E_2$-P and release of $Na^+$. b: Time course of ouabain binding detected by observation of anthroylouabain (AO) fluorescence. AO binding was induced by the addition of ATP to a solution containing 25 mM Na, 10 mM Mg, 25 mM histidine, 0.5 mM EDTA, about 0.9 mg/ml membrane fragments, 1 µM AO and the indicated concentrations of hydrophobic ions ($T$ = 37°C).

indicated that only a minor part of the $Na^+$ ions were released from their binding sites after the conformational transition. Inhibition of the enzyme by ouabain induced $Na^+$ release and thus a fluorescence increase. In the presence of $TPP^+$ the ATP-induced fluorescence increase was significantly larger due to an enhanced $Na^+$ release. Ouabain addition under this condition resulted in $Na^+$ binding to the enzyme, inducing a decrease of fluorescence. In Fig. 4b the influence of $TPP^+$ and $TPB^-$ on anthroylouabain binding is shown. The kinetics of this process is determined by the population of the state P-$E_2(Na_2)$ which is controlled by the binding affinity for sodium. Since $TPB^-$ enhances the affinity of $Na^+$, binding of AO should be faster. $TPP^+$ decreases $Na^+$-binding and correspondingly AO binding should be slowed down. The fluorescence amplitude at long times reflects the amount of AO bound to the enzyme. The experiments shown in Fig. 4b support this expectation.

## 4. DISCUSSION

Accepting that the styryl dye RH 421 detects changes in electric field strength, interesting conclusions are suggested by these fluorescence experiments. The final fluorescence amplitude after addition of ouabain to the phosphorylated enzyme is independent of the buffer concentration of $Na^+$ and indicates that 2 $Na^+$ ions are bound per pump. This estimate fits well to published findings [3,17,18], that two $Na^+$ (or $K^+$) ions were occluded per $\alpha\beta$-subunit in ouabain inhibited enzyme.

The finding, that at high $Na^+$ concentrations (> 0.5 M), charge is released from the enzyme upon ouabain binding, whereas at low $Na^+$ concentration (< 0.5 M), charge is bound to the enzyme, indicates that there are two electrogenic steps in the reaction sequence $(Na_3)E_1$-P $\rightleftarrows$ P-$E_2(Na_2)$ $\rightleftarrows$ P-$E_2$. The first electrogenic step presumably consists in the release of 1 $Na^+$ ion to the extracellular side. Since the ouabain-enzyme complex displays $E_2$-like digestion patterns [17], this step probably is correlated with the $(Na_3)E_1$-P $\rightleftarrows$ P-$E_2(Na_2)$ conformational transition. The second electrogenic step consists in the release of the remaining $Na^+$ ions from their binding sites. This interpretation is in agreement

with the assumption, that the (negatively charged) binding sites for $Na^+$ and $K^+$ are connected with the aqueous medium by a narrow access channel or 'ionwell' [1,19,20]. Thus, the binding kinetics of cardiac glycosides on $Na^+$- ATPase should be affected by the electric field in the protein (i.e. transmembrane voltage). The experiments in Fig. 4b support this interpretation.

Altogether, this fluorescence method provides valuable insight in mechanism of cardiac glycoside binding and in electrogenic ion translocation and is a convenient tool to characterize cardiac glycoside binding to the Na,K-pump.

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