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Charge Translocation by the Na,K-Pump: II. Ion Binding and Release at the Extracellular Face

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Summary. In the first part of the paper, evidence has been presented that electrochromic styryl dyes, such as RH 421, incorporate into Na,K-ATPase membranes isolated from mammalian kidney and respond to changes of local electric field strength. In this second part of the paper, fluorescence studies with RH-421-labeled membranes are described, which were carried out to obtain information on the nature of charge-translocating reaction steps in the pumping cycle. Experiments with normal and chymotrypsin-modified membranes show that phosphorylation by ATP and occlusion of Na^+ are electroneutral steps, and that release of Na^+ from the occluded state to the extracellular side is associated with translocation of charge. Fluorescence signals observed in the presence of K^+ indicate that binding and occlusion of K^- at the extracellular face of the pump is another major electrogenic reaction step. The finding that the fluorescence signals are insensitive to changes of ionic strength leads to the conclusion that the binding pocket accommodating Na^+ or K^+ is buried in the membrane dielectric. This corresponds to the notion that the binding sites are connected with the extracellular medium by a narrow access channel ("ion well"). This notion is further supported by experiments with lipophilic ions, such as tetraphenylphosphonium (TPP^+) or tetraphenylborate (TPB^-), which are known to bind to lipid bilayers and to change the electrostatic potential inside the membrane. Addition of TPP^+ leads to a decrease of binding affinity for Na^+ and K^+, which is thought to result from the TPP^+-induced change of electric field strength in the access channel.

Key Words Na,K-ATPase · ion pumps · electrogenic transport · potentiometric dyes

Introduction

Operation of the Na,K-pump involves a sequence of conformational transitions and ion binding and release reactions (Glynn, 1985; Jørgensen & Andersen, 1988). According to the Post-Albers reaction scheme (Fig. 1), the enzyme can assume two principal conformations E_1 and E_2 with inward-facing (E_1) and outward-facing ion-binding sites (E_2). When the protein is phosphorylated in state E_1 by ATP, Na^+ becomes "occluded," i.e., trapped inside the protein ($Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1$-P). Transition to conformation E_2 leads to release of Na^+ and binding of K^+. This results in dephosphorylation of the protein and occlusion of K^+. The cycle is completed by transition to conformation E_1 and release of K^+ to the cytoplasmic side.

In the course of the pumping cycle, net charge is translocated across the membrane. An important problem in the mechanistic understanding of the pump is the question in which steps of the cycle charge is actually moved inside the pump molecule (De Weer, Gadsby & Rakowski, 1988; Apell, 1989). Studies of transient currents in heart cells (Nakao & Gadsby, 1986) and in reconstituted membrane systems (Fendler et al., 1985; Borlinghaus, Apell & Läuger, 1987) have shown that reactions in the sodium limb of the cycle are associated with translocation of electric charge. The same conclusion has been drawn from studies of reconstituted vesicles (Goldshlegger et al., 1987; Goldshlegger, Shahak & Karlish, 1990; Apell, Häring & Roudna, 1990). Experiments with chymotrypsin-modified Na,K-ATPase have given evidence that the first steps in the Na part of the cycle, *viz.*, phosphorylation by ATP and occlusion of Na^+, are electrically silent and that deocclusion and release of Na^+ to the extracellular medium are major electrogenic events (Apell, Borlinghaus, & Läuger, 1987, Borlinghaus et al., 1987).

The interpretation of these findings raises the question how charge translocation in the membrane-embedded pump protein can be understood at the microscopic level. Since it seems unlikely that charge moves in a single step over the entire hydrophobic thickness of the membrane, it has been proposed that ion pumps have access channels that

* Deceased (September 13, 1990).

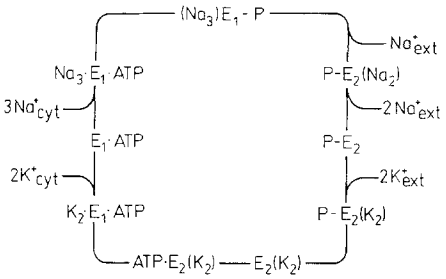

Fig. 1. Post-Albers scheme for the pumping cycle of Na,K-ATPase, adapted from Glynn (1985), Forbush (1988) and Jørgensen and Andersen (1988). E_1 and E_2 are conformations of the enzyme with ion binding sites facing the cytoplasm and the extracellular medium, respectively. In the "occluded" states $(Na_3)E_1$-P, P-$E_2(Na_2)$, P-$E_2(K_2)$ and $E_2(K_2)$, the bound ions are unable to exchange with the aqueous phase. Dashes indicate covalent bonds and dots indicate noncovalent bonds

connect the ion-binding sites to the aqueous medium (Tanford, 1983; Läuger & Apell, 1986). Within the access channel ions may migrate by a diffusion-type mechanism. For the structure of an access channel two limiting cases may be distinguished: (i) The access channel may consist of a wide opening (or vestibule) into which water and all kinds of ions are allowed to enter freely. Under this condition the electrical conductance of the access channel is large, meaning that the field strength resulting from a transmembrane voltage is low within the channel. (ii) In the other extreme case, the channel is narrow and specific for the transported ions, so that part of the transmembrane voltage drops across the length of the channel. This corresponds to the "ion well" model introduced by Mitchell (Mitchell & Moyle, 1974). In the presence of an ion well, an ion moving from the aqueous medium to the binding site has to traverse part of the transmembrane electric field, so that the effective ion affinity of the site becomes voltage dependent.

The existence of ion wells in the Na,K-ATPase has been discussed previously as a possibility to explain effects of transmembrane voltage on stationary and nonstationary pump currents (Läuger & Apell, 1986, 1988; Nakao & Gadsby, 1986; Rakowski, Vasilets & Schwarz, 1990; Rakowski et al., 1991). Goldshlegger et al. (1987) have considered the possibility that the experimentally observed voltage effect on the affinity for cytoplasmic Na^+ is caused by a shallow ion well at the cytoplasmic face of the pump. Direct evidence for the presence of ion wells in the Na,K-pump is lacking so far, however.

In the first part of this paper (Bühler et al., 1991) we have described the use of electrochromic styryl dyes for the detection of changes of electric field strength in Na,K-ATPase membranes. In this second part of the paper, we describe the application of styryl dyes for investigating charge translocation associated with binding and release of K^+ and Na^+ at the extracellular face of the pump. The experiments were carried out with open membrane fragments containing a high density (10^3–10^4 per μm^2) of oriented Na,K-ATPase molecules (Deguchi, Jørgensen & Maunsbach, 1977). If a binding site for Na^+ or K^+ in the Na,K-ATPase is connected by an ion well to the aqueous medium, ion binding or release should give rise to a change of electric field strength in the membrane, which may be sensed by the electrochromic dye. The results of these experiments are consistent with the notion that an ion well exists at the extracellular face of the pump. This notion is further supported by experiments in which lipophilic cations or anions were added to the aqueous medium. Partitioning of lipophilic ions into the membrane leads to a change of binding affinity for Na^+ and K^+. This affinity change is thought to result from the electrostatic potential created by the lipophilic ion inside the lipid bilayer.

Materials and Methods

Materials

α-Chymotrypsin, apyrase VI and ouabain were purchased from Sigma. 5-Iodoacetamidofluorescein (IAF) and RH 421 were obtained from Molecular Probes (Eugene, OR). The purity of the dyes was checked by thin-layer chromatography. Sodium tetraphenylborate (TPB^-) and tetraphenylphosphonium chloride (TPP^+) were from Merck (Darmstadt). For the experiments in the nominal absence of K^+, NaCl was used in Suprapur quality (Merck). All other reagents were analytical grade. Choline chloride was obtained form Aldrich ("Goldmarke") or from Sigma (3× recrystallized). P^3-1-(2-nitro) phenylethyladenosine-5'-triphosphate ("caged" ATP) was synthesized by K. Janko using a modified version of the method of Kaplan, Forbush and Hoffmann (1978). The purity of the product was checked by HPLC. The compound was stored as tetramethylammonium salt in the dark at −40°C. Acetylphosphate (AcP) was obtained from Boehringer, Mannheim, as a Li,K salt. The compound was converted to the Tris salt by ionic-exchanger chromatography with Dowex 50W-X8, as described by Rephaeli, Richards and Karlish (1986).

Preparation of Na,K-ATPase Membranes and Fluorescence Labeling

Na,K-ATPase was prepared from the outer medulla of rabbit kidneys using procedure C of Jørgensen (1974), as described in part I of this paper (Bühler et al., 1991). This method yields purified enzyme in the form of open membrane fragments containing about 0.8 mg phospholipid and 0.2 mg cholesterol per mg protein. For most preparations the specific activity was in the range between 1500 and 2200 μmol P_i per hr and mg protein at 37°C, corresponding to a turnover rate of 120–170 sec^{-1} (based

on a molar mass of 280,000 g/mol). Labeling of the enzyme with IAF was carried out as described previously (Bühler et al., 1991). In the experiments with RH 421, the dye was added from an ethanolic stock solution (concentration 0.2 mM) to the aqueous suspension of membrane fragments.

Recording of Steady-State and Transient Fluorescence Signals

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 15 nm) and the emission wavelength to 650 nm (slit width 15 nm). If not otherwise indicated, the experiments were carried out at 20°C.

Transient fluorescence signals after photochemical release of ATP were performed as described previously (Stürmer et al., 1989; Bühler et al., 1991). ATP was released from "caged" ATP in the sample cell by light flashes (wavelength 308 nm, total energy 150 mJ, duration 10 nsec) generated by an EMG 100 excimer laser (Lambda Physics, Göttingen). At pH 7.0, ATP is released with a time constant of 4.6 msec. If not otherwise stated, the concentration of caged ATP was 100 μM. About 20 μM ATP were released in a single flash, corresponding to a photochemical yield of 20%. In order to remove traces of free ATP contained in the sample of caged ATP, a small amount of apyrase VI (10^{-3} units/ml) and 2 mM Mg^{2+} were added to the membrane suspension prior to the flash experiment.

Results

Fluorescence Signals Associated with (Na^+ + ATP)-Induced Phosphorylation and K^+-Induced Desphosphorylation

In the experiment represented in Fig. 2*A*, Na,K-ATPase membrane fragments (9 μg protein/ml) were equilibrated with styryl dye RH 421 in the presence of 20 mM Na^+ and 5 mM Mg^{2+}. Under the given experimental conditions, about 80% of the dye binds to the membranes (Bühler et al., 1991). Upon addition of 10 μM ATP, the fluorescence of the dye increases by about 100%. This fluorescence increase is not observed when ATP is added to the membrane fragments in the absence of Mg^{2+}, or when the Na,K-ATPase is inhibited by ouabain or vanadate. When thereafter 20 mM K^+ are added to the medium, the fluorescence decreases again to a value about 15% above the level prior to the addition of ATP.

The sign of the fluorescence change upon addition of ATP ($\Delta F > 0$) corresponds to the creation of a negative electrostatic potential inside the membrane dielectric. This follows from the comparison with fluorescence signals induced by adsorption of lipophilic cations and anions to Na,K-ATPase membranes (Bühler et al., 1991).

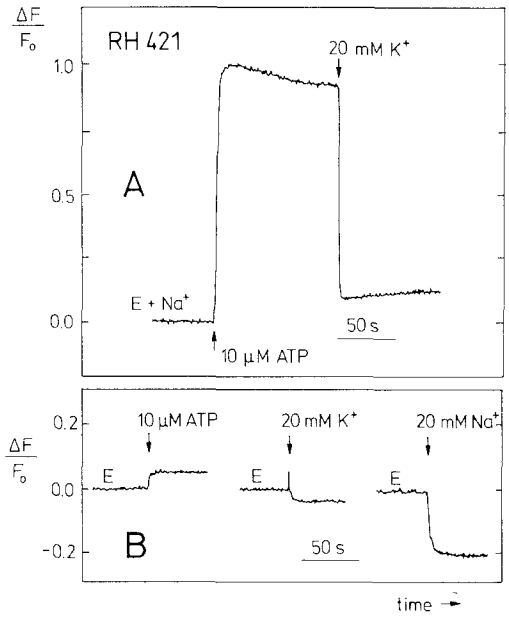

Fig. 2. Fluorescence changes of styryl dye RH 421 bound to Na,K-ATPase membranes. (*A*) Open membrane fragments (9 μg protein/ml) were equilibrated with 0.23 μM RH 421 (total concentration) in the presence of 20 mM NaCl, 5 mM $MgSO_4$, 1 mM EDTA, and 30 mM imidazole sulfate, pH 7.2. The temperature was 20 ± 0.2°C. The fluorescence was excited at 580 nm and recorded at 650 nm. 10 μM ATP and 20 mM K^+ were successively added to the medium. $\Delta F/F_0$ is the relative fluorescence change referred to the fluorescence intensity F_0 prior to the addition of ATP. (*B*) As in *A*, but without Na^+ initially present in the medium. Either 10 μM ATP or 20 mM K^+ or 20 mM Na^+ were added to a suspension of membrane fragments

Experiments in which ATP, Na^+, or K^+ were added separately to the enzyme are shown in Fig. 2*B*. Upon addition of ATP or of K^+, the fluorescence remained virtually unchanged, whereas addition of Na^+ led to a distinct fluorescence decrease. The possible meaning of these results will be discussed later.

The observed fluorescence changes (Fig. 2*A*) may be compared with the predictions of the Post-Albers reaction scheme of the Na,K-ATPase (Fig. 1). In the presence of Na^+, the enzyme is in conformation E_1 with inward-facing ion-binding sites. Addition of ATP leads to phosphorylation and occlusion of Na^+

$$E + 3Na^+_{cyt} + ATP \rightarrow Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1\text{-}P + ADP. \quad (1)$$

The occluded state undergoes a transition to conformation E_2 with outward-facing binding sites, followed by release of Na^+

$$(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 + 3Na^+_{ext}. \quad (2)$$

This reaction is likely to proceed through an intermediate state P-$E_2(Na_2)$ with two occluded Na^+ ions (Yoda & Yoda, 1987; Jørgensen & Andersen, 1988; Nørby & Klodos, 1988). In the absence of K^+, dephosphorylation of the phosphoenzyme in state P-E_2 is extremely slow, but is greatly accelerated upon addition of K^+

$$P\text{-}E_2 + 2K^+_{ext} \rightarrow P\text{-}E_2(K_2) \rightarrow E_2(K_2) + P_i. \quad (3)$$

Evidence for the existence of the phosphoenzyme form P-$E_2(K_2)$ with occluded K^+ has been obtained by Forbush (1988). Thus, according to the reaction scheme in Fig. 1, it may be assumed that the fluorescence increase observed upon addition of ATP (Fig. 2*A*) reflects the transition from state $Na_3 \cdot E_1$ to state P-E_2, whereas the fluorescence decrease observed upon addition of K^+ reflects the transition from state P-E_2 to state $E_2(K_2)$.

Phosphorylation by Acetylphosphate

The assumption that the fluorescence increase observed upon addition of ATP (Fig. 2*A*) results from phosphorylation of the enzyme and transition to state P-E_2 was further tested in an experiment in which ATP was replaced by acetylphosphate (AcP). AcP is known to phosphorylate Na,K-ATPase in the presence of Na^+, yielding a phosphoenzyme which is indistinguishable from the phosphoenzyme formed by ATP (Beaugé et al., 1985; Rephaeli et al., 1986). Addition of AcP in saturating concentration (1 mM) in the presence of 20 mM Na^+ gave rise to a fluorescence signal which was only 10% smaller than the signal observed upon addition of ATP (*data not shown*). The reduction in signal amplitude is likely to result from the low rate of phosphorylation by AcP which leads to a reduction in the steady-state level of the phosphoenzyme. (From stopped-flow experiments, the maximal phosphorylation rate by AcP was estimated to be about 2 sec^{-1} at 20°C.) When 1 mM K^+ was added after phosphorylation by AcP, the fluorescence signal decreased again to a low level, as in the experiment shown in Fig. 2*A*.

Reversibility of the ATP-Induced Fluorescence Change

The reversibility of the phosphorylation-induced fluorescence change was tested in the experiment shown in Fig. 3. Membrane fragments were initially equilibrated with 10 mM Na^+ and 0.25 μM RH 421. When a small amount of ATP (1 μM) was added, the fluorescence immediately increased (as in Fig. 2*A*), but decreased again steeply after about 100 sec to nearly the original level. This fluorescence decrease is thought to result from the consumption of ATP in the solution in the course of the Na-ATPase reaction. (Under the given experimental conditions (absence of K^+), the enzyme operates when at about 10–20% of its normal activity.) When the addition of 1 μM ATP was repeated, essentially the same fluorescence signal was observed again (Fig. 3).

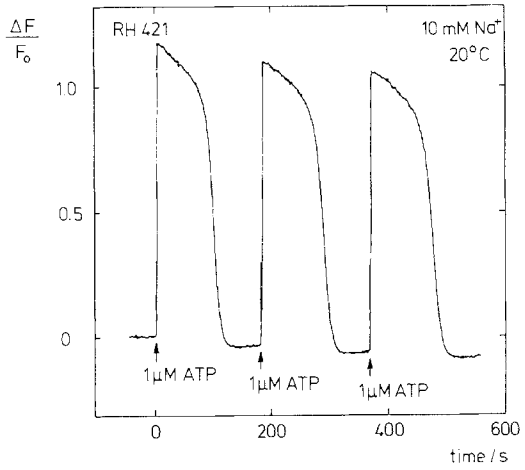

Fig. 3. Reversibility of the phosphorylation-induced fluorescence change. The experiment was carried out under similar conditions as in Fig. 2*A*, but with a ten times smaller amount of ATP added to the cuvette. The membrane fragments (9 μg protein/ml) were equilibrated with 10 mM NaCl, 5 mM $MgCl_2$, 1 m EDTA and 0.25 μM RH 421 prior to the addition of ATP. After the fluorescence has returned to nearly the original level, addition of a further amount of ATP elicits a new fluorescence signal of virtually identical amplitude. The fluorescence changes are referred to the fluorescence F_o prior to the addition of ATP

Influence of Ionic Strength

A possible origin of the fluorescence signals represented in Fig. 2*A* is the change of local field strength resulting from release of Na^+ or binding of K^+ at the extracellular binding sites. If the binding sites are located at the surface of the protein, the amplitude of the signals should decrease with increasing ionic strength of the medium. To test this possibility, the experiment of Fig. 2*A* was repeated, varying the ionic strength by addition of choline chloride to the solution. The relative fluorescence amplitudes $a_X \equiv \Delta_X/F_o$, which are referred to the fluorescence F_o of the dye-labeled membranes in the presence of 20 mM Na^+, are plotted in Fig. 4 as a function of choline chloride concentration. It is seen that the fluorescence amplitudes change only insignificantly (by less

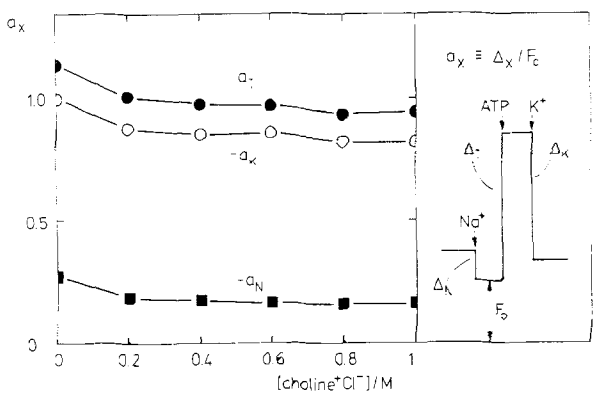

Fig. 4. Influence of ionic strength on the amplitudes of fluorescence signals observed under the conditions of the experiment of *Fig. 2A*. The concentration of choline chloride was varied between 0 and 1 M, corresponding to a variation of the total ionic strength between 40 mM and 1.04 M. Membrane fragments (9 μg protein/ml) were equilibrated with 5 mM $MgSO_4$, 1 mM EDTA, 30 mM imidazole sulfate, pH 7.2, and 0.25 μM RH 421. 20 mM Na^+, 10 μM ATP and 20 mM K^+ were successively added. The temperature was 20 ± 0.2°C. The fluorescence changes are referred to the fluorescence F_0 recorded in the presence of 20 mM Na^+ prior to the addition of ATP

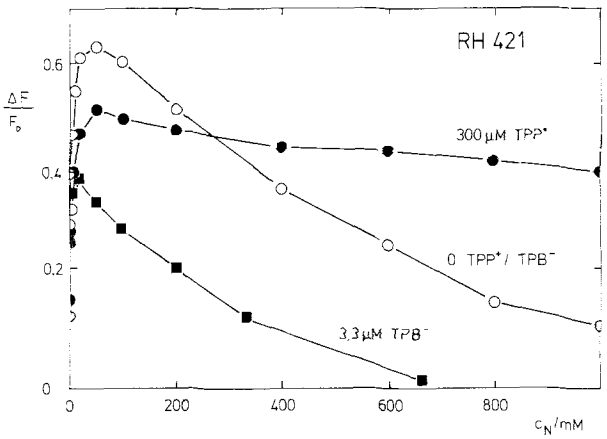

Fig. 5. Change of fluorescence of RH 421 upon ATP-induced phosphorylation (Fig. 2*A*), as a function of Na^+ concentration c_N in the presence and in the absence of tetraphenylphosphonium (TPP^+) or tetraphenylborate (TPB^-). The fluorescence change ΔF is referred to the fluorescence F_0 prior to addition of ATP. ΔF was evaluated from time-resolved fluorescence experiments (ΔF is the stationary amplitude which is reached at long times). The ionic strength was held constant by addition of choline chloride. A suspension of membrane fragments (30 mg protein/ml) was added to a solution of 0.7 μM RH 421, 20 μM "caged" ATP, 10 mM $MgCl_2$, 1 M (NaCl + choline chloride), 1 mM EDTA, 0 or 300 μM TPP^+ or 3.3 μM TPB^- and 30 mM imidazole chloride, pH 7.2. About 5 μM ATP were released by a 308-nm flash of 10 nsec duration. The temperature was 20°C

than 20%) when the choline chloride concentration is increased from 0 to 1 M, corresponding to a variation of total ionic strength from about 40 mM to 1.04 M. This finding argues against the possibility that the observed fluorescence signals reflect changes of surface-charge density. The results represented in Fig. 4 are thus consistent with the view that the styryl dye responds to charge movements in the interior of the membrane dielectric.

Chymotrypsin-Modified Na,K-ATPase

Further information on the origin of the fluorescence increase observed upon phosphorylation of the enzyme (Fig. 2*A*) can be obtained from experiments in which the Na,K-ATPase is modified by α-chymotrypsin. Treatment of Na,K-ATPase with α-chymotrypsin in the presence of Na^+ at low ionic strength leads to cleavage of a single peptide bond in the α-subunit; the split is located in the cytoplasmic portion of the protein between Leu-266 and Ala-267 (Jørgensen & Collins, 1986). Under suitable reaction conditions, secondary cleavage is negligible. In the chymotrypsin-treated enzyme, phosphorylation by ATP and occlusion of Na^+ are preserved, while Na,K-pumping is abolished (Glynn, Hara & Richards, 1984; Jørgensen & Petersen, 1985). These findings indicate that modification by chymotrypsin stabilizes the Na,K-ATPase in state $(Na_3)E_1$-P by preventing the transition to state P-E_2 (Fig. 1).

Membrane fragments were pretreated with chymotrypsin for 60 min at 37°C in a medium containing 5 μg/ml α-chymotrypsin, 100 μg/ml Na,K-ATPase, 10 mM NaCl, 1 mM EDTA and 30 mM imidazole, pH 7.2. Under this condition the proteolytic reaction may be expected to be selective, consisting of the cleavage of a single peptide bond, as discussed above. When the experiment in Fig. 2*A* was repeated with chymotrypsin-modified Na,K-ATPase, fluorescence changes were no longer observed upon addition of ATP or K^+. This means that the ATP-induced fluorescence increase shown in Fig. 2*A* occurs during deocclusion and release of Na^+ to the medium ($(Na_3)E_1$-P → P-E_2 + 3Na).

Na^+-Concentration Dependence of the ATP-Induced Fluorescence Signals

Fluorescence of RH 421. In Fig. 5, the amplitude of the fluorescence signal of RH 421, which is observed upon phosphorylation by ATP (Fig. 2*A*) is plotted as a function of Na^+ concentration c_N (curve labeled *0 TPP^+/TBP^-*). The ionic strength was held constant at 1 M by addition of choline chloride. It is seen that the relative fluorescence change $\Delta F/F_0$ reaches a maximum at $c_N \approx 40$ mM and declines at higher Na^+ concentrations. This decrease of $\Delta F/F_0$ is likely

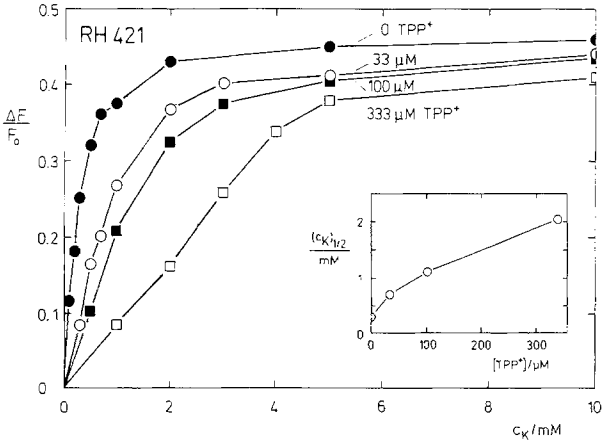

Fig. 6. Change of fluorescence of RH 421 upon addition of K^+ to the phosphoenzyme (Fig. 2*A*), as a function of K^+ concentration c_K. The fluorescence change ΔF is referred to the fluorescence F_o prior to release of ATP. ΔF was evaluated from time-resolved fluorescence experiments and taken as the difference of the stationary signal amplitudes at long times after addition of ATP with and without addition of K^+. A suspension of membrane fragments (30 μg protein/ml) was added to a solution of 0.7 μM RH 421, 20 μM "caged" ATP, 10 mM $MgCl_2$, 50 mM NaCl, 1 M choline chloride, 1 mM EDTA, 30 mM imidazole chloride, pH 7.2, and various concentrations of KCl and tetraphenylphosphonium (TPP^+). In the absence of choline chloride, the results were virtually identical. About 5 μM ATP were released by a 308 nm flash of 10 nsec duration. The temperature was 20°C

to result from a Na^+-induced shift of the distribution between states P-E_2, P-$E_2(Na_2)$ and $(Na_3)E_1$-P

$$(Na_3)E_1\text{-}P \rightleftarrows P\text{-}E_2(Na_2) \rightleftarrows P\text{-}E_2. \quad (4)$$

At high Na^+ concentration, the reaction sequence is shifted to the left, so that the enzyme mainly remains in the states $(Na_3)E_1$-P and P-$E_2(Na_2)$.

Fluorescence of IAF. In parallel experiments, the fluorescence of membrane fragments labeled with 5-iodoacetamidofluorescein (IAF) was recorded after photochemical release of ATP from "caged" ATP (Stürmer et al., 1989). From previous studies it is known that the fluorescence of IAF-labeled Na,K-ATPase decreases during the transition from $(Na_3)E_1$-P to P-E_2 (Kapakos & Steinberg, 1986; Stürmer et al., 1989). In the present experiments, the amplitude of this fluorescence change was studied as a function of Na^+ concentration c_N up to c_N = 1 M. The amplitude of fluorescence change was found to decrease with increasing Na^+-concentration c_N above 40 mM (*not shown*), in a similar way as the fluorescence change of RH 421-labeled membranes decreased with increasing c_N (Fig. 5). The experiments with IAF-labeled enzyme thus provide further support for the notion that the reaction sequence (4) is shifted to the left at high Na^+ concentrations.

Fluorescence Change upon Addition of K^+

The fluorescence change observed upon addition of K^+ to the phosphoenzyme (Fig. 2*A*) is plotted as a function of K^+ concentration c_K in Fig. 6 (curve labeled *0 TPP*$^+$). $\Delta F/F_o$ is found to saturate at high K^+-concentrations, with a half-saturation concentration $(c_K)_{1/2}$ of about 0.25 mM. This value is similar to the apparent dissociation constant of K^+ at the extracellular side (0.1–0.2 mM) which is obtained from the dependence of ATP-hydrolysis rate on K^+ concentration (Robinson & Flashner, 1979).

This agreement between $(c_K)_{1/2}$ from Fig. 6 and the apparent affinity for extracellular K^+ indicates that the signal observed upon addition of K^+ in Fig. 2*A* results from binding of K^+ to form P-E_2 of the enzyme, followed by occlusion of K^+ and release of P_i. (In the experiment shown in Fig. 2*A*, the ATP concentration (1 μM) was so small that binding of ATP to $E_2(K_2)$ is negligible.) The assumption that RH 421 responds to local field changes in the membrane (Bühler et al., 1991), thus leads to the conclusion that one of the following two reactions, or both, are associated with translocation of charge in the protein

$$P\text{-}E_2 + 2K^+_{ext} \rightarrow P\text{-}E_2(K_2) \quad (5)$$

$$P\text{-}E_2(K_2) \rightarrow E_2(K_2) + P_i. \quad (6)$$

In experiments with reconstituted vesicles, Goldshleger et al. (1987, 1990) observed that K,K-exchange in the presence of ATP and P_i and at saturating extracellular K^+ concentration is voltage independent. This indicates that the dephosphorylation step (reaction 6) is electrically silent. On the other hand, since reaction (5) is strongly poised to the right at high $(c_K)_{ext}$, the experiments of Goldshleger et al. do not exclude the possibility that reaction (5) is electrogenic. The fluorescence change observed upon addition of K^+ to the phosphorylated enzyme (Fig. 2*A*) thus indicates that binding of K^+ to P-E_2 followed by occlusion (reaction 5) is a major electrogenic reaction step. A likely explanation for the electrogenic nature of reaction (5) consists in the assumption that K^+ ions have to traverse part of the membrane dielectric to reach the occlusion sites from the extracellular medium in state P-E_2 of the enzyme. This corresponds to the "ion well" model introduced by Mitchell and Moyle (1974).

Transient Fluorescence Changes Associated with Release of Na^+ and Binding of K^+ after Phosphorylation

When ATP is photochemically released from caged ATP in the presence of both Na^+ and K^+, the reaction sequence $Na_3 \cdot E_1 \rightarrow Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1$-

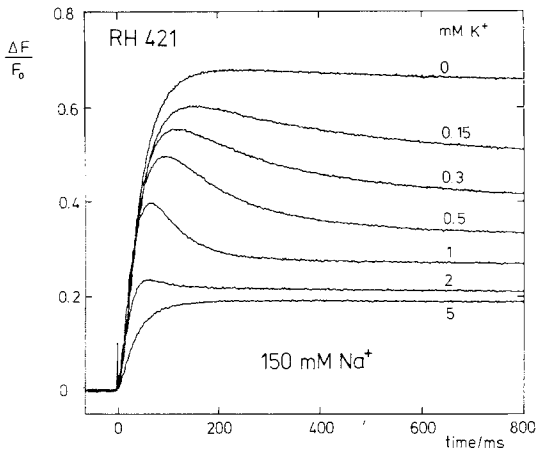

Fig. 7. Time course of the relative fluorescence change $\Delta F/F_0$ of RH 421-labeled membrane fragments after phosphorylation by ATP in the presence of Na^+ and K^+. About 2 μM ATP were photochemically released at time $t = 0$. F_0 is the fluorescence intensity prior to the light flash. A suspension of membrane fragments (30 μg protein/ml) was equilibrated with 0.7 μM RH 421, 10 μM "caged" ATP, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, 30 mM imidadzole chloride, pH 7.2, and various concentrations of KCl. The temperature was 20°C

$P \rightarrow P\text{-}E_2 \rightarrow P\text{-}E_2(K_2) \rightarrow E_2(K_2)$ is initiated. If the concentration of released ATP is much smaller than the apparent dissociation constant of ATP at the low-affinity binding site ($K_m \approx 20$ μM), the enzyme remains essentially in state $E_2(K_2)$. Assuming that the reaction steps $Na_3 \cdot E_1 \rightarrow Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1\text{-}P$ and $P\text{-}E_2(K_2) \rightarrow E_2(K_2)$ are not rate limiting (Glynn, 1985; Stürmer et al., 1989), the overall reaction can be approximately described by the following transitions:

$$(Na_3)E_1\text{-}P \xrightarrow{k_A} P\text{-}E_2 \xrightarrow{k_B} E_2(K_2). \qquad (7)$$

Implicit in this reaction scheme is the assumption that the concentrations of Na^+ and P_i are low enough so that both steps are virtually irreversible. The rate constant k_B depends on the potassium concentration in the medium. In the absence of K^+ ($k_B = 0$), a monotonous fluorescence increase should be observed, corresponding to the transition $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2$. In the presence of a high concentration of K^+, the second step is fast, so that, again, a monotonous fluorescence change is expected. At intermediate K^+ concentrations at which k_A and k_B are of comparable magnitude, the fluorescence signal should be nonmonotonous, reflecting the transition from the weekly fluorescent state $(Na_3)E_1\text{-}P$ to the strongly fluorescent state $P\text{-}E_2$, followed by the transition to state $E_2(K_2)$ which has again a low fluorescence. These predictions are borne out by the experiment shown in Fig. 7. Both in the absence of K^+ and in the presence of 5 mM K^+, a nearly monoexponential fluorescence increase is observed, whereas at intermediate K^+ concentrations, the fluorescence signal reaches a maximum and thereafter decreases towards a quasistationary level. The large fluorescence amplitude in the absence of K^- and the much smaller fluorescence amplitude at 5 mM K^+ approximately correspond to the fluorescence levels observed in the experiment of Fig. 2*A* in which ATP and K^+ have been added successively to the medium. The biphasic behavior shown in Fig. 7 agrees with similar results obtained with IAF-labeled enzyme (Stürmer et al., 1989).

The observation that the quasistationary fluorescence levels $a_\infty \equiv \Delta F(t \rightarrow \infty)/F_0$ depend on K^+ concentration c_K (Fig. 7) is not accounted for by the simple reaction sequence (7). A likely explanation of the dependence of a_∞ on c_K consists in the assumption that state $E_2(K_2)$ can undergo a transition to state E_1 from which a new phosphorylation/dephosphorylation cycle can start

$$E_2(K_2) \rightleftarrows K_2 \cdot E_1 \rightleftarrows E_1 \cdots \rightarrow (Na_3)E_1\text{-}P. \qquad (8)$$

To test this expectation, a numerical simulation of the whole reaction cycle (Fig. 1) including reaction sequence (8) was carried out, using the values of kinetic parameters taken from the literature (Stürmer et al., 1989). The fluorescence levels of the individual states of the cycle were assigned as will be described in the next section. By the numerical simulation, the fluorescence signals represented in Fig. 7 could be qualitatively reproduced (*data not shown*).

Effects of Lipophilic Ions

Lipophilic cations and anions, such as tetraphenylborate (TPB^-) or tetraphenylphosphonium (TPP^+) adsorb to lipid bilayers; the adsorption plane is located a few tenths of a nanometer away from the membrane-solution interface (McLaughlin, 1977; Andersen et al., 1978). Accordingly, binding of lipophilic ions creates a local electric field which is restricted to a narrow zone on the lipid side of the interface (Bühler et al., 1991). In the following we describe experiments in which TPP^+ or TPB^- are used to study effects of electric fields on single reaction steps of the pumping cycle.

Effect of TPP^+ and TPB^- on Na^+ Release after Phosphorylation by ATP

When the enzyme is phosphorylated by ATP in the absence of K^+ at high Na^+ concentration ($c_N \approx 1$ M), the phosphoenzyme may be expected to be present

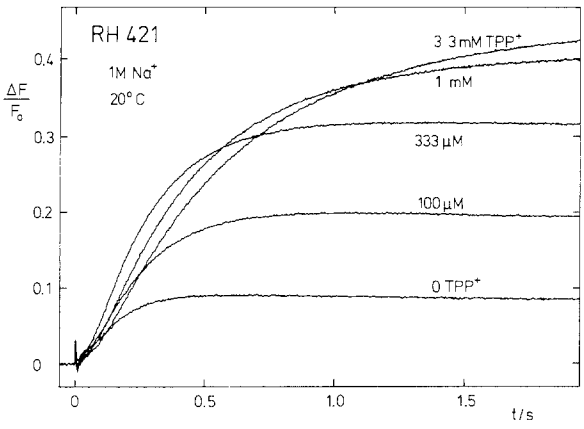

Fig. 8. Fluorescence change of RH 421 upon phosphorylation by ATP in the presence of 1 M Na^+ and various concentrations of tetraphenylphosphonium (TPP^+). At time $t = 0$, about 4 µM ATP were liberated from caged ATP by a 308-nm flash of 10 nsec duration. The fluorescence change ΔF is referred to the fluorescence F_0 prior to ATP release. A suspension of membrane fragments (30 µg protein/ml) was added to a solution of 0.7 µM RH 421, 20 µM "caged" ATP, 10 mM $MgCl_2$, 1 M NaCl, 1 mM EDTA, 30 mM imidazole chloride, pH 7.2, and various concentrations of TPP^+. The temperature was 20°C

mainly in states $(Na_3)E_1$-P and P-$E_2(Na_2)$ in which three or two Na^+ ions are still bound. This expectation is consistent with the finding that the electrochromic fluorescence signal which is thought to result from deocclusion and release of Na^+, declines at large Na^+ concentrations (Fig. 5). When lipophilic cations bind to the lipid bilayer, a positive electrostatic potential is created in the membrane dielectric. Accordingly, release of Na^+ ions should be enhanced in the presence of a positive potential. This means that the electrochromic fluorescence signal, which is reduced at high Na^+ concentration, should increase again upon addition of TPP^+.

This expectation is borne out by the time-resolved fluorescence measurements shown in Fig. 8. At time $t = 0$, ATP is released from "caged" ATP by a light flash in the presence of 1 M Na^+ and in the absence of K^+. The fluorescence signal (curve labeled *0 TPP*$^+$) has a low amplitude ($\Delta F/F_0 \approx 0.08$), as already shown in Fig. 4. When increasing amounts of tetraphenylphosphonium (TPP^+) are added to the suspension, the amplitude strongly increases, approaching saturation at TPP^+ concentrations near 1 mM.

As seen from Fig. 8, addition of TPP^+ not only increases the amplitude of the fluorescence change, but also slows down the rise of the fluorescence signal. A possible explanation of this finding consists in the assumption that the adsorption plane of TPP^+ is located between the membrane-solution interface and the Na^+-binding sites. This would mean that rate of release of Na^+ from the binding sites is reduced by the presence of the electrostatic energy barrier created by adsorption of TPP^+. On the other hand, the apparent binding affinity of Na^+ is always decreased by the presence of TPP^+, irrespective of the location of the adsorption plane.

The signal amplitude $\Delta F/F_0$ in the presence of 300 µM TPP^+ is represented in Fig. 5 as a function of Na^+ concentration c_N. It is seen that in the presence of the lipophilic cation, the decline of $\Delta F/F_0$ at high Na^+ concentrations is much reduced, as may be expected from the results presented in Fig. 8. In the TPP^+ experiments shown in Fig. 5, the ionic strength was held constant by addition of corresponding amounts of choline chloride. However, the results did not appreciably change when choline chloride was omitted.

Figure 5 further shows that addition of TPP^+ lowers the maximum value of $\Delta F/F_0$ by about 20%. This reduction of $\Delta F/F_0$ at low sodium concentrations is likely to result from a field-induced change in the distribution of TPP^+ between membrane and water. As discussed in part I of the paper (Bühler et al., 1991), lipophilic ions may redistribute between the lipid bilayer and the aqueous medium in response to pump-mediated charge movements in the membrane. This leads to a reduction of the electric field strength in the membrane and to a concomitant decrease of the fluorescence signal of the dye.

Addition of the lipophilic anion tetraphenylborate (TPB^-) creates a negative electrostatic potential inside the lipid bilayer, which should lead to an increase of apparent affinity for Na^+ at the extracellular binding sites. This is indeed the case, as Fig. 5 shows. In the presence of TPB^-, the decline of the fluorescence signal with increasing c_N, which is thought to reflect a shift of the $(Na_3)E_1$-P/P-E_2 equilibrium back to $(Na_3)E_1$-P, is steeper than under control conditions. In the absence of TPB^-, the half-maximal fluorescence amplitude is reached at $(c_N)_{1/2} \approx 500$ mM, whereas in the presence of TPB^-, $(c_N)_{1/2}$ is about 250 mM. (Compared to the control signal in the absence of lipophilic ions, the fluorescence observed in the presence of TPB^- is reduced at all Na^+ concentrations. As discussed above, this decrease of $\Delta F/F_0$ is likely to result from a redistribution of lipophilic ions within the lipid bilayer.)

Effects of TPP^+ on K^+ Binding to the Phosphoenzyme

TPP^+ strongly affects the fluorescence signal observed upon addition of K^+ to the phosphoenzyme (Fig. 2*A*). This is shown in Fig. 6 in which the signal amplitude $-\Delta F/F_0$ is plotted as a function of K^+

concentration c_K. In the presence of lipophilic cations, the signal amplitude is decreased. This decrease of $-\Delta F/F_o$ is associated with a shift of the half-saturation concentration $(c_K)_{1/2}$, as shown in the inset of Fig. 6; $(c_K)_{1/2}$ is the K^+ concentration at which $-\Delta F/F_o$ in Fig. 6 becomes half-maximal. The experiments represented in Fig. 6 were carried out at high ionic strength by addition of 1 M choline chloride. Omission of choline chloride did not appreciably change the results.

As seen from Fig. 6 (inset), the apparent affinity $1/(c_K)_{1/2}$ for K^- decreases with increasing concentration of TPP^+. This observation is consistent with the assumption that a positive electrostatic potential created by adsorption of TPP^+ to the membrane renders binding of K^+ to the extracellular sites energetically less favorable. This decrease of K^+ affinity thus parallels the decrease of Na^+ affinity observed in the presence of TPP^- (Fig. 5).

An opposite effect on the affinity of K^+ may be expected when the lipophilic ion TPB^- is adsorbed to the membrane. Experiments in the presence of TPB^- and K^+ could not be carried out, however, since the solubility of TPB^- in potassium solutions is extremely low.

Control Experiments

A possible objection against the interpretation of the experiments shown in Fig. 6 is that the effect of TPP^+ on K^+ binding is only apparent and reflects in reality an interaction between TPP^+ and the potentiometric dye. To test for this possibility, two sets of control experiments were carried out. In one set of experiments, the transition from P-E_2 to $E_2(K_2)$ was studied using IAF-labeled Na,K-ATPase. ATP was photochemically released from "caged" ATP in the presence of 50 mM Na^+ and various concentrations of K^-. In Fig. 9 the asymptotic value ($t \rightarrow \infty$) of the relative fluorescence amplitude $\Delta F/F_o$ is plotted as a function of K^+ concentration c_K. At $c_K = 0$, the fluorescence amplitude $-\Delta F/F_o$ is small, corresponding to the small difference in the IAF fluorescence of states $Na_3 \cdot E_1 \cdot ATP$ and P-E_2 (Kapakos & Steinberg, 1986; Stürmer et al., 1989). With increasing K^+ concentration, the fluorescence amplitude $-\Delta F/F_o$ increases, reflecting the transition to state $E_2(K_2)$ which has a much lower IAF fluorescence than state P-E_2. In the absence of TPP^+, the half-maximal effect of K^+ is reached at $c_K^{1/2} \approx 0.2$ mM (curve labeled *0 TPP*$^+$ in Fig. 9) which approximately agrees with the $c_K^{1/2}$ value evaluated from the RH 421 experiments ($c_K^{1/2} \approx 0.3$ mM). Addition of 100 μM TPP^+ leads to a three- to fourfold increase of the half-saturation concentration of K^+, as Fig. 9 shows.

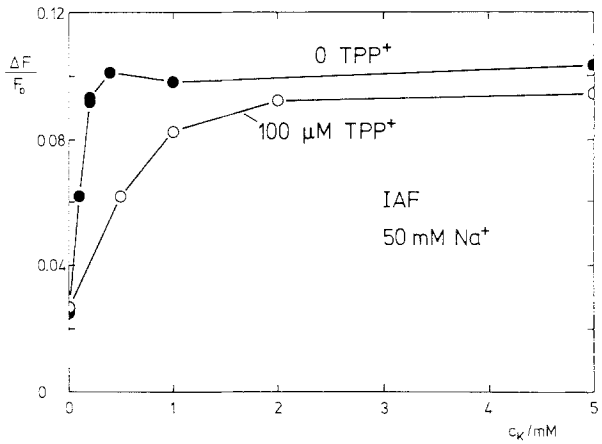

Fig. 9. Fluorescence change of IAF-labeled Na,K-ATPase after photochemical release of ATP from "caged" ATP, as a function of K^- concentration c_K. $\Delta F/F_o$ is the asymptotic value ($t \rightarrow \infty$) of the relative fluorescence change; F_o is the fluorescence prior to the liberation of ATP. IAF-labeled membrane fragments (6 μg protein/ml) were equilibrated with 50 mM NaCl, various concentrations of K^+, 0 or 100 μM tetraphenylphosphonium chloride (TPP^-), 10 mM $MgCl_2$, 1 mM EDTA, 20 μM caged ATP and 30 mM imidazole chloride, pH 7.2. About 4 μM ATP were released by the laser flash. The temperature was 20°C

This finding represents independent evidence for the notion that the electrostatic potential created by adsorption of TPP^+ inhibits binding of K^+ at the extracellular face of the pump.

In a second set of control experiments, the enzymatic activity of the Na,K-ATPase was measured as a function of K^+ concentration at a fixed Na^+ concentration of 50 mM with and without TPP^+. Addition of 100 μM TPP^+ was found to shift the half-saturation concentration of K^+ from ≈ 0.5 to ≈ 2 mM (*data not shown*).

Assignment of Fluorescence Levels to Individual Pump States

The results of the fluorescence experiments may be conveniently expressed by assigning relative fluorescence levels to the individual states of the pump. For this purpose we consider the case that all pump molecules are in the same state E_i and denote the fluorescence of the membrane suspension observed under this condition by F_i. If N_D is the total number of membrane-incorporated dye molecules, and φ_i the average contribution of the single dye molecule to F_i, the relation

$$F_i = N_D \varphi_i \tag{9}$$

holds. The quantities φ_i which reflect the electrostatic interaction between protein charges and dye

Table. Relative fluorescence amplitudes f_i [Eq. (10)] in different states of the Na,K-pump (Fig. 1), referred to state $Na_3 \cdot E_1$.*

States	$Na_3 \cdot E_1$	$(Na_3)E_1$-P	P-E_2	$E_2(K_2)$	E_1	$E_1 \cdot ATP$
f_i	0[a]	≈0[b]	1.06[c]	0.2[c]	0.26[d]	0.31[d]

*The values of f_i were evaluated from an enzyme preparation exhibiting a specific activity of 2500 μmol P_i per hour and mg protein at 37°C. The concentration of membrane fragments in the suspension was 9 μg protein/ml, the total dye concentration was 225 nM. The solution contained 5 mM $MgCl_2$, 1 mM EDTA and 30 mM imidazole chloride, pH 7.2. Excitation wavelength: 580 nm (slit width, 15 nm), emission wavelength: 650 nm (slit width, 15 nm). The temperature was 20°C.

[a] The fluorescence amplitude of state $Na_3 \cdot E_1$ is zero by definition.

[b] Based on the finding that the ATP-induced fluorescence change is abolished in the chymotrypsin-modified enzyme.

[c] *Compare* Fig. 2*A*.

[d] *Compare* Fig. 2*B*. In the absence of Na^+, nucleotides and P_i, the enzyme is thought to be predominantly in state E_1.

molecules may be expected to depend on the average distance between protein molecules and dye molecules and thus on the density of pumps in the membrane fragment. In the following, all fluorescence levels are referred to state 1 which is chosen to be state $Na_3 \cdot E_1$. The relative fluorescence amplitude f_i of state i with respect to state 1 is then given by

$$f_i = \frac{\varphi_i - \varphi_1}{\varphi_1} = \frac{F_i - F_1}{F_1}. \qquad (10)$$

The amplitudes f_i were found to vary somewhat from enzyme preparation to enzyme preparation, exhibiting a positive correlation with the specific activity of the Na,K-ATPase. Furthermore, at a fixed concentration of membrane fragments, the f_i were found to decrease with increasing dye concentration. This could mean that the dye binds to saturable sites on the protein, or that the membrane-bound dye, at high concentration, forms aggregates that no longer respond to changes of electric field strength. To minimize errors resulting from variations in enzyme properties, dye concentration and spectral parameters of the fluorescence measurement, the f_i values given below were evaluated from the same enzyme preparation at fixed total concentrations of dye and membrane fragments and with fixed excitation and emission wavelengths.

By definition, the relative fluorescence amplitude of state $Na_3 \cdot E_1$ is zero. The fluorescence amplitudes of states $(Na_3)E_1$-P, P-E_2, · · ·, which were evaluated from experiments of the kind shown in Fig. 2 at saturating ion and ligand concentrations, are given in the Table. The fluorescence amplitudes of states $(Na_3)E_1$-P and $Na_3 \cdot E_1$ are nearly identical, as indicated by the experiments with chymotrypsin-modified enzyme. The largest fluorescence change (about 106%) occurs in the transition from $Na_3 \cdot E_1$ to P-E_2 which is associated with release of three Na^+ ions from the protein.

Since the enzyme may be assumed to be predominantly in state E_1 in the absence of alkali ions and P_i, the fluorescence change observed upon addition of Na^+ in the experiment of Fig. 2*B* reflects the transition from E_1 to $Na_3 \cdot E_1$; this yields a value of $f \approx 0.26$ for the relative fluorescence amplitude of state E_1 (Table). A tentative explanation for the difference in the fluorescence levels of states E_1 and $Na_3 \cdot E_1$ consists in the assumption that binding of Na^+ at the cytoplasmic face of the pump changes the electric field strength in the membrane by a minor ion-well effect. The existence of a shallow ion-well at the cytoplasmic side has already been discussed by Goldshlegger et al. (1987) as a possible explanation for the influence of transmembrane voltage on apparent Na^+ affinity observed in experiments with reconstituted Na,K-ATPase.

Discussion

In this study, we have investigated the fluorescence of RH-421-labeled membranes to obtain information on electrogenic events associated with binding and release of Na^+ and K^+ at the extracellular face of the Na,K-pump. The main results may be summarized in the following way.

i) Phosphorylation-induced transition from state $Na_3 \cdot E_1$ to state P-E_2 leads to a large increase of dye fluorescence (Fig. 2). A similar observation has been reported before by Klodos and Forbush (1988) from experiments with another styryl dye, RH 160. The sign of the fluorescence change corresponds to the creation of a negative electrostatic potential inside the membrane dielectric. After chymotrypsin-modification of the Na,K-ATPase, the fluorescence signal is abolished, indicating that the charge-translocating step occurs after the formation of the occluded state $(Na_3)E_1$-P.

ii) At high Na^+ concentration ($c_N > 100$ mM), the ATP-induced fluorescence change is diminished, consistent with the assumption that the reaction $(Na_3)E_1\text{-P} \rightleftarrows \text{P-}E_2 + 3Na^+_{ext}$ is shifted to the left at large c_N (Fig. 5). This effect of Na^+ is strongly reduced in the presence of lipophilic cations (TPP^+) which are known to create a positive electrostatic potential inside a lipid bilayer (Figs. 5 and 8).

iii) The transition from P-E_2 to $E_2(K_2)$, which is induced by addition of K^+ to the phosphorylated enzyme (Fig. 2*A*), is associated with a decrease of

fluorescence, corresponding to the build-up of a positive potential inside the membrane. The half-saturation concentration of K^+ in this experiment, $c_K^{1/2} \approx 0.25$ mM, approximately agrees with the apparent affinity for extracellular K^+ known from transport studies. $c_K^{1/2}$ is shifted towards larger values in the presence of TPP^+ (Fig. 6).

iv) The fluorescence changes associated with release of Na^+ or binding of K^+ are largely independent of ionic strength (Fig. 4).

Distinction between Surface Charges and Buried Charges

An obvious possibility to explain these experimental findings consists in the notion that the potentiometric dye responds to variations of electric field strength resulting from changes in the charge state of the protein. When, after phosphorylation, Na^+ is released at the extracellular face of the pump, the net charge of the protein nominally decreases by $3e_o$ (e_o is the elementary charge). When in a subsequent step K^+ is bound to the phosphoenzyme form $P\text{-}E_2$, the net charge increases by $2\ e_o$. Important for the understanding of the mechanism of ion binding and release is the question where these changes of electric charge occur. The ion-binding sites may be located at the surface of the protein, i.e., at the interface between the highly conducting aqueous medium and the poorly conducting protein matrix. Or, the binding sites may be buried in the interior of the protein.

These possibilities may be distinguished by studying the effects of ionic strength on the fluorescence response of the dye. At high ionic strength, surface charges are electrostatically shielded by counter ions in the aqueous medium. On the other hand, buried charges cannot be completely shielded by counter ions, even at high ionic strength. The finding that the fluorescence signals associated with release of Na^+ from $(Na_3)E_1\text{-}P$ and with binding of K^+ to $P\text{-}E_2$ remain virtually unchanged by addition of 1 M choline chloride to the medium (Fig. 4), leads to the conclusion that the ion-binding sites are buried inside the protein.

A highly idealized electrostatic model for the interaction between protein charges, counter ions and potentiometric dye is shown in Fig. 10. The model is based on the following assumptions: (i) The protein forms a two-dimensional lattice, with the dye molecules located in the interstices at a distance h from the center of the nearest protein. Since in reality the number of dye molecules is variable (with respect to the number of protein molecules), the length h should be considered as an effective dye-protein distance. (ii) Electric charges which are bound to or released from the protein are located at a distance r from the membrane-water interface. (iii) The counter ions neutralizing the protein charges are located at a distance l_D from the interface on the aqueous side. The Debye length l_D is proportional to $(J)^{-1/2}$, where J is the ionic strength of the solution. (iv) The dielectric properties of the protein-lipid-water system are described by an effective dielectric constant ε (v) The potentiometric dye is located in the membrane-solution interface ($x = 0$) and senses the component E_x of the electric field strength in the direction normal to the interface.

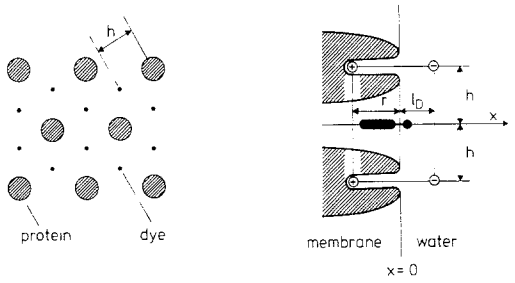

Fig. 10. Electrostatic model for the interaction between potentiometric dye and charges on the protein. Pump molecules are assumed to be arranged in a two-dimensional lattice with dye molecules located in the interstices. Protein charges resulting from binding or release of ions are located at a distance r from the membrane-water interface. The charges on the protein are screened by counter ions in the aqueous solution located at a distance l_D from the interface. l_D is the Debye length

As may be shown by elementary electrostatic considerations, the change of the field strength E_x upon binding or release of ions is, under the simplifying assumptions introduced above, given by

$$\Delta E_x = P \frac{n\Delta z}{\varepsilon} \left[\frac{r/h}{(1 + r^2/h^2)^{3/2}} + \frac{l_D/h}{(1 + l_D^2/h^2)^{3/2}} \right] \quad (11)$$

$$P \equiv \frac{e_o}{4\pi\varepsilon_o h^2} \approx 3.0 \times 10^7\ \mathrm{V/m} \qquad (h = 7\ \mathrm{nm}) \quad (12)$$

$$l_D \approx \frac{9.6\ \mathrm{nm}}{\sqrt{J/\mathrm{m}}}. \quad (13)$$

n is the effective number of pump molecules interacting with the dye, Δz the change in the number of bound charges per pump molecule, $e_o \approx 1.6 \times 10^{-19}$ C the elementary charge, and $\varepsilon_o \approx 8.85 \times 10^{-12}$ C $V^{-1}m^{-1}$ the permittivity of the vacuum. The parameter P has been calculated assuming a lattice constant $h = 7$ nm, corresponding to a protein density of about $10^4\ \mu m^{-2}$. With $h \gtrsim 7$ nm, the relations $r < h$ and $l_D < h$ hold. (Since the ionic strength J in our experiments was always larger than 30 mM, an upper limit of the Debye length is given by $l_D < 1.8$ nm.)

Under this condition, Eq. (11) can be replaced by the approximation

$$\Delta E_x \approx P \frac{n\Delta z}{\varepsilon h}(r + l_D). \tag{14}$$

This equation predicts that ΔE_x is linearly related to the Debye length l_D. In the experiments represented in Fig. 4, the fluorescence signal was found to be insensitive to changes of ionic strength J above $J \approx$ 200 mM, corresponding to $l_D \approx 0.7$ nm (J is the total ionic strength including buffer ions). According to Eq. (14) this means that r must be substantially larger than l_D, indicating that Na^+ and K^+ are bound at a depth $r > 0.7$ nm from the extracellular face of the protein.

With $n = 10$, $\Delta z = 3$, $\varepsilon = 5$, $h = 7$ nm and $(r + l_D) = 3$ nm, the change of field strength is estimated from Eq. (14) to be $\Delta E_x \approx 8 \times 10^7$ V/m, corresponding (with a membrane thickness of 5 nm) to a transmembrane voltage of about 400 mV. In view of the uncertainties in the values of r, h and ε, this value of ΔE_x should be considered as an estimate to the order of magnitude. The calculation shows, however, that ion binding and release may lead to large changes of electric field strength in the membrane.

Effects of Lipophilic Ions

The observed effects of lipophilic ions on Na^+ release and K^+ binding at the extracellular side agree with the electrostatic model discussed above. Lipophilic ions such as tetraphenylborate (TPB^-) or tetraphenylphosphonium (TPP^+) are known to adsorb to lipid bilayers on the apolar side close to the membrane-solution interface and to create a potential difference between the adsorption plane and the aqueous medium (Andersen et al., 1978; Bühler et al., 1991). In the experiments with Na,K-ATPase membranes, TPP^+ was found to strongly reduce the affinity for Na^+ and K^+ (Figs. 5, 6 and 8). A trivial explanation of this observation would be that TPP^+ acts by a Gouy-Chapman effect, i.e., by a change of the interfacial potential. This possibility can be excluded, however, since the effects of TPP^+ were observed at high ionic strength ($J > 1$ M) at which interfacial potentials are negligible. The most likely interpretation of the effects of TPP^+ therefore consists in the notion that the change of electric potential in the membrane resulting from adsorption of hydrophobic ions affects the electrostatic energy of Na^+ and K^+ at binding sites buried in the protein.

Ion Binding and Release at the Extracellular Face of the Pump

The results of this study indicate that binding and release of Na^+ and K^+ at the extracellular face of the pump are associated with translocation of electric charge. A straightforward interpretation of these findings consists in the notion that the binding sites for Na^+ and K^+ at the extracellular side are connected with the aqueous medium by narrow access channels, or "ion wells."

Binding and occlusion of K^+ at the extracellular side is sometimes assumed to proceed through an intermediate state $P\text{-}E_2 \cdot K_2$, followed by transition to the occluded state $P\text{-}E_2(K_2)$ (Glynn, 1985). In state $P\text{-}E_2 \cdot K_2$ the bound K^+ is assumed to exchange freely with the extracellular solution ($P\text{-}E_2 \cdot K_2 \rightleftarrows P\text{-}E_2 + 2K^+_{ext}$). Since direct evidence for the existence of a state $P\text{-}E_2 \cdot K_2$ is lacking, it seems preferable to treat binding of extracellular K^+ as a simple two-step process in which entry of K^+ into the binding site is immediately followed by occlusion (Forbush, 1988)

$$P\text{-}E_2 \rightarrow P\text{-}E_2(K) \rightarrow P\text{-}E_2(K_2). \tag{15}$$

From kinetic studies, Forbush (1987) obtained evidence that deocclusion of the two K^+ ions is an ordered process, which could mean that the two sites in the binding pocket are arranged in series. Forbush proposed that the rates of occlusion and deocclusion are determined by the rates of opening and closing of a "flickering" gate. This model is compatible with our results if it is assumed that opening and closing of the gate is electrically silent and that exchange of ions between the binding pocket and the aqueous medium is associated with translocation of charge.

Release of Na^+ from the occluded state $(Na_3)E_1\text{-}P$ is likely to proceed through a (long-lived) intermediate state $P\text{-}E_2(Na_2)$ with two occluded Na^+ ions (Nørby, 1987; Yoda & Yoda, 1987; Jørgensen & Andersen, 1988). It is feasible that state $P\text{-}E_2(Na_2)$ (or $P\text{-}E^*(Na_2)$ in the notation of Yoda & Yoda) is structurally similar to the K^+-occluded state $P\text{-}E_2 \cdot K_2$. On the other hand, a major conformational change may occur in the transition from $(Na_3)E_1\text{-}P$ to $P\text{-}E_2(Na_2)$. In analogy to the binding of K^+, release of Na^+ from the phosphoenzyme form $P\text{-}E_2(Na_2)$ may be considered as a simple two-step process, since evidence for an intermediate state $P\text{-}E_2 \cdot Na_2$ does not exist

$$P\text{-}E_2(Na_2) \rightarrow P\text{-}E_2(Na) \rightarrow P\text{-}E_2. \tag{16}$$

Experimentally, it cannot be distinguished so far whether the transition from $(Na_3)E_1$-P to P-$E_2(Na_2)$, or reaction (16), or both contribute to charge translocation.

Comparison with Electrophysiological Studies and Vesicle Experiments

Nakao and Gadsby (1989) studied the current-voltage characteristic of the Na,K-pump in cardiac myocytes as a function of extracellular K^+ concentration. The shape of the $I(V)$ curve was found to be independent of $[K^-]_{ext}$ (apart from a scaling factor), meaning that the apparent affinity for K^+_{ext} is voltage independent. On the other hand, in studies of Na,K-pump currents of *Xenopus* oocytes, Rakowski et al. (1990, 1991) observed a distinct dependence of the $I(V)$ characteristic on extracellular K^+ concentration. At $[K^+]_{ext} = 5$ mM, the pump current was a monotonous function of voltage, whereas at $[K^+]_{ext} < 2$ mM the $I(V)$ curve exhibited a maximum and a negative slope at (cytoplasmic-side) positive voltages. This result can be explained by the assumption that part of the transmembrane voltage drops between the K^+ binding sites and the extracellular medium, so that K^+ binding is inhibited at positive potentials. This interpretation (Rakowski et al., 1990, 1991) agrees with the results of our fluorescence experiments with Na,K-ATPase membranes from kidney medulla. A possible explanation of the difference between the results of Bahinski, Nakao and Gadsby (1988) and Nakao and Gadsby (1989) and of Rakowski et al. (1990, 1991) consists in the assumption that the Na,K-ATPases of cardiac myocytes and of *Xenopus* oocytes are intrinsically different in their kinetic behavior. The affinity evaluated from the current-voltage characteristic is an apparent affinity which depends not only on the equilibrium constant of ion binding, but also on the rate constants of the transport cycle. Another possibility is that the Na,K-ATPases in cardiac cells and in *Xenopus* oocytes differ in the dielectric properties of the extracellular access channel.

Goldshleger et al. (1987, 1990) studied (ATP + P_i)-activated exchange of Rb^+ (acting as a K^+ congener) in reconstituted vesicles and found that the exchange was potential insensitive and unable to create a transmembrane voltage. These observations agree with the results of voltage-jump studies of cardiac cells in which no transient pump-current was observed in the presence of K^+ and absence of Na^+ (Rakowski & Paxson, 1988). Since all these experiments were done at saturating extracellular $K^+(Rb^+)$ concentration, they do not exclude the possibility of voltage-dependent binding of K^+_{ext}.

Charge translocation in the Na^+ limb of the pumping cycle has been demonstrated by several techniques and in different systems. Transient pump currents in the presence of Na^+ and absence of K^+ have been observed in experiments with cardiac cells (Nakao & Gadsby, 1986) and with Na,K-ATPase membranes bound to planar lipid bilayers (Fendler et al., 1985; Borlinghaus et al., 1987). Further evidence for the electrogenic nature of Na^+ transport was obtained in experiments with Na,K-ATPase in reconstituted vesicles (Rephaeli et al., 1986; Goldshleger et al., 1987, 1990; Cornelius, 1989; Apell et al., 1990). Studies with chymotrypsin-modified Na,K-ATPase membranes (Borlinghaus et al., 1987; Apell, Borlinghaus & Läuger, 1987) have shown that phosphorylation and occlusion of Na^+ ($Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1$-P) is electroneutral and that the major electrogenic event in the Na^+-transport route is release of Na^+ from the occluded state. This conclusion agrees with the results reported here.

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References

Andersen, O.S., Feldberg, S., Nakadomari, H., Levy, S. McLaughlin, S. 1978. Electrostatic interactions among hydrophobic ions in lipid bilayer membranes. *Biophys. J.* **21**:35–70

Apell, H.-J. 1989. Electrogenic properties of the Na,K pump. *J Membrane Biol.* **110**:103–114

Apell, H.-J., Borlinghaus, R, Läuger, P. 1987. Fast charge translocations associated with partial reactions of the Na,K pump: II. Microscopic analysis of transient currents. *J. Membrane Biol.* **97**:179–191

Apell, H.-J., Häring, V., Roudna, M. 1990. Na,K-ATPase in artificial lipid vesicles: Comparison of Na,K and Na-only pumping mode. *Biochim. Biophys. Acta* **1023**:81–90

Bahinski, A., Nakao, M., Gadsby, D.C. 1988. Potassium translocation by the Na/K pump is voltage insensitive. *Proc. Natl. Acad. Sci. USA* **85**:3412–3416

Beaugé, L., Berberian, G., Campos, M., Pedemonte, C. 1985. Substrate role of acetyl phosphate on Na,K-ATPase. *In*: The Sodium Pump. I. Glynn and C. Ellory, editors. pp. 321–333. Company of Biologists, Cambridge

Borlinghaus, R., Apell, H.-J., Läuger, P. 1987. Fast charge translocations associated with partial reactions of the Na,K-pump: I. Current and voltage transients after photochemical release of ATP. *J. Membrane Biol.* **97**:161–178

Bühler, R., Stürmer, W., Apell, H.-J., Läuger, P. 1991. Charge translocation by the Na,K-pump: I. Kineticts of local field changes studies by time-resolved fluorescence measurement. *J. Membrane Biol.* **121**:141–161

Cornelius, F. 1989. Uncoupled Na^+-efflux on reconstituted shark

Na,K-ATPase is electrogenic. *Biochem. Biophys. Res. Commun.* **160:**801–807

Deguchi, N., Jørgensen, P.L., Maunsbach, A.B. 1977. Ultrastructure of the sodium pump. Comparison of thin sectioning, negative staining, and freeze-fracture of purified, membrane-bound (Na^+,K^+)-ATPase. *J. Cell Biol.* **75:**619–634

De Weer, P., Gadsby, D.C., Rakowski, R.F. 1988. Voltage dependence of the Na-K pump. *Annu. Rev. Physiol.* **50:**225–241

Fendler, K., Grell, E., Haubs, M., Bamberg, E. 1985. Pump currents generated by the purified Na^+,K^+-ATPase from kidney on black lipid membranes. *EMBO J.* **4:**3079–3085

Forbush, B., III. 1987. Rapid release of ^{42}K and ^{86}Rb from two distinct transport sites on the Na,K-pump in the presence of P_i or vanadate. *J. Biol. Chem.* **262:**11116-11127

Forbush, B., III. 1988. Occluded ions and Na,K-ATPase. *In*: Na^+,K^+-Pump. Part A: Molecular Aspects. J.C. Skou, J.G. Nørby, A.B. Maunsbach, and M. Esmann, editors. pp. 229–248. A.R. Liss, New York

Glynn, I.M. 1985. The Na^+,K^+-transporting adenosine triphosphatase. *In*: The Enzymes of Biological Membranes. (2nd ed.) Vol. 3, pp. 35–114. A.N. Martonosi, editor. Plenum, New York

Glynn, I.M., Hara, Y., Richards, D.E. 1984. The occlusion of sodium ions within the mammalian sodium-potassium pump: Its role in sodium transport. *J. Physiol.* **351:**531–547

Goldshleger, R., Shahak, Y., Karlish, S.J.D. 1990. Electrogenic and electroneutral transport modes of renal Na/K ATPase reconstituted into proteoliposomes. *J. Membrane Biol.* **113:**139–154

Goldshlegger, R., Karlish, S.J.D., Rephaeli, A., Stein, W.D. 1987. The effect of membrane potential on the mammalian sodium-potassium pump reconstituted into phospholipid vesicles. *J. Physiol.* **387:**331–355

Jørgensen, P.L. 1974. Isolation of the (Na^+ + K^+)-ATPase. *Methods Enzymol.* **32:**277–290

Jørgensen, P.L., Andersen, J.P. 1988. Structural basis for E_1–E_2 conformational transitions in Na,K-pump and Ca-pump proteins. *J. Membrane Biol.* **103:**95–120

Jørgensen, P.L., Collins, J.H. 1986. Tryptic and chymotryptic cleavage sites in the sequence of α-subunit of (Na^+ + K^+)-ATPase from outer medulla of mammalian kidney. *Biochim. Biophys. Acta* **860:**570–576

Jørgensen, P.L., Petersen, J. 1985. Chymotryptic cleavage of α-subunit in E_1-forms of renal (Na^+ + K^+)-ATPase: Effects on enzymatic properties, ligand binding and cation exchange. *Biochim. Biophys. Acta* **821:**319–333

Kapakos, J.G., Steinberg, M. 1986. Ligand binding to (Na,K)-ATPase labeled with 5-iodoacetamidofluorescein. *J. Biol. Chem.* **261:**2084–2089

Kaplan, J.H., Forbush, B., III, Hoffman, J.F. 1978. Rapid photolytic release of adenosine-5'-triphosphate from a protected analogue: Utilization by the Na:K pump of human red blood cell ghosts. *Biochemistry* **17:**1929–1935

Klodos, I., Forbush, B., III. 1988. Rapid conformational changes of the Na/K pump revealed by a fluorescent dye, RH-160. *J. Gen. Physiol.* **92:**46*a* (*abstr.*)

Läuger, P., Apell, H.-J. 1986. A microscopic model for the current-voltage behavior of the Na,K-pump. *Eur. Biophys. J.* **13:**305–321

Läuger, P., Apell, H.-J. 1988. Transient behaviour of the Na,K-pump: Microscopic analysis of nonstationary ion-translocation. *Biochim. Biophys. Acta* **944:**451–464

Mitchell, P., Moyle, J. 1974. The mechanism of proton translocation in reversible proton-translocating adenosine triphosphatases. *Biochem. Soc.* (Spec. Publ.) **4:**91–111

Nakao, M., Gadsby, D.C. 1986. Voltage dependence of Na translocation by the Na/K pump. *Nature* **323:**628–630

Nakao, M., Gadsby, D.C. 1989. [Na] and [K] dependence of the Na/K pump current-voltage relationship in guinea pig ventricular myocytes. *J. Gen. Physiol.* **94:**539–565

Nørby, J.G. 1987. Na,K-ATPase: Structure and kinetics. Comparison with other ion transport systems. *Chem. Scripta* **27B:**119–129

Nørby, J.G., Klodos, I. 1988. The phosphointermediates of Na,K-ATPase. *In*: The Na^+,K^+-Pump. Part A: Molecular Aspects. J.C. Skou, J.G. Nørby, A.B. Maunsbach, and M. Esmann, editors. pp. 249–270. A.R. Liss, New York

Rakowski, R.F., Paxson, C.L. 1988. Voltage dependence of Na/K pump current in *Xenopus* oocytes, *J. Membrane Biol.* **106:**173–182

Rakowski, R.F., Vasilets, L.A., LaTona, J., Schwarz, W. 1991. A negative slope in the current-voltage relationship of the Na^+/K^+ pump in *Xenopus* oocytes produced by reduction of external [K^+]. *J. Membrane Biol.* **121:**177–187

Rakowski, R.F., Vasilets, L.A., Schwarz, W. 1990. Conditions for a negative slope in the current-voltage relationship of the Na/K pump in *Xenopus* oocytes. *Biophys. J.* **57:**182*a*

Rephaeli, A., Richards, D., Karlish, S.J.D. 1986. Electrical potential accelerates the $E_1P(Na)$-E_2P conformational transition of (Na,K)ATPase is reconstituted vesicles. *J. Biol Chem.* **261:**12,437–12,440

Robinson, J.D., Flashner, M.S. 1979. The (Na^+ + K^+)-activated ATPase. Enzymatic and transport properties. *Biochim. Biophys. Acta* **549:**145–176

Stürmer, W., Apell, H.-J., Wuddel, I., Läuger, P. 1989. Conformational transitions and charge translocation by the Na,K pump: Comparison of optical and electrical transients elicited by ATP-concentration jumps. *J. Membrane Biol.* **110:**67–86

Tanford, C. 1983. Mechanism of free energy coupling in active transport. *Annu. Rev. Biochem.* **52:**379–409

Yoda, A., Yoda, S. 1987. Two different phosphorylation-dephosphorylation cycles of Na,K-ATPase proteoliposomes accompanying Na^+ transport in the absence of K^+. *J. Biol. Chem.* **262:**110–115

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