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Charge Translocation by the Na,K-Pump: I. Kinetics of Local Field Changes Studied by Time-Resolved Fluorescence Measurements

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Summary. Membrane fragments containing a high density of Na,K-ATPase can be noncovalently labeled with amphiphilic styryl dyes (e.g., RH 421). Phosphorylation of the Na,K-ATPase by ATP in the presence of Na^+ and in the absence of K^+ leads to a large increase of the fluorescence of RH 421 (up to 100%). In this paper evidence is presented that the styryl dye mainly responds to changes of the electric field strength in the membrane, resulting from charge movements during the pumping cycle: (i) The spectral characteristic of the ATP-induced dye response essentially agrees with the predictions for an electrochromic shift of the absorption peak. (ii) Adsorption of lipophilic anions to Na,K-ATPase membranes leads to an increase, adsorption of lipophilic cations to the decrease of dye fluorescence. These ions are known to bind to the hydrophobic interior of the membrane and to change the electric field strength in the boundary layer close to the interface. (iii) The fluorescence change that is normally observed upon phosphorylation by ATP is abolished at high concentrations of lipophilic ions. Lipophilic ions are thought to redistribute between the adsorption sites and water and to neutralize in this way the change of field strength caused by ion translocation in the pump protein. (iv) Changes of the fluorescence of RH 421 correlate with known electrogenic transitions in the pumping cycle, whereas transitions that are known to be electrically silent do not lead to fluorescence changes. The information obtained from experiments with amphiphilic styryl dyes is complementary to the results of electrophysiological investigations in which pump currents are measured as a function of transmembrane voltage. In particular, electrochromic dyes can be used for studying electrogenic processes in microsomal membrane preparations which are not amenable to electrophysiological techniques.

Key Words Na,K-ATPase · ion pumps · electrogenic transport · voltage-sensitive dyes · electrochromic effects

Introduction

Ion transport by the Na,K-pump is associated with translocations of net charge across the membrane (Glynn, 1984; De Weer, 1986; De Weer, Gadsby & Rakowski, 1988; Apell, 1989). Electrogenic properties of the pump have been investigated in cellular systems such as squid giant axon (Rakowski, Gadsby & De Weer, 1989), cardiocytes (Gadsby & Nakao, 1989; Glitsch, Krahn & Pusch, 1989; Nakao & Gadsby, 1989) or oocytes (Rakowski & Paxson, 1988; Schweigert, Lafaire & Schwarz, 1988). From such electrophysiological studies, the voltage dependence of steady-state and transient pump currents can be evaluated at different intra- and extracellular ion concentrations.

In its normal mode of operation, the Na,K-pump performs a cycle of conformational transitions and ion-binding and release steps (Cantley, 1981; Glynn, 1985; Jørgensen & Andersen, 1988). In the course of the cycle, the enzyme is thought to assume two principal conformations, E_1 and E_2, with inward-facing (E_1) and outward-facing ion-binding sites (E_2). Phosphorylation by ATP in state E_1 in the presence of intracellular Na^+ leads to a transition to state E_2 via an intermediate "occluded" state $(Na_3)E_1$-P in which Na^+ ions are trapped inside the protein. In conformation E_2, sodium ions are released to the extracellular side. After binding of extracellular K^+, the protein is dephosphorylated, leading to occlusion of K^+, followed by a transition back to state E_1 and release of K^+ at the cytoplasmic side.

An important problem in the understanding of the pumping mechanism is the question in which step(s) of the cycle charge is actually moved, and how charge-translocating events are correlated with conformational transitions of the protein. For studying this problem, cell-free systems offer distinct advantages. The pump protein may be isolated from kidney outer medulla in form of open membrane fragments containing a high density (10^3–10^4 per μm^2) of oriented Na,K-ATPase molecules (Deguchi, Jørgensen & Maunsbach, 1977). These membrane fragments may be bound to a planar lipid bilayer acting as a capacitive electrode; activation of the pump by an ATP-concentration jump elicits charge

* Deceased (September 13, 1990).

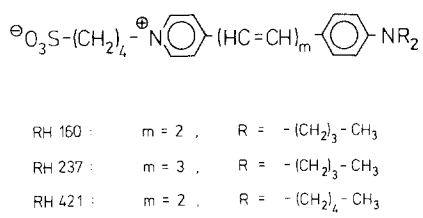

Fig. 1. Structure of styryl dyes, after Grinvald et al. (1982)

movements in the protein, which can be recorded as a transient current in the external measuring circuit (Fendler et al., 1985; Borlinghaus, Apell & Läuger, 1987). In more recent experiments (Stürmer et al., 1989), the transient charge movements could be correlated with conformational transitions by recording, in parallel to the current measurement, the fluorescence of Na,K-ATPase labeled with 5-iodoacetamidofluorescein (IAF). Comparison of the time-course of the fluorescence change after the ATP-concentration jump with the time course of the translocated charge in the electrical experiment led to the conclusion that the major charge-carrying step in the reaction cycle is the deocclusion of Na^+, followed by release of Na^+ to the extracellular side.

Recently, Klodos and Forbush (1988) reported that a fluorescent styryl dye, RH 160, incorporates into Na,K-ATPase membranes and monitors transitions between states of the pumping cycle. Fluorescent styryl dyes have been introduced by Loew and coworkers (Loew et al., 1979; Fluhler, Burnham & Loew, 1985) and by Grinvald and coworkers (Grinvald et al., 1982, 1983) as electrochromic dyes for the optical recording of membrane potentials (Ehrenberg, Meiri & Loew, 1984; Gross, Loew & Webb, 1986; Müller, Windisch & Tritthart, 1986; Grinvald et al., 1987). The dyes of the RH series (Fig. 1) have a delocalized positive charge in the pyridinium moiety and a localized negative charge on the terminal sulfo group. According to their amphiphilic structure, RH dyes have been proposed to insert into lipid bilayers with the sulfo group facing the aqueous medium and the rest of the molecule buried in the apolar interior of the membrane (Loew & Simpson, 1981; Grinvald et al., 1982). The semi-rigid, rod-like dye molecule is likely to be oriented perpendicular to the plane of the membrane, an arrangement which is favorable for the occurrence of large electrochromic spectral shifts in response to change of transmembrane voltage.

When open membrane fragments containing Na,K-ATPase are suspended in aqueous electrolyte solution, the two sides of the membrane are shortcircuited by the electrically conducting medium so that the transmembrane voltage remains always zero under quasi-stationary conditions. Fluorescence signals from RH dyes bound to the membrane fragments, which are observed upon activation of the pump may have at least two different origins (Klodos & Forbush, 1988): (i) The dye may bind directly to the ATPase and in this way may become sensitive to conformational changes of the protein. (ii) Dye molecules incorporated into the lipid bilayers regions of the membrane fragments may sense local changes of electric field strength resulting from charge movements in the protein. In part I of this paper, we present evidence that the RH dye responds mainly to local changes of field strength in the membrane. This conclusion is based, in part, on a comparison of fluorescence signals with electrical signals recorded from membrane fragments bound to planar bilayers. Further evidence for the electrostatic origin of the observed fluorescence signals comes from experiments in which local field changes in the membrane are induced by adsorption of lipophilic cations and anions.

In part II of the paper (Stürmer et al., 1991), we describe an experimental study, using the styryl dye RH 421, of charge movements associated with binding and release of cations at the extracellular face of the pump.

Materials and Methods

Materials

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, luciferin, luciferase, NADH and ATP (disodium salt, Sonderqualität) were from Boehringer (Mannheim). α-chymotrypsin (type II), apyrase VI and ouabain were purchased from Sigma. 5-iodoacetamidofluorescein (IAF), RH 237 and RH 421 were 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). In the experiments in the nominal absence of K^+, NaCl was used in Suprapur quality (Merck). All other reagents were analytical grade. Sephadex G25 was obtained from Serva (Heidelberg). 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 Hoffman (1978). The purity of the product was checked by HPLC. The compound was stored as tetramethylammonium salt in the dark at −40°C.

Enzyme Preparation and Fluorescence Labeling

Na,K-ATPase was prepared from the outer medulla of rabbit kidneys using procedure C of Jørgensen (1974*b*). This method yields purified enzyme in the form of membrane fragments con-

taining about 0.8 mg phospholipid and 0.2 mg cholesterol per mg protein (Jørgensen, 1974*a*, 1982). The specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay (Schwartz et al., 1971) and the protein concentration 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 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). The suspension of Na,K-ATPase-rich membrane fragments (about 3 mg protein per ml) in buffer (25 mM imidazole sulfate, pH 7.5, 1 mM EDTA, 10 mg/ml saccharose) was frozen in samples of 100 μl; in this form the preparation could be stored for several months at −70°C without significant loss of activity.

Fluorescence labeling of the enzyme was performed by incubating 200–300 μg of the enzyme for 48 hr at 4°C with a solution containing 100 μM IAF, 10 mM K_2SO_4 and 50 mM imidazole sulfate, pH 7.5 (Kapakos & Steinberg, 1982). The labeled enzyme was separated from unbound dye by passing the reaction mixture through a 3-cm long Sephadex G-25 column. In the experiments with RH 421 and RH 237, the dye was added from an ethanolic stock solution (concentration 0.2 mM) to the aqueous suspension of membrane fragments.

Determination of Lipid Content of Membrane Fragments

The phospholipid content of the Na,K-ATPase membranes was determined according to Broekhuyse (1968). Analysis for cholesterol was carried out by the cholesterol-oxidase method (Boehringer, Cat. No. 139050). Average values of 0.82 mg phospholipid per mg protein and 0.22 mg cholesterol per mg protein were obtained. These values approximately agree with the results of Jørgensen (1974*b*) and Peters et al. (1981) obtained from similar preparations. According to Peters et al. (1981), a correction term of 0.12 mg per mg protein was added in order to account for residual lipid other than cholesterol and phospholipids. In this way the total lipid content was determined to be 1.16 mg per mg protein.

Reconstituted Vesicles

Reconstituted vesicles with membrane-incorporated Na,K-ATPase were prepared as described previously (Apell et al., 1985). Na,K-ATPase membranes were solubilized in sodium cholate. After addition of dioleoylphosphatidylcholine in sodium cholate, the detergent was removed by dialysis at 4°C for 60 hr. This yields unilamellar vesicles with a diameter of about 90 nm.

Steady-State Fluorescence and Absorbance Measurements

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). Absorbance measurements were performed with a Perkin-Elmer Lambda 5 spectrophotometer. If not otherwise indicated, the experiments were carried out at 20°C.

Measurement of Transient Fluorescence Signals after Photochemical Release of ATP

The method for the measurement of time-dependent fluorescence signals after photolytic ATP-release was described previously (Stürmer et al., 1989). The optical cell was filled with 200–300 μl of a suspension of membrane fragments (usually 5–30 μg protein per ml) in a medium containing 30 mM imidazole buffer, pH 7.2, and 1 mM EDTA. The fluorescence was excited by light from a 250-W tungsten-halogen lamp. Fluorescence light emitted from the sample cell was collected by an ellipsoidal mirror and focussed onto the cathode of the photomultiplier. Excitation and emission wavelength were selected by interference filters (half-width about 13 nm).

The current signal of the photomultiplier was converted to voltage and amplified in three stages. The input stages of the amplifiers were protected by diodes against overload. This was necessary since the 308 nm flash excited strong fluorescence and phosphorescence in some of the optical elements (lenses and cuvette). The total recovery time of the recording system after the flash was given by the decay time constant of the phosphorescence (~1 msec). For the compensation of the stationary fluorescence signal prior to the flash, a differential amplifier was used. The output signal of the differential amplifier was amplified ten times and passed through an active low-pass filter. In most experiments the bandwidth was 1 kHz. The filtered signal was digitized with a 12-bit analog-to-digital converter (RC-Electronics, Santa Barbara, CA, Mod. ISC-16) and stored on the hard disk of the Compaq-386 computer.

ATP was released from "caged" ATP in the sample cell by light flashes (wavelength: 308 nm, total energy: 150 mJ, duration: 10 nsec) generated with a EMG 100 excimer laser (Lambda Physics, Göttingen). At pH 7.0, ATP is liberated from "caged" ATP (cg-ATP) with a time constant of 4.6 msec (McCray et al., 1980). The concentration of released ATP was determined by the luciferin/luciferase test, which was calibrated using solutions of known ATP concentration (De Luca & McElroy, 1978; Ernst, Böhme & Böger, 1983). If not otherwise stated, the concentration of cg-ATP was 100 μM. About 15–25 μM ATP were released in a single flash, corresponding to a photochemical yield of 15–25%. 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

Spectral Properties and Membrane/Water Partition Coefficient of RH 421

When a suspension of Na,K-ATPase membranes is added to an aqueous solution of RH 421, a strong increase of fluorescence is observed. The maximum ratio of the fluorescence intensities in the presence and in the absence of membrane fragments is about 70. This fluorescence increase is thought to result from binding of the dye to membrane fragments.

Binding of dye to the Na,K-ATPase membranes

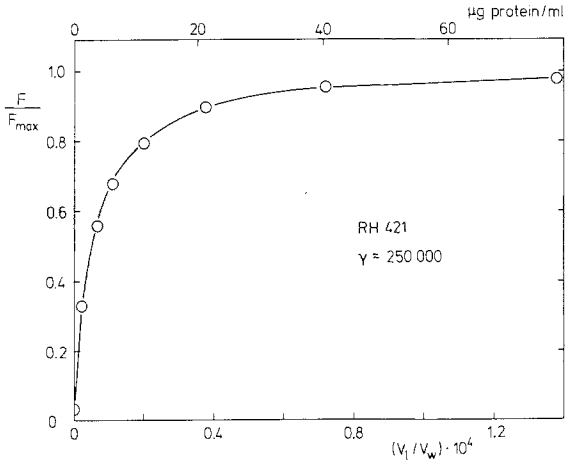

Fig. 2. Fluorescence change of RH 421 upon binding to Na,K-ATPase membranes. To an aqueous solution of 225 nM RH 421 in 30 mM imidazole, pH 7.2, and 1 mM EDTA, small amounts of a suspension of membrane fragments were successively added. The membrane concentration is given in μg protein/ml (upper abscissa) or as the lipid/water volume fraction V_l/V_w (lower abscissa). For the determination of V_l, *see* test. The fluorescence intensity F is referred to the saturation value F_{max} in the limit of large V_l/V_w. The fluorescence was excited at λ_{ex} = 520 nm and recorded at λ_{em} = 620 nm. The continuous line represents a fit of Eq. (2) to the experimental data points, yielding a lipid/water partition coefficient $\gamma \approx 250{,}000$

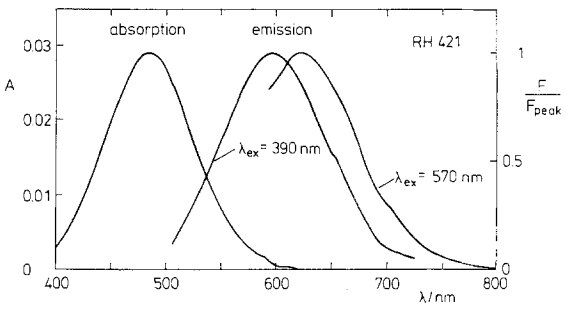

Fig. 3. Absorption and emission spectra of RH 421 bound to Na,K-ATPase membranes. To a suspension of membrane fragments containing 75 μg protein/ml (absorption) or 30 μg/ml (emission) in aqueous buffer (30 mM imidazole, pH 7.2, and 1 mM EDTA), 2.8 μM RH 421 (absorption) or 1.1 μM RH 421 (emission) were added from an ethanolic stock solution. Under these conditions, about 90% of the dye is bound to the membrane fragments. The temperature was 20°C. The absorption spectrum was corrected for background light scattering by measuring the transmission of the cell prior to dye addition. The emission was excited at λ_{ex} = 390 or 570 nm (slit width 7 nm) and recorded with a fixed slit width of 7 nm. Under the given experimental conditions, effects of self-absorption and scattering were negligible

is described by a partition coefficient γ which is defined by

$$\gamma = \frac{n_l/V_l}{n_w/V_w} \tag{1}$$

n_l is the amount of dye (in mol) bound to membranes and n_w the amount of dye present in water; V_l and V_w are the total volumes of lipid and water in the aqueous suspension of membrane fragments. (The reason for referring the dye concentration in the membrane to the lipid volume V_l and not to the total membrane volume V_m is the notion that the dye binds predominantly to the lipid domains of the membrane. If the partition coefficient γ is referred to V_m, the value of γ becomes smaller by a factor $V_l/V_m \approx 0.72$, see below). For the determination of γ, the fluorescence was measured as a function of V_l at constant total dye concentration, as described previously (Apell & Bersch, 1987). γ was evaluated from the fluorescence data (Fig. 2) using the relation

$$\frac{F}{F_{max}} \approx \frac{\gamma V_l/V_w}{1 + \gamma V_l/V_w} \tag{2}$$

F_{max} is the extrapolated limiting fluorescence at high lipid content ($V_l \rightarrow \infty$). Equation (2) follows from Eq. (3) of Apell and Bersch (1987) under the condition of negligible dye fluorescence in the aqueous phase. V_l was obtained from the amount of protein in the cuvette and from protein : lipid mass ratio m_p/m_l of the membrane fragments, using a value of $\rho_l \approx 1.01$ g/ml for the density of the lipid. From determinations of the total lipid content of the membrane fragments (described in Materials and Methods), an average value of $m_p/m_l \approx 0.86$ was obtained. With a ratio of $\rho_p/\rho_l \approx 1.24$ for the densities of protein and lipid, the volume fraction of lipid in the membrane fragments is estimated to be $V_l/V_m \approx 0.72$. As seen from Fig. 2, Eq. (2) satisfactorily describes the dependence of fluorescence on lipid concentration. In this way, a lipid/water partition coefficient of RH 421 of $\gamma \approx 2.5 \times 10^5$ is obtained. This high value of γ means that under the normal conditions of our experiments (30 μg protein/ml $\hat{=}$ 35 μg lipid/ml), about 90% of the dye is bound to the membrane. With a total dye concentration of 0.5 μM, the molar ratio of dye : lipid in the membrane is estimated to be about 1 : 90. Referred to a molar mass of the protein of 140,000 g/mol (corresponding to a single $\alpha\beta$ unit), the molar ratio of dye : protein is approximately 3.

Absorption and emission spectra of RH 421 bound to Na,K-ATPase membranes are shown in Fig. 3. Nearly identical peak positions are observed when the dye interacts with dioleoyllecithin vesicles, whereas in ethanol the absorption and emission

Table. Peak wavelengths (in nm) for absorption and emission of RH 237 and RH 421 in ethanol, in dioleoyllecithin vesicles and in Na,K-ATPase membranes[a]

	Ethanol	Vesicles	Membranes
	Absorption wavelength (nm)		
RH 237	534	501	504
RH 421	506	482	482
	Emission wavelength (nm)		
RH 237	757	666	656
RH 421	685	605	600

[a] The total dye concentration was 2.8 μM in the absorption experiments, and 225 nM in the emission experiments with vesicles and membrane fragments. The lipid concentration was 250 μg/ml (absorption) and 30 μg/ml (emission) in the vesicle experiments and 87 μg/ml (absorption) and 10 μg/ml (emission) in the experiments with membrane fragments. The fluorescence was excited at wavelengths in the vicinity of the absorption peak. The slit widths for excitation and emission were 10 and 5 nm, respectively. The error in the determination of the peak positions was estimated to be about ±5 nm.

bands are shifted to longer wavelengths (Table). The observed peak positions for absorption and emission in vesicles and in ethanol approximately agree with the results reported by Müller et al. (1986).

The emission spectrum of RH 421 in membrane fragments is found to depend on the excitation wavelength λ_{ex} (Fig. 3). When the fluorescence is excited at the red (long-wavelength) edge of the absorption band, the emission spectrum is red shifted with respect to the emission spectrum for short-wavelength excitation. Nearly the same dependence of the emission spectrum on λ_{ex} is observed with dye bound to lipid vesicles (*not shown*). Such a "red-edge-excitation red-shift" (Demchenko, 1986; Demchenko & Ladokhin, 1988) can occur when the energy difference between ground state and excited state depends on polar interactions of the dye with neighboring molecules, and when individual dye molecules differ by the polarity of their environments. Under these conditions, long-wavelength radiation preferentially excites chromophores with low excitation energy. When dipole reorientation in the environment of the dye is slow within the lifetime of the excited state, emission will occur predominantly at long wavelengths. A possible explanation for the observed red-shift of the emission spectrum upon long-wavelength excitation thus consists in the assumption that the membrane represents a semi-rigid environment for the bound dye. The finding that the red shift is nearly the same in lipid vesicles and in membrane fragments is consistent with the notion that the dye binds to the lipid domains of the Na,K-ATPase membranes.

The emission spectrum of RH 421 in ethanol

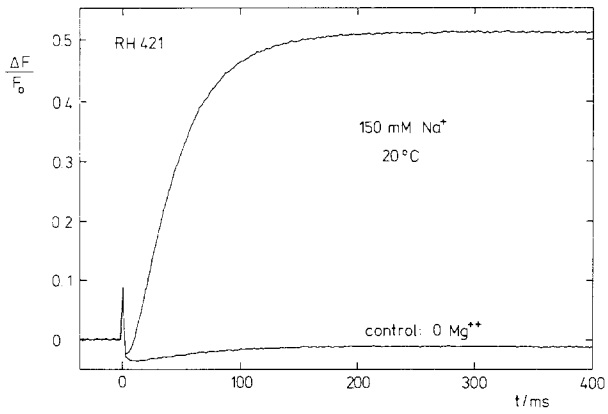

Fig. 4. Relative fluorescence change $\Delta F/F_0 \equiv (F - F_0)/F_0$ in the presence of RH 421, as a function of time t after photochemical ATP release at $t = 0$; F_0 is the fluorescence intensity prior to the flash. The solution contained 150 mM Na^+ but no K^+. A suspension of membrane fragments (30 μg protein per ml) was added to a solution of 0.7 μM RH 421, 20 μM "caged" ATP, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA and 30 mM imidazole chloride, pH 7.2. About 5 μM ATP were released by a 308-nm light flash of 10 nsec duration. The temperature was 20°C. The lower signal was observed in a control experiment in the absence of Mg^{2+} (Mg^{2+} is required as a cofactor for phosphorylation by ATP). The fluorescence of RH 421 was excited at 580 nm and recorded at 660 nm

measured at an excitation wavelength of 592 nm exhibits a symmetrical peak at 685 nm (*not shown*). When the fluorescence is excited at $\lambda_{ex} = 426$ nm, the peak position remains virtually unchanged, but a prominent shoulder appears at 620 nm. The origin of this effect of excitation wavelength on the shape of the emission spectrum is not clear. A tentative explanation consists in the assumption that in ethanolic solution the dye is present as a mixture of *cis* and *trans* isomers which are differently excited at 426 and 620 nm.

Fluorescence Changes upon Pump Activation in the Presence of Na^+ and Absence of K^+

The time course of the relative fluorescence change which is observed upon photochemical ATP release in the presence of 150 mM Na^+ and in the absence of K^+ is shown in Fig. 4 (upper curve). A total concentration of 0.7 μM RH 421 dye was added to the suspension of membrane fragments (30 μg protein per ml) prior to the experiment; from the membrane/water partition coefficient of RH 421, $\gamma \approx 250{,}000$, the fraction of dye bound to membrane fragments is estimated to be ≈0.94 under the given conditions. After release of about 5 μM ATP from caged ATP at time $t = 0$, the fluorescence increases

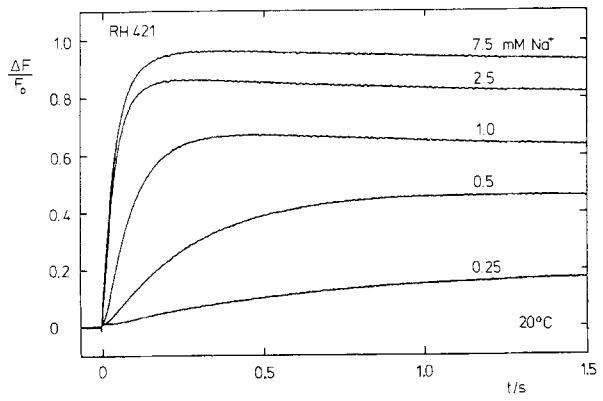

Fig. 5. Time course of the fluorescence change ΔF after photochemical ATP release in the absence of K^+ and in the presence of different concentrations of Na^+. F_0 is the fluorescence prior to the flash; $\Delta F(t)$ was corrected for the flash artifact. Except for the concentrations of ATP (20 μM) and Na^+ (variable), the experiment conditions were the same as in Fig. 4

with a half-time of ≈40 msec to about 1.5 times the original value ($\Delta F/F_0 \approx 0.5$).

The lower curve in Fig. 4 represents a control experiment in which Mg^{2+} was omitted from the solution (Mg^{2+} is a necessary cofactor for phosphorylation by ATP). In the control experiment, a small fluorescence decrease by about 3% occurs after the flash, which decays to nearly zero within 200 msec. This residual signal is also observed in the absence of caged ATP; it presumably results from an effect of the 308-nm light flash on the RH dye (Grinvald et al., 1982). Virtually identical control signals were observed when Na^+ or caged ATP instead of Mg^{2+} were omitted from the solution. The amplitude of the flash artifact increases with decreasing wavelength λ_{ex} of the light used for fluorescence excitation. Therefore, in order to minimize the flash artifact, all experiments were carried out with an excitation wavelength at the red edge of the absorption band (usually λ_{ex} = 581 nm). All fluorescence signals were corrected by digitally subtracting the signal observed under control conditions.

Fluorescence signals (corrected for the flash artifact) observed in the absence of K^+ are shown in Fig. 5 for different concentrations of Na^+. With increasing Na^+ concentration, the risetime of the signal decreases and the signal amplitude increases, approaching a maximal value at $[Na^+] \approx 20$ mM. At Na^+ concentrations higher than 20 mM, the signal amplitude starts to decrease again. The fluorescence behavior at high Na^+ concentrations will be analyzed in more detail in part II of this paper (Stürmer et al., 1990).

The fluorescence signals measured at 1, 2.5 and 7.5 mM Na^+ (Fig. 5) exhibit a marginal maximum and tend to decrease at long times. The origin of this "overshoot" (which is also seen in Figs. 7 and 12) is not clear so far. Possibly, the slow fluorescence decrease results from the formation of a Na-occluded state $P\text{-}E_2(Na_2)$ in which Na^+ acts as a substitute for K^+ (Apell, Häring & Roudna, 1990).

To test whether the styryl dye affects the kinetic properties of the pump, the enzymatic activity of Na,K-ATPase membranes was measured in the presence of RH 421, as described under Materials and Methods. Up to dye concentrations of 1 μM (the highest concentration used in the fluorescence experiments), the change of enzymatic activity was less than 5%.

In a further series of control experiments, we used 5-IAF labeled membrane fragments to test whether the dye RH 421 has any effect on rate constants of conformational changes or on the apparent affinities of ATP, Na^+ and K^+. None of these experiments revealed a significant effect of the dye with respect to the properties of the enzyme.

Under the experimental conditions of Figs. 4 and 5, i.e., in the presence of Na^+ and absence of K^+, the Na,K-ATPase is known to become phosphorylated by ATP (Glynn, 1985). This leads to a transition from state E_1 to the occluded state $(Na_3)E_1\text{-}P$

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

At low Na^+ concentrations in the medium, Na^+ is released from the protein

$$P\text{-}E_2(Na_3) \rightarrow P\text{-}E_2 + 3Na^+_{ext}. \quad (4)$$

In the absence of K^+, dephosphorylation is extremely slow, so that most of the enzyme remains in state $P\text{-}E_2$.

At saturating concentrations of Na^+ and ATP, phosphorylation of the enzyme is known to be fast, the rate constant of the reaction $Na_3 \cdot E_1 \cdot ATP \rightarrow (Na_3)E_1\text{-}P$ being about 180 sec^{-1} at 21°C (Mårdh & Zetterquist, 1974). It is therefore likely to assume that the rate-limiting step in the experiment shown in Figs. 4 and 5 is, at saturating Na^+ concentration, the E_1/E_2 conformational transition, followed by release of Na^+. The half-time of the fluorescence increase at 150 mM Na^+ is about 40 msec. This value has to be compared with the time constant of 24 msec, which was determined by Klodos and Forbush (1988) from dog kidney enzyme in a similar experiment. It is close to the time constant for release of $^{22}Na^+$ after phosphorylation of the enzyme, which is about 30 msec at 20°C (Forbush, 1984). A somewhat larger value for the half-time of the reaction $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2$, $t_{1/2} \approx 60$ msec, has been esti-

mated from experiments with iodoacetamidofluorescein-labeled enzyme (Stürmer et al., 1989).

Previous studies of transient currents in Na,K-ATPase membranes bound to lipid bilayers have shown that the transition $(Na_3)E_1$-P→ P-E_2 is associated with translocation of electric charge in the protein (Borlinghaus et al., 1987). A possible explanation of the fluorescence signals shown in Fig. 5 therefore consists in the assumption that the dye responds to changes of local field strength associated with charge translocation in the sodium limb of the pumping cycle (Klodos & Forbush, 1988). In the following, we describe different experimental approaches to test this hypothesis.

Spectral Characteristics of ATP-Induced Fluorescence Changes

Information on the origin of the fluorescence signals can be obtained from the wavelength dependence of the absorption and fluorescence changes. The absorption spectrum of RH 421-labeled Na,K-ATPase membranes before and after addition of ATP in the presence of 50 mM Na^+ and in the absence of K^+ is shown in Fig. 6*A*. It is seen that pump activation leads to a red shift and to a slight broadening of the absorption band. No spectral change was seen when ATP was added in the absence of Mg^{2+}, i.e., under conditions where phosphorylation of the enzyme is inhibited.

In Fig. 6*B*, the steady-state fluorescence change ΔF upon ATP addition is represented as a function of excitation wavelength λ. ΔF corresponds to the difference $F(t\rightarrow\infty) - F(t = 0)$ in the kinetic experiment of Fig. 5 in which ATP was released from "caged" ATP. As may be expected from the red shift of the absorption spectrum (Fig. 6*A*), ΔF is positive at long wavelengths (corresponding to the enhanced absorption at the red edge of the absorption band) and negative at short wavelengths. Also shown in Fig. 6*B* is the relative absorption change, $\Delta A/A_o$ which is taken from Fig. 6*A*. It is seen that $\Delta F/F_o$ and $\Delta A/A_o$ nearly coincide at short wavelength, whereas above 550 nm, $\Delta F/F_o$ tends to be larger than $\Delta A/A_o$. This result can be used to obtain information about the origin of the spectral changes.

The finding that $\Delta A/A_o$ as well as $\Delta F/F_o$ change sign upon variation of the (excitation) wavelength is consistent with the notion that at least part of the spectral change results from an electrochromic effect (Loew, 1982). An electrochromic shift of the absorption band occurs when the energy difference between ground state and excited state depends on electric field strength. In addition to the electrochromic absorbance change ΔA, the electric field

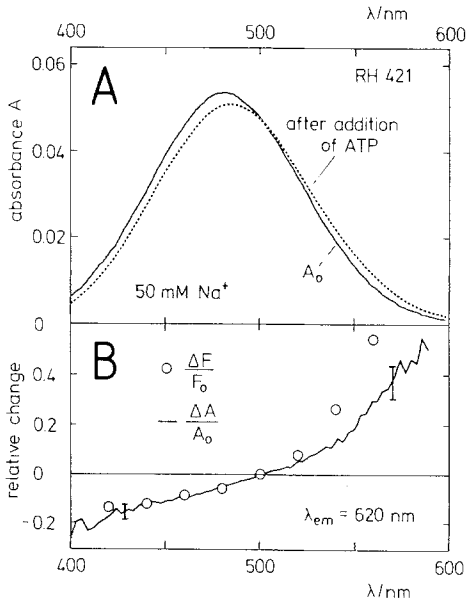

Fig. 6. Spectral characteristics of ATP-induced absorbance and fluorescence changes. (*A*) Absorbance *A* as a function of wavelength λ. To a suspension of Na,K-ATPase membranes (90 μg protein/ml) with bound RH 421 (total concentration 4.5 μM), 0.5 mM ATP were added in the presence of 50 mM NaCl. A_o is the absorption prior to addition of ATP. The solution contained 5 mM $MgCl_2$, 1 mM EDTA and 30 mM imidazole chloride, pH 7.2. The temperature was 20°C. The bars indicate the estimated error range of the absorption measurements. The dilution effect due to ATP addition is negligible. (*B*) Relative fluorescence change $\Delta F/F_o$ observed upon addition of 0.5 mM ATP, as a function of excitation wavelength λ. F_o is the fluorescence intensity prior to the addition of ATP. The fluorescence was measured at a fixed emission wavelength λ_{em} = 620 nm. The other experimental conditions were the same as in Fig. 6*A*. Each data point was obtained from a separate experiment. For comparison, the relative absorption change $\Delta A/A_o$, as taken from Fig. 6*A*, is also shown. Because of the small amplitude of the absorption change, the value of $\Delta A/A_o$ is subjected to large errors

may affect the fluorescence quantum yield q. The fluorescence intensity F is proportional to the product of the absorbance A, times the quantum yield q (Waggoner & Grinvald, 1977), so that the relative fluorescence change is given by

$$\frac{\Delta F}{F_o} = \frac{\Delta A}{A_o} + \frac{\Delta q}{q_o} + \frac{\Delta A}{A_o}\cdot\frac{\Delta q}{q_o}. \tag{5}$$

Values of $\Delta F/F_o$ larger than $\Delta A/A_o$ (Fig. 6*A*) indicate that the electrochromic band shift is superimposed by a change of quantum yield q, which becomes prominent at long wavelengths. Such a change of quantum yield may result from a field effect on the rate of radiationless desactivation (Ephardt & Fromherz, 1989).

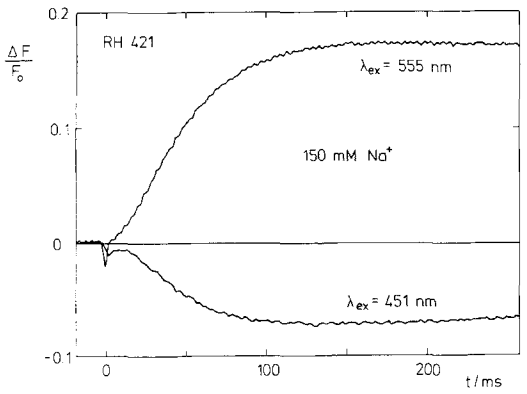

Fig. 7. Time course of the fluorescence change after photochemical ATP release, measured at two different excitation wavelengths, λ_{ex} = 555 nm and λ_{ex} = 451 nm. Both signals were recorded at the same emission wavelength, λ_{em} = 620 nm. The solution contained 150 mM NaCl. The other experimental conditions were the same as in Fig. 4

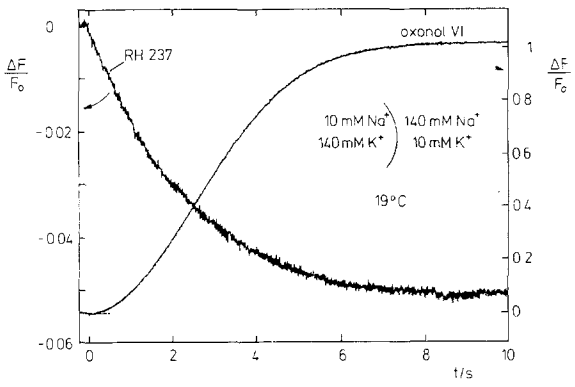

Fig. 8. Optical recording of pump-generated membrane potentials in reconstituted dioleoyllecithin vesicles. Vesicles were prepared by detergent dialysis in an aqueous buffer containing 30 mM imidazole, pH 7.2, 1 mM EDTA, 10 mM $MgSO_4$, 5 mM Na_2SO_4 and 70 mM K_2SO_4. The vesicles were suspended in a medium containing 70 mM Na_2SO_4, 5 mM K_2SO_4, 30 mM imidazole chloride, pH 7.2, 1 mM EDTA, 10 mM $MgSO_4$ and either 1 μM RH 237 or 30 nM oxonol VI. At time t = 0, 20 μM ATP were released from caged ATP. The (inside positive) membrane potential was monitored with RH 237 (left ordinate) or, in a parallel experiment, with oxonol VI (right ordinate). The excitation wavelengths were 580 nm (RH 237 and oxonol VI). From previous calibrations of the oxonol VI signal (Apell & Bersch, 1987), the steady-state membrane potential is estimated to be about 110 mV

Figure 7 represents an experiment in which the fluorescence signal after photochemical ATP release has been recorded at two different excitation wavelengths. In agreement with the steady-state experiments of Fig. 6*B*, the fluorescence increases at λ_{ex} = 555 nm and decreases at λ_{ex} = 451 nm. Apart from the sign of the fluorescence change, the time course of both signals is virtually identical, the time for half-maximal fluorescence change being about 40 msec at both wavelengths. This indicates that the optical signals recorded at the long- and at the short-wavelength side of the absorption band result from the same molecular process.

In a further series of experiments, the emission spectrum of RH 421 bound to Na,K-ATPase membranes was studied at a constant excitation wavelength of 530 nm before and after addition of ATP. Pump activation by ATP was found to result in a small red shift by about 7 nm of the emission spectrum (*not shown*). This red shift which parallels the red shift of the absorption spectrum (Fig. 6) has to be expected if the energy difference between excited state and ground state is decreased by the pump-induced change of electric field strength.

Fluorescence Signals from Reconstituted Vesicles

In order to correlate the sign of the fluorescence change with the sign of the electric field change, experiments with reconstituted vesicles were carried out. Solubilized Na,K-ATPase was incorporated into the membrane of dioleoyllecithin vesicles by detergent dialysis, as described in Materials and Methods. The dialysis procedure yields unilamellar vesicles with a diameter of about 90 nm in which the enzyme is incorporated in random orientation (Apell et al., 1985; Rey, Moosmayer & Anner, 1987). Addition of caged ATP (which may be assumed to be virtually membrane-impermeable) to the suspension medium and photochemical release of ATP activates only those pump molecules which have the ATP-binding site facing outward. In the presence of extravesicular (cytoplasmic) Na^+ and of intravesicular (extracellular) K^+, the pump operates in the Na,K-exchange mode, which is associated with the build-up of an inside-positive transmembrane voltage (Apell et al., 1985, 1990).

A fluorescence experiment with reconstituted vesicles labeled with RH 237 is shown in Fig. 8. The dye was added to the vesicle suspension 2 min prior to the experiment. When at time t = 0, ATP was photochemically released in the medium, the fluorescence excited at λ_{ex} = 580 nm started to decrease, approaching a stationary value with a half-time of about 2 sec. A similar, but smaller, fluorescence signal was observed with RH 421 (λ_{ex} = 580 nm) instead of RH 237.

Figure 8 also shows the result of an experiment carried out under virtually identical conditions, but with oxonol VI as potential-sensitive dye. Oxonol VI, which distributes between membrane and water

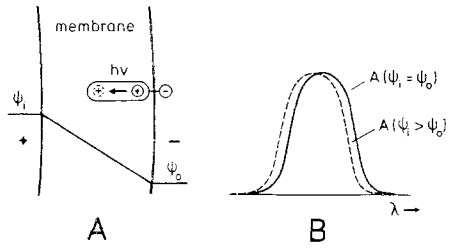

Fig. 9. Electrochromic response of a styryl dye bound to the outer lipid monolayer of a vesicle membrane. The size of the dye molecule is exaggerated with respect to the thickness of the membrane. In the excited state of the dye, positive charge is shifted toward the interior of the membrane (*A*); an inside-positive potential ($\psi_i > \psi_a$) therefore leads to a blue shift of the absorption spectrum (*B*)

in a potential-dependent manner, is known to respond to a potential change (vesicle-interior positive) with an increase of fluorescence (Apell & Bersch, 1987). In contrast to the "fast" styryl dyes which respond to a change of field strength within less than 0.5 msec, (Müller et al., 1986), the time constant of oxonol VI is of the order of 300 msec (Clarke & Apell, 1989). The finite rise-time of the oxonol signal is clearly evident from Fig. 8.

The comparison of the signals observed with the styryl dye and with oxonol VI shows that with long-wavelength excitation, the styryl dye responds to an *inside-positive* potential with a fluorescence *decrease*. The sign of the fluorescence change can be understood in the following way: Since RH 237 and RH 421 contain a terminal sulfo group with a localized negative charge, it is likely to assume that the dye binds from the extravesicular medium to the outer monolayer of the vesicle, without penetrating the membrane (Fig. 9*A*). Furthermore, it may be assumed that the dye molecule becomes oriented with its long axis more or less parallel to the hydrocarbon chains of the lipid (Loew & Simpson, 1981). An inside-positive potential ($\psi_i > \psi_a$) then leads to an increase of the energy difference between excited state and ground state and to a blue shift of the absorption spectrum (Fig. 9*B*). This results in a fluorescence decrease when the fluorescence is excited at the long-wavelength side of the absorption band.

The sign of the fluorescence change as observed here with RH 237 and RH 421 in lipid vesicles agrees with the results obtained by Loew and Simpson (1981) with a similar styryl dye [4-(*p*-aminostyryl-pyridiniumpropylsulfonate)] in hemispherical lipid bilayers. Furthermore, the sign of the fluorescence change observed in the vesicle experiments is in agreement with studies of excitable cells stained with RH 237 or RH 421 (Grinvald et al., 1982, 1983; Müller et al., 1986). From the magnitude of the oxonol signal in Fig. 8, the voltage built up across the vesicle membrane may be estimated to be about 110 mV (Apell & Bersch, 1987). Grinvald et al. (1982, 1983) reported fluorescence changes of 14 and 21% per 100 mV for RH 237 and RH 421 from experiments with nerve cells. The voltage sensitivity of the dyes may be expected to vary considerably from preparation to preparation due to differences in background fluorescence. For this reason the absolute magnitudes of the fluorescence changes observed in different systems are difficult to compare.

Fluorescence Changes upon Binding of Lipophilic Ions

Lipophilic ions such as tetraphenylborate (TPB^-) or tetraphenylphosphonium(TPP^+) are known to partition between water and lipid membranes (McLaughlin, 1977; Läuger et al., 1981; Altenbach & Seelig, 1985; Flewelling & Hubbell, 1986). From conductance studies with planar bilayers it can be inferred that the lipophilic ion adsorbs to the apolar region of the membrane a few tenths of a nanometer away from the membrane/water interface (Andersen et al., 1978). In this way a potential drop is created between the adsorption plane and the adjacent aqueous solution.

The ability of lipophilic ions to modify the electrostatic potential in the lipid/water interface may be used to test the hypothesis that styryl dyes respond to changes of local electric fields in the membrane. For this purpose, fluorescence experiments were carried out in which the concentration of TPB^- or TPP^+ in a suspension of dye-labeled Na,K-ATPase membranes was increased stepwise by successive additions of small volumes of a concentrated solution of the lipophilic ion. The results of these experiments are summarized in Fig. 10. Addition of the lipophilic anion TPB^- in micromolar concentrations leads to large increases of fluorescence (up to $\Delta F/F_o \approx 2.2$). A change in opposite direction, i.e., a fluorescence decrease, is observed in the presence of the lipophilic cation TPP^+. An about 10^3-fold higher concentration of TPP^+ was required, however, to produce a fluorescence change of similar absolute magnitude as the fluorescence change obtained with TPB^-. This finding is consistent with the observation that the potency of TPP^+ in increasing the electrical conductance of planar lipid bilayers is, depending on the lipid, 10^3–10^7 times lower than that of TPB^- (Pickar & Benz, 1978). This large difference in the behavior of the two structurally similar lipophilic ions is thought to result from the existence of oriented dipolar layers in the membrane/water interface, leading to a positive electrostatic potential

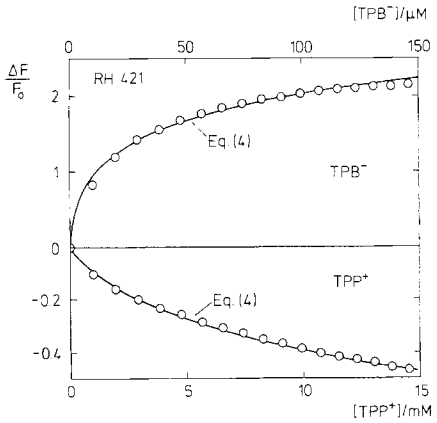

Fig. 10. Fluorescence changes ΔF of dye-labeled Na,K-ATPase membranes upon binding of lipophilic ions. A suspension of membrane fragments (9 μg protein/ml) in aqueous buffer (30 mM imidazole chloride, pH 7.2 and 1 mM EDTA) was equilibrated in the fluorescence cell with 0.5 μM RH 421. Small volumes of a concentrated solution of either tetraphenylborate (TPB^-) or tetraphenylphosphonium (TPP^+) were successively added. This resulted in stepwise fluorescence changes. The experimental data (circles) have been corrected for the successive dilution of the suspension. F_o is the fluorescence intensity prior to the addition of lipophilic ions. [TPB^-] and [TPP^+] are the total concentrations in the cuvette which are nearly identical with the free aqueous concentrations. Note the different concentration scales for TPB^- and TPP^+. The continuous curves have been obtained by fitting Eq. (6) to the experimental values of $\Delta F/F_o$, using the following parameter set: $H = 0.71$, $c_o = 2.02$ μM for TPB^-, and $H = 0.38$, $c_o = 3.42$ mM for TPP^+

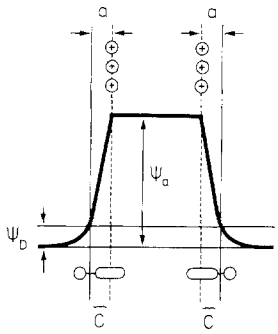

Fig. 11. Three-capacitor model for the treatment of adsorption of lipophilic ions to a lipid bilayer. Lipophilic cations are assumed to adsorb to a plane that is located at the distance a from the membrane-solution interface. This creates a potential drop which is sensed by bound dye molecules. C is the capacitance of the adsorption layer and ψ_a the electric potential in the adsorption plane with respect to the aqueous solution. ψ_D is the potential in the membrane-solution interface due to the ionic double layer. At high ionic strength, ψ_D is much smaller than ψ_a

in the membrane interior (Szabo, 1974; Flewelling & Hubbell, 1986).

The fluorescence measurements represented in Fig. 10 were carried out at an ionic strength of about 30 mM. A number of experiments in the presence of TPP^+ were also performed, in which the ionic strength was increased to 0.5 M by addition of choline chloride. Under these conditions a similar but somewhat less steep decrease of $\Delta F/F_o$ was observed. (Analogous experiments with TPB^- could not be carried out because of the limited solubility of TPB^- in aqueous solutions of high ionic strength.) The finding that the fluorescence change induced by addition of TPP^+ is only weakly voltage-dependent is consistent with the notion that the binding sites of the lipophilic ions are located in the interior of the lipid bilayer some distance away from the membrane-solution interface.

The observation that the fluorescence of RH 421 bound to membrane fragments is increased by TPB^-, but decreased by TPP^+, is consistent with the notion that the dye senses the change of electric field strength brought about by adsorption of the lipophilic ion (Fig. 11). Binding of TPP^+ to the membrane creates a positive potential in the interior of the membrane (Fig. 11); this leads to a blue shift of the absorption band and (with red-edge excitation) to a decrease of fluorescence intensity (Fig. 9). Conversely, binding of TPB^- is predicted to result in a fluorescence increase, as observed.

A trivial explanation of the effect of TPB^- on fluorescence intensity would be that the partition coefficient γ of the styryl dye is increased by binding of TPB^- to the membrane. The possibility that the fluorescence increase results from an increase of γ can be excluded, however, since in the experiments of Fig. 10 about 85% of the dye is already bound to membranes. In this case a further increase of the partition coefficient would not appreciably change the fluorescence intensity.

The experimentally observed dependence of fluorescence intensity on the concentrations of TPB^- and TPP^+ (Fig. 10) can be compared with theoretical predictions. Neglecting effects of diffuse double-layers (McLaughlin, 1977), binding of lipophilic ions to the lipid bilayer can be approximately described by a three-capacitor model (Markin, Grigor'ev & Yermishkin, 1971; Andersen et al., 1978), as indicated in Fig. 11. According to this model, the interfacial concentration N of adsorbed lipophilic ions is expressed by $N = \beta c \cdot \exp(-z\psi_a e_o/kT)$, where β is the interfacial partition coefficient, c the aqueous concentration, z the valency, ψ_a the electric potential in the adsorption plane, e_o is the elementary charge, k the Boltzmann constant and T the absolute temperature. (The interfacial partition coefficient β is related to the bulk partition coefficient γ according to $\beta = \gamma d/2$, where

d is the membrane thickness.) If C is the specific electrical capacitance of the adsorption layer, the potential ψ_a is given by $\psi_a = ze_oN/C$. Assuming that the fluorescence change ΔF is proportional to the change of electric field strength in the interface, $\Delta F/F_o$ is predicted to be (with $y = z\psi_a e_o/kT$)

$$\frac{\Delta F}{F_o} = Hy;$$

$$y = \frac{c}{zc_o} \cdot \exp(-zy) \tag{6}$$

$$c_o \equiv \frac{kTC}{z^2e_o^2\beta}; \qquad H \equiv \frac{pkTC}{e_o\varepsilon_o\varepsilon_a}. \tag{7}$$

p is an empirical coefficient describing the field effect on the fluorescence [Eq. (A11)], ε_o the permittivity of the vacuum, and ε_a the dielectric constant of the adsorption layer; for the derivation of Eqs. (6) and (7), *see* Appendix. H and c_o are formal parameters and may be determined by fitting Eq. (6) to the experimentally observed concentration dependence of $\Delta F/F_o$; for this purpose, the implicit equation for y has to be solved numerically.

Theoretical curves which have been calculated in this way are represented in Fig. 10 together with the experimental data points. It is seen that a satisfactory fit of Eq. (6) to the experimental results is possible, when c_o is chosen to be 2.02 μM for TPB^- and 3.42 mM for TPP^+. The value of c_o for TPB^- may be compared with the value predicted from Eq. (7). From conductance studies with planar bilayers in the presence of TPB^- (Andersen et al., 1978; Benz, Läuger & Janko, 1976), C and β have been estimated to be $C \approx 40$ μF/cm^2 and $\beta \approx 0.02$ cm. This yields, according to Eq. (8), $c_o \approx 0.5$ μM. The fact that the value of c_o obtained from the fit is about four times larger, is likely to result from differences in lipid composition. The experiments with planar bilayers have been carried out with neutral phospholipids, whereas Na,K-ATPase membranes are known to contain negatively charged lipids (De Pont, van Prooijen-van Eeden & Bonting, 1978). Fixed negative surface charges in the membrane fragments would lead to a decrease of the partition coefficient β of TPB^- and, according to Eq. (7), to an increase of c_o.

Values of C and β for TPP^+ are not available from independent experiments; they may be estimated, however, from the empirically determined values of H and c_o. Using the notation $H(TPP^+) \equiv H_+$, etc., the following estimates are obtained from Eq. (7):

$$\frac{C_+}{C_-} = \frac{H_+}{H_-} \approx 0.54 \tag{8}$$

$$\frac{\beta_+}{\beta_-} = \frac{c_o^- H_+}{c_o^+ H_-} \approx 3.2 \times 10^{-4}. \tag{9}$$

Implicit in relations (8) and (9) is the assumption that the experiments with TPP^+ and TPB^- can be described by the same coefficient p. This may be only approximately true, since the distribution of the electric field with respect to the dye molecule may be different in both cases. With $C_- \approx 40$ μF/cm^2 and $\beta_- \approx 0.02$ cm, C_+ and β_+ are estimated to be $C_+ \approx 20$ μF/cm^2 and $\beta_+ \approx 6 \times 10^{-6}$ cm. This indicates that TPP^+ binds to an adsorption plane which is located, as compared to TPB^-, more towards the membrane interior. With an estimated effective dielectric constant of $\varepsilon_a \approx 10$, the distance a of the adsorption plane from the interface becomes about 0.2 nm for TPB^- and about 0.4 nm for TPP^+. The partition coefficient of TPP^+ is found to be smaller by the factor ≈ 3000 than the partition coefficient of TPB^-. This is consistent with the known differences in the conductance behavior of TPP^+ and TPB^- in planar bilayers (Pikar & Benz, 1978). In view of the simplifying assumptions introduced into the derivation of Eqs. (6) and (7), the numerical values obtained from the relations (8) and (9) should be considered as estimates to the order of magnitude.

Effect of Lipophilic Ions on ATP-Induced Fluorescence Signals

The hypothesis that the fluorescence signals observed upon pump activation (Fig. 5) reflect changes of local field strength in the membrane may be further tested by ATP-release experiments in the presence of lipophilic ions. If TPB^- or TPP^+ are adsorbed to the membrane, charge movements in the pump protein should lead to a redistribution of lipophilic ions between water and the two adsorption layers. When the concentration of adsorbed lipophilic ions is sufficiently high, this redistribution may be expected to neutralize the effect of ATP-induced charge translocation in the membrane by "clamping" the electric field strength in the interface to a virtually constant value (for a more rigorous discussion, *see* Appendix).

The expectation that the fluorescence change should be abolished at high concentration of the lipophilic ion is borne out by ATP-release experiments in which TPB^- was added to the membrane

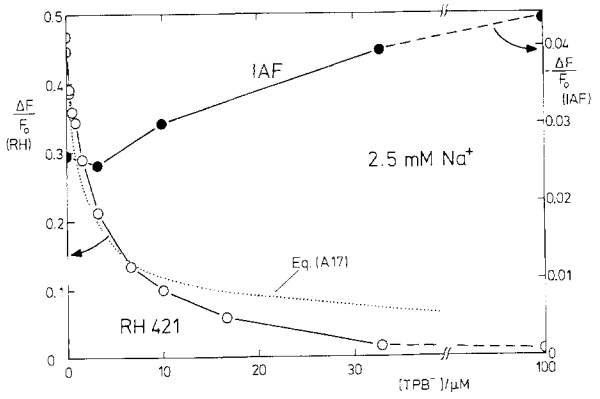

Fig. 12. Relative amplitude, $\Delta F/F_o \equiv (F_\infty - F_o)/F_o$, of the RH 421- and IAF-fluorescence signals after flash release of ATP at time $t = 0$ in a medium containing 2.5 mM Na^+, as a function of TPB^- concentration. F_o and F_∞ are the fluorescence intensities at times $t \leq 0$ and $t \to \infty$, respectively. Except for the Na^+ concentration, the experimental conditions were the same as in Fig. 4. The dotted line represents a fit of Eqs. (A17)–(A19) to the fluorescence signal observed with RH 421, using the following parameter values: $c_o = 2.0$ μM, $H = 0.481$, $s_p^o = 0.58$, $s_p^\infty = 0$

suspension. As seen from Fig. 12, the amplitude of the fluorescence signal from the styryl dye recorded after pump activation in a Na^+ medium is reduced to about one-half in the presence of 4 μM TPB^-, and is virtually abolished at 30 μM TPB^-. On the other hand, the time behavior of the fluorescence signal was not significantly changed. A similar, but smaller, effect on the fluorescence signal was observed with TPP^+; in this case the signal amplitude, measured at a Na^+ concentration of 20 mM, was reduced by about 20% when 300 μM TPP^+ were present in the medium (*not shown*).

To avoid an effect of TPB^- on the apparent Na^+-affinity at the extracellular binding site, the experiments shown in Fig. 12 were carried out at small Na^+ concentration (2.5 mM). As will be discussed in part II of this paper, the apparent affinity of the phosphorylated enzyme ($P\text{-}E_2$) for Na^+ is affected by the electric field in the membrane. This field effect leads to an increase of the apparent affinity for Na^+ when TPB^- binds to the membrane. The apparent dissociation constant of Na^+ at the extracellular binding site in state $P\text{-}E_2$ is larger than 100 mM (Taniguchi & Post, 1975). Since in the experiments of Fig. 12, the Na^+ concentration was much less than K''_N, dissociation of Na^+ at the extracellular side can be assumed to be complete even in the presence of TPB^-. A field effect at the cytoplasmic side, on the other hand, cannot be excluded *a priori*. An electrostatic influence of adsorbed TPB^- on Na^+ binding by an "ion-well" effect at the cytoplasmic site in state E_1, however, would increase the apparent Na^+ affinity and thus would favor phosphorylation. This would result in an increase of the fluorescence signal with increasing TPB^- concentration, contrary to the experimental result (Fig. 12).

The experimentally observed decrease of $\Delta F/F_o$ with increasing concentration TPB^- (Fig. 12) can be compared with theoretical predictions. For the three-capacitor model analyzed in the Appendix, the dependence of $\Delta F/F_o$ on the concentration of TPB^- is approximately given by Eqs. (A17)–(A19). These equations contain the same parameters c_o and H introduced above (Eq. 7) and, in addition, the quantities s_p^o and s_p^∞ which depend on the number of translocated charges in the protein [Eq. (A9)]. An approximate fit of Eqs. (A17)–(A19) to the experimental data (RH 421) in Fig. 12 at low TPB^- concentration was possible with similar values of c_o and H as used before ($c_o = 2.0$ μM, $H = 0.481$), together with $s_p^o = 0.58$ and $s_p^\infty = 0$ (dotted line in Fig. 12). This agreement between the theoretical prediction and the experimental data in Fig. 12 supports the assumption that the decline of dye fluorescence at high TPB^- concentration essentially results from redistribution of hydrophobic ions between membrane and aqueous medium.

To check whether TPB^- has an inhibitory effect on the pump, two control experiments were carried out. In one experiment, the Na,K-ATPase in the membrane fragments was labeled with 5-iodoacetamidofluorescein (IAF). This dye is known to bind to a cystein residue of the α-subunit of the enzyme and to respond to the $E_1 \to E_2$ conformational change by a decrease of fluorescence (Kapakos & Steinberg, 1982, 1986*a,b*: Glynn et al., 1987; Steinberg & Karlish, 1989; Stürmer et al., 1989; Tyson et al., 1989). As seen from Fig. 12, in the same concentration range of TPB^- in which the RH 421 signal is nearly abolished, the amplitude of the IAF signal is only moderately affected, and in the opposite direction. Furthermore, the time course of the IAF signal was found to be virtually unchanged in the presence of TPB^- up to a TPB^- concentration of 5 μM. These observations indicate that TPB^- does not interfere with phosphorylation of the enzyme and the subsequent $E_1 \to E_2$ conformational transition.

In a second set of control experiments, the enzymatic activity of Na,K-ATPase membranes was measured in the presence of TPB^-. At concentrations of TPB^- up to 100 μM, and at saturating concentrations of Na^+ and K^+, only a small reduction (by about 13%) of ATP hydrolysis rate was observed.

Comparison of Electrical and Optical Signals

To obtain further information on the possible origin of the fluorescence signal observed upon pump acti-

vation, we compare in the following the time course of $\Delta F/F_o$ with the time course of transient charge movements in the pump molecule. For the recording of transient pump currents, membrane fragments were bound to one side (*cis* side) of a planar lipid bilayer, as described previously (Borlinghaus et al., 1987; Stürmer et al., 1989). The solution on the *cis* side of the lipid bilayer contained, in addition to the membrane fragments, 240 μM caged ATP and 150 mM Na^+, but no K^+. A light flash of 40 μsec duration which liberated about 30 μM ATP on the *cis* side elicited a transient electric current $I(t)$, corresponding to a translocation of positive charge from the solution towards the bilayer. In these experiments the planar bilayer acts as a capacitive element that couples charge movements in the protein layer to the external measuring circuit. The transient current is generated by those membrane fragments that are bound to the lipid bilayer with the cytoplasmic side facing the aqueous medium. From the recorded short-circuit current $I(t)$ or from the open-circuit voltage $V(t)$, the intrinsic pump current $I_p(t)$ can be evaluated (Borlinghaus et al., 1987). $I_p(t)$ rises within about 30 msec and decays with a time constant of about 40 msec to a small quasistationary current I_p^∞ (*not shown*). Labeling of the membrane fragments with RH 421 was found to have virtually no influence on the time course of I_p. It has been demonstrated previously that the transient pump current I_p is associated with deocclusion of Na^+, followed by release to the extracellular side ($(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2 + 3Na^+_{ext}$). The small quasistationary current I_p^∞ is likely to result from pump molecules undergoing spontaneous dephosphorylation ($P\text{-}E_2 \rightarrow E_1$) and reentering the cycle again (Apell, Borlinghaus & Läuger, 1987).

If the hypothesis is correct that the styryl dye responds to the local electric field strength generated in the membrane by pump-mediated charge movement, then the time course of the fluorescence signal $\Delta F/F_o$ should agree with the time course of the translocated charge Q, since the field strength is proportional to Q. The total charge Q which has moved until time t after release of ATP at time $t = 0$ is given by

$$Q(t) = \int_o^t (I_p - I_p^\infty)dt. \tag{10}$$

In Fig. 13 the time course of the fluorescence signal $\Delta F/F_o$ is compared with the time course of the translocated charge Q. Both $\Delta F(t)/F_o$ and $Q(t)$ were determined from experiments carried out in the presence of Na^+ and absence of K^+ under virtually identical conditions. It is seen from Fig. 13 that the time behavior of $\Delta F(t)/F_o$ and $Q(t)$ is very similar, the time constants for half-maximal signal amplitudes being about 40 msec for $\Delta F(t)/F_o$ and 60 msec for $Q(t)$. This close agreement between $\Delta F(t)/F_o$ and $Q(t)$ is consistent with the notion that the fluorescence dye responds to local field changes in the membrane.

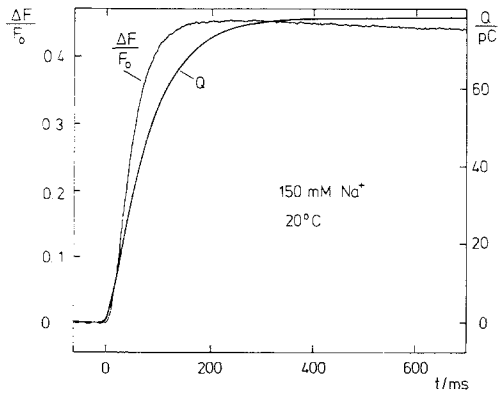

Fig. 13. Comparison of ATP-induced electrical and optical signals measured in parallel experiments under nearly identical conditions (150 mM Na^+, 30 mM imidazole chloride, pH 7.2, 1 mM EDTA, 10 mM Mg^{2+}, T = 21–22°C). $\Delta F/F_o$ is the relative fluorescence signal and Q the translocated charge [Eq. (9)]; 1 pC $\equiv 10^{-12}$ coulomb. Q has been measured according to Stürmer et al. (1989). In the fluorescence experiment the concentration of caged ATP was 100 μM and the concentration of released ATP approximately 20 mM. In the current measurement the concentration of caged ATP was 240 μM and the concentration of released ATP about 30 μM

Fluorescence Signals in the Presence of K^+ and Absence of Na^+

From previous work (Glynn, 1985) it is known that in the presence of millimolar concentrations of K^+ and in the absence of Na^+, inorganic phosphate and nucleotides, the enzyme is predominantly in state $E_2(K_2)$ with occluded K^+. When under these conditions ATP is released to the medium, a shift from $E_2(K_2)$ to $E_1 \cdot$ ATP may be expected to occur, since the equilibrium between ATP $\cdot$ $E_2(K_2)$ and $K_2 \cdot E_1 \cdot$ ATP is strongly poised toward the side of E_1 (Glynn, 1985)

$$E_2(K_2) + ATP \rightarrow ATP \cdot E_2(K_2) \rightarrow K_2 \cdot E_1 \cdot ATP \rightarrow E_1 \cdot ATP + 2K^+_{cyt}. \tag{11}$$

The result of a fluorescence experiment carried out under these conditions (0 Na^+, 1 mM K^+) is shown in Fig. 14*A* (lower curve). The fluorescence signal recorded after ATP release is found to be very small, much smaller than the signal in the presence of Na^+ and absence of K^+ (upper curve in Fig. 14*A*).

For comparison, in Fig. 14*B* fluorescence experiments are represented which were carried out with

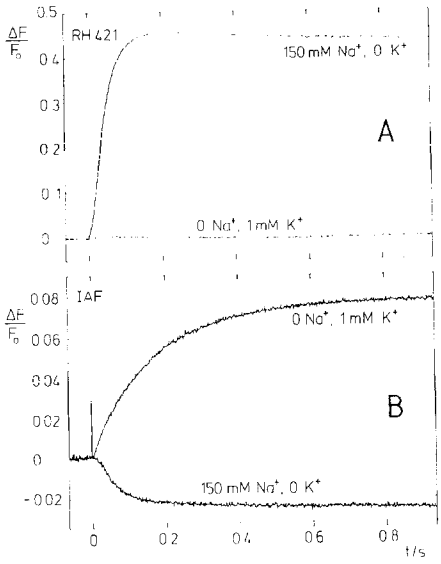

Fig. 14. Relative fluorescence change $\Delta F/F_o$ of IAF-labeled or RH 421-labeled membrane fragments, as a function of time t. At $t = 0$, ATP was photochemically released from caged ATP in the presence of either 150 mM Na^+ or 1 mM K^+; $T = 20°C$. (*A*) A suspension of membrane fragments (30 μg protein per ml) was added to a solution of 0.7 μM RH 421, 20 μM caged ATP, 10 mM $MgCl_2$, 1 mM EDTA, 30 mM imidazole chloride, pH 7.2, 150 mM or 0 mM NaCl and 1 mM or 0 mM KCl. About 5 μM ATP were released by a 308-nm flash of 10 nsec duration, (*B*) A suspension of IAF-labeled membrane fragments (5 μg protein per ml) was added to a solution of 100 μM caged ATP, 10 mM $MgCl_2$, 1 mM EDTA, 30 mM imidazole, pH 7.2, 150 mM or 0 mM NaCl and 1 mM or 0 mM KCl. About 20 μM ATP were released to the solution from caged ATP

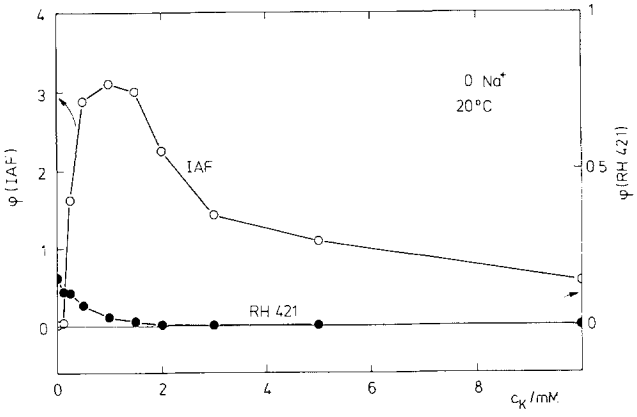

Fig. 15. Fluorescence change φ of IAF- or RH 421-labeled membrane fragments upon photochemical ATP release, as a function of K^+ concentration c_K. φ is referred to the fluorescence change observed in the presence of 150 mM Na^+ and absence of K^+ [Eq. (12)]. φ was evaluated from the experiments shown in Fig. 12 and from analogous experiments at other K^+ concentrations. Note the different ordinate scales for the IAF and RH 421 experiments

enzyme labeled with 5-iodoacetamidofluorescein (IAF), as described above. As seen from Fig. 14*B* (upper curve), a large change of fluorescence occurs upon ATP release, reflecting a substantial shift of the $E_2(K_2)/E_1$ conformational equilibrium (Stürmer et al., 1989).

Fluorescence experiments such as shown in Fig. 14 were carried out at different K^+ concentrations. It is convenient to represent the results in normalized form by introducing the quantity φ defined by

$$\varphi \equiv \frac{[\Delta F_{max}/F_o]_{c_N=0,\ c_K>0}}{[\Delta F_{max}/F_o]_{c_N=150\ mM,\ c_K=0}}. \qquad (12)$$

$\Delta F_{max}/F_o$ is the relative fluorescence change, and c_N and c_K are the concentrations of Na^+ and K^+, respectively. (ΔF_{max} is taken either as the limiting value of ΔF for $t \to \infty$, or, in cases in which ΔF declines at long times (Fig. 5), as the peak value of ΔF.) φ represents the relative fluorescence change at a given K^+ concentration, referred to the relative fluorescence change in the presence of 150 mM Na^+.

Values of φ for IAF- and RH 421-labeled membranes are plotted in Fig. 15 as a function of potassium concentration c_K. It is seen that, apart from a spurious effect at low c_K, the fluorescence change φ of the RH dye is vanishingly small in the whole range of K^+ concentrations. (The small fluorescence change seen at low c_K may result from a weak sodium-like effect of imidazole (Schuurmans Stekhoven et al., 1988) which is suppressed at higher concentrations of K^+.)

The finding that the fluorescence of RH 421 is virtually insensitive to ATP-induced conformational transitions in the potassium limb of the cycle may be compared with other experimental results. Studies of transient pump currents in cardiac cells (Bahinski, Nakao & Gadsby, 1988) and in reconstituted membrane systems (Fendler et al., 1985; Borlinghaus et al., 1987) have shown that transitions between the potassium-liganded states ($K_2 \cdot E_1 \rightleftarrows E_2(K_2) \rightleftarrows P - E_2 \cdot K_2$) are not associated with translocation of electric charge. The same conclusion has been drawn by Goldshlegger et al. (1987) from studies of ion fluxes mediated by Na,K-ATPase in reconstituted vesicles. The results represented in Fig. 15 thus support the notion that the bound IAF is sensitive to conformational changes of the enzyme, whereas RH 421 mainly responds to changes of electric field strength in the membrane.

Discussion

Styryl compounds have originally been introduced by Loew and coworkers (Loew et al., 1979) and by Grinvald and coworkers (Grinvald et al., 1982) as

electrochromic dyes for the optical recording of membrane voltages. Klodos and Forbush (1988) have recently described the use of styryl dye RH 160 for studying kinetic properties of the Na,K-pump. In this paper we have shown that certain styryl dyes, such RH 421 or RH 237, respond to ATP- or cation-induced transitions between states of the pump with large fluorescence signals (relative fluorescence changes up to 100%), and we have investigated the possible origin of these fluorescence changes.

Fluorescence changes of the membrane-bound dye upon pump activation can arise in at least two different ways. (i) Dye molecules which are bound to the protein may directly respond to changes of protein conformation. Experimental findings indicating that potential-sensitive dyes may monitor conformational changes of membrane proteins has been described by Lüdi, Oetliker and Brodbeck (1981) and by Nagel, Slayman and Klodos (1989). (ii) The dye may respond to changes of the electrostatic potential in the membrane resulting from charge movements in the protein. Although we cannot completely rule out the possibility that the observed fluorescence changes contain a component resulting from direct dye-protein interaction, the most likely explanation of our experimental findings consists in the assumption that the dye acts as a field sensor, as will be discussed in the following.

Evidence for the Electrostatic Origin of Fluorescence Changes

The notion that the styryl dye responds mainly to local electric fields in the membrane is based on several lines of evidence.

Spectral Characteristic of Dye Response

An electrochromic effect, i.e., a field-induced change of the energy difference between the excited state and the ground state of the dye, leads to a shift of the absorption peak to smaller or larger wavelengths (Loew, 1982). If the absorption spectrum is red shifted, the fluorescence change is predicted to be positive when the fluorescence is excited at the long-wavelength edge of the spectrum and negative when the fluorescence is excited at the short-wavelength edge (Fig. 6). The shift of the absorption spectrum of RH 421 observed upon pump activation and the wavelength-dependent sign of the concomitant fluorescence change (Fig. 6) are consistent with a (partially) electrochromic origin of the dye signal. In the spectral range below 520 nm, the relative changes of absorption and fluorescence closely agree, as expected for an electrochromic mechanism (Waggoner & Grinvald, 1977; Loew & Simpson, 1981; Loew, 1982; Fluhler et al., 1985). At longer wavelengths, however, the relative fluorescence change tends to be larger than the relative absorption change (Fig. 6). This indicates that, in addition to an electrochromic shift, the quantum yield of fluorescence is changed. The possibility that the voltage response of styryl dyes contains nonelectrochromic components has also been discussed by Fluhler et al. (1985) and by Müller et al. (1986). Ephardt and Fromherz (1989) have recently shown that the fluorescence yield of (dibutylamino) stilbazolium butylsulfonate (RH 364), a styryl dye similar to RH 421, is highly sensitive to changes of polarity and viscosity of the environment, and they have proposed that the voltage sensitivity of styryl dyes is partly determined by changes of the fluorecence yield.

Fluorescence Changes upon Adsorption of Lipophilic Ions

Further evidence in favor of an electrostatic origin of the dye response comes from experiments in which lipophilic cations (TPP^+) or anions (TPB^-) are allowed to adsorb to Na,K-ATPase membranes. Adsorption of TPB^- leads to an increase and adsorption of TPP^+ to a decrease of fluorescence (Fig. 10). These ions are known to bind to the hydrophobic interior of the membrane a few tenths of a nanometer away from the membrane-solution interface (Andersen et al., 1978). In this way they change the electrical field strength in a narrow zone close to the interface (Fig. 11). Assuming a linear relationship between fluorescence change ΔF and field strength, the dependence of $\Delta F/F_o$ on the concentration of lipophilic ions (Fig. 10) can be satisfactorily described by the three-capacitor model which has been used previously to describe adsorption of lipophilic ions to planar lipid bilayers (Andersen et al., 1978).

Suppression of Pump-Linked Dye Signals at High Concentration of Lipophilic Ions

The fluorescence change that is normally observed upon phosphorylation by ATP is abolished at high concentrations of TPB^- (Fig. 12). A likely explanation of this finding consists in the assumption that lipophilic ions redistribute between the adsorption sites and water and in this way neutralize the change of field strength caused by ion movement in the protein.

Correlation Between Fluorescence Changes and Known Electrogenic Reaction Steps in the Pumping Cycle

Fluorescence changes of RH 421 correlate with electrogenic transport reactions of the pump. From previous studies it is known that phosphorylation in the presence of Na^+ and absence of K^+ initiates translocation of electric charge (Fendler et al., 1985; Borlinghaus et al., 1987); under the same experimental conditions, large changes of the fluorescence of Rh 421 are observed (Fig. 5). On the other hand, the ATP-induced conformational change in the presence of K^+, which is known to be electrically silent (Bahinski et al., 1988; Stürmer et al., 1989), is found not to be accompanied by a fluorescence change (Figs. 14 and 15). The close correlation between dye response and electrogenic event is immediately apparent from Fig. 13 in which the time course of the fluorescence signal is compared with the time course of charge translocation.

Electric Properties of Na,K-ATPase Membranes

When in a suspension of Na,K-ATPase membranes the pump is activated by photochemical release of ATP, a transient voltage is built up across the membrane sheet. This voltage decays by ionic conduction in the electrolyte solution surrounding the membrane. The time constant τ of the voltage decay may be easily estimated to the order of magnitude; if C_m is the specific capacitance of the membrane, D the average diameter of the approximately circular membrane and ρ the specific resistance of the solution, the relation $\tau \approx \rho D C_m$ holds. With $C_m \approx 1\ \mu F/cm^2$, $D \approx 1\ \mu m$ and $\rho < 10^3\ \Omega$ cm, an upper limit, $\tau < 0.1\ \mu sec$, may be estimated under the conditions of our experiments. This means that the membrane fragments may be considered to be short circuited in the whole experimental time range.

Even without pump activation, internal electric fields are, in general, present in the membrane. Internal fields may result from an asymmetric distribution of surface charges of the protein and the lipid (Honig, 1986; McLaughlin, 1989; Hubbell, 1990). By the fluorescence method described above, only changes of field strength in the membrane can be detected.

It is useful to estimate the order of magnitude of the changes of field strength which may arise from pump-mediated charge movements. We assume that n_p elementary charges per unit area are located in the membrane at a distance r from the left-hand interface (Fig. A1). If these charges are released to the solution, the field strength in the left-hand interface changes by the amount ΔE

$$\Delta E = \frac{n_p e_o}{\varepsilon_o \varepsilon}\left(1 - \frac{r}{d}\right). \tag{13}$$

This relation follows from Eqs.(A7) and (A8) with $c = 0$. e_o is the elementary charge, ε_o the permittivity of the vacuum, ε the dielectric constant of the membrane, and d the membrane thickness. (Implicit in Eq. (13) is the assumption that the ionic strength in the aqueous medium is high, so that double-layer effects can be neglected.) With n_p taken to be equal to the upper limit of the density of Na,K-ATPase molecules in the membrane ($\approx 10^4\ \mu m^{-2}$) (Deguchi et al., 1977), and with $r \approx d/2$ and $\varepsilon \approx 2$, ΔE becomes about 5×10^7 V/m,which is equivalent (with $d = 5$ nm) to a transmembrane potential drop of 250 mV. An absolute upper limit of ΔE is obtained assuming that three Na^+ ions are released per Na,K-ATPase ($n_p \approx 3 \times 10^4\ \mu m^{-2}$) and that the ion-binding sites are located close to the dielectric interface ($r \approx 0$). This yields $\Delta E \approx 3 \times 10^8$ V/m, corresponding to a transmembrane potential drop of 1.5 V.

A calibration of the fluorescence change $\Delta F/F_o$ as a function of ΔE is not easily possible. A rough estimate of the relation between $\Delta F/F_o$ and ΔE may be obtained, however, from the experiments with membrane fragments in the presence of hydrophobic ions. With $|\Delta E| = |\psi_d/a|$ (*compare* Fig. A1), the following relation is obtained from Eqs. (A14) and (A16):

$$|\Delta E| = \frac{kT}{e_o a H}|\Delta F/F|_o = \frac{1}{p}|\Delta F/F_o|. \tag{14}$$

With the values $a \approx 0.4$ nm and $H \approx 0.38$ estimated above for TPP^+, one obtains $p \approx 5.8 \times 10^{-9}$ m/V. At a TPP^+ concentration of 5 mM, the relative fluorescence change $\Delta F/F_o$ resulting from adsorption of TPP^+ is about 0.27 (Fig. 10). With $p \approx 5.8 \times 10^{-9}$m/V, a change of field strength of $|\Delta E| \approx 4.7 \times 10^7$ V/m is estimated, equivalent to a transmembrane voltage drop of 230 mV.

The magnitude of the proportionality factor p relating the fluorescence change $\Delta F/F_o$ to the field strength ΔE, $p \approx 6 \times 10^{-9}$ m/V, may be compared with theoretical predictions of the size of electrochromic signals of styryl dyes. Loew (1982) has estimated that the maximum fluorescence change which may be expected from an electrochromic response of a styryl dye is about 20% per 100 mV transmembrane voltage. This corresponds (with a membrane thickness of 5 nm) to a value of $p \approx 10^{-8}$ m/V, which is about twice as large as the experimentally determined value of 5.8×10^{-9} m/V.

It is pertinent to mention that the voltage sensitivity of styryl dyes in reconstituted vesicles was

much lower than in Na,K-ATPase membranes. From the experiment shown in Fig. 8, a fluorescence change of $\Delta F/F_o \approx 0.05$ per 100 mV is estimated for RH 237, corresponding to $p \approx 2.5 \times 10^{-9}$ m/V. An even lower value, $p \approx 10^{-9}$ m/V is obtained for RH 421. The origin of this difference of the behavior of the styryl dyes in vesicles and in membrane fragments is not clear so far. Large variations in the voltage sensitivity of RH 421 in different membrane preparations have also been reported from other studies (Grinvald et al., 1983; Fluhler et al., 1985).

The largest values of $\Delta F/F_o$ which we have observed in the ATP-release experiments in the presence of Na^+ (Fig. 5) are of the order of 1.0. With $p \approx 5.8 \times 10^{-9}$ m/V, the corresponding change of field strength is estimated to be about 2×10^8 V/m. As discussed above, values of ΔE of this order of magnitude can indeed be expected for membrane fragments containing $\approx 10^4$ ATPase molecules per μm^2.

The treatment of the electrostatic properties of Na,K-ATPase membranes given above is strongly simplified by the use of a continuum model of the membrane in which all charges are assumed to be homogeneously distributed in planes parallel to the membrane surface. In reality, the membrane is a mosaic of bilayer domains and embedded proteins, and the dielectric properties of the membrane are inhomogeneous. With an estimated pump density of $\leq 10^4\ \mu m^{-2}$, the lower limit of the average center-to-center distance of pump molecules in the membrane is 10 nm. While theoretical methods are available by which effects of discrete charges and of inhomogeneous dielectric properties of membranes can be described (*see*, for instance, Thorne & Duniec, 1983; McLaughlin, 1989; Zimányi & Garab, 1989), it is not possible so far to apply these methods to Na,K-ATPase membranes, since information on the structure of the membrane is still scanty.

It cannot be excluded that dye molecules bind preferentially to the interface between protein and lipid; these dye molecules would be exposed to particularly large field changes. On the other hand, as discussed above, there is no evidence that the styryl dye, by binding to the protein, senses conformational changes directly; it is pertinent to mention that RH 421 does not respond to the electrically silent transition between states $E_2(K_2)$ and E_1, whereas the IAF label, which is sensitive to conformational changes, exhibits a large fluorescence change during the $E_2(K_2)/E_1$ transition.

Evidence that potential-sensitive dyes may respond to local field changes which are not necessarily correlated to changes of transmembrane voltage has been obtained also in other investigations. For instance, Aiuchi and Kobatake (1979) have found that merocyanine 540 is sensitive to changes of surface potential in mitochondrial membranes. More recently, Heiny and Jong (1990) observed that the merocyanine dye WW-375 responds in a unexpected nonlinear fashion to voltage changes across the T-tubular membrane and proposed that the dye is sensitive to local electrostatic potential changes resulting from charge movements in the membrane. *See also* Beeler, Farmen and Martonosi (1981) and Krasne (1983) for related observations.

Conclusion

The use of electrochromic dyes of high voltage sensitivity offers new possibilities for studying electrogenic properties of ion pumps. With amphiphilic styryl dyes which bind to the membrane-solution interface changes of local electric field strength can be monitored. The information obtained from such experiments is complementary to the results of electrophysiological investigations in which pump currents are measured as a function of transmembrane voltage. In particular, electrochromic dyes can be used for studying electrogenic processes in microsomal membrane preparations which are not amenable to electrophysiological techniques.

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Appendix

Fluorescence Change Associated with Charge Translocation in the Membrane in the Presence of Lipophilic Ions

We consider a simplified electrosatic model of the Na,K-ATPase membrane (Fig. A1) which is based on the following assumptions: (i) The membrane consists of a symmetric lipid bilayer into which protein molecules are embedded at random. (ii) Lipophilic ions adsorb to planes located in the membrane at a distance a from interface. The charge of the lipophilic ions is homogeneously distributed in the adsorption plane and is described by a charge density σ (expressed in C/cm^2). Discreteness-of-charge effects are neglected (McLaughlin, 1989; Zimányi & Garab, 1989). (iii) The mobile charges in the protein, i.e., the charges that are translocated upon pump activation, are homogeneously distributed in a plane located at a distance r from the left interface (Fig. A1). Upon pump activation, r may change from an initial value r_o to a final value r_∞. The charge density σ_p corresponding to this homogeneous distribution has to be considered as an effective charge density, since in reality the mobile charges are discrete and are located in the protein, whereas lipophilic ions and dye molecules are located in the bilayer domains. (iv) Specific interactions between lipophilic ions and dye molecules and between dye molecules and protein are neglected. (v) The electrolyte concentration in the aqueous medium is high, so that effects of diffuse double layers can be neglected.

From elementary electrostatic considerations, the surface charge densities σ'_s and σ''_s (Fig. A1) are obtained as

$$\sigma'_s = -(1-\alpha)\sigma'_a - (1-\rho)\sigma_p - \alpha\sigma''_a \tag{A1}$$

$$\sigma''_s = -\alpha\sigma'_a - \rho\sigma_p - (1-\alpha)\sigma''_a \tag{A2}$$

$$\alpha \equiv a/d; \qquad \rho \equiv r/d. \tag{A3}$$

d is the membrane thickness. If the membrane is an inhomogeneous dielectric, the quantities α and ρ have to be considered as fractional dielectric distances which may be different from geometrical distances. Equation (A1) and (A2) fulfill the condition for electroneutrality

$$\sigma'_s + \sigma''_s + \sigma'_a + \sigma''_a + \sigma_p = 0. \tag{A4}$$

If $C = \varepsilon_a\varepsilon_o/a$ is the specific electric capacitance of the adsorption layer ($\varepsilon_o \approx 8.85 \times 10^{-12}$ C V^{-1} m^{-1} is the permittivity of the vacuum and ε_a the dielectric constant of the adsorption layer), the electric potentials ψ'_a and ψ''_a (Fig. A1) in the adsorption planes are given by

$$\psi'_a = -\sigma'_s/C; \qquad \psi''_a = -\sigma''_s/C$$
$$C = \varepsilon_o\varepsilon_a/a. \tag{A5}$$

The concentrations N' and N'' of lipophilic ions (referred to unit area) in the adsorption planes are connected with the aqueous concentration c by the relations

$$N' = \beta c \cdot \exp(-z\psi'_a e_o/kT); \quad N'' = \beta c \cdot \exp(-z\psi''_a e_o/kT). \tag{A6}$$

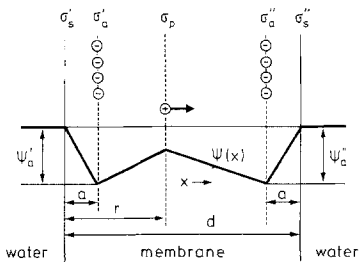

Fig. A1. Electrostatic model of the Na,K-ATPase membrane. Lipophilic anions are adsorbed in planes located at a distance a from the interface. Mobile charges in the protein are localized in a plane at a distance r from the left interface. σ'_s, σ''_s, σ'_a, σ''_a and σ_p are charge densities (C/m^2). $\psi(x)$ is the electrical potential and d the membrane thickness. ′ and ″ refer to the cytoplasmic and extracellular faces of the membrane, respectively

β is the interfacial partition coefficient, z the valency of the lipophilic ion, e_o the elementary charge, k Boltzmann's constant, and T the absolute temperature. The Boltzmann factor $\exp(-z\psi_a e_o/kT)$ accounts for electrostatic repulsion by the charges in the adsorption plane. Equations (A1), (A2), (A5) and (A6) yield (with $y \equiv \psi_a e_o/kT$)

$$y' + y'' = \frac{c}{zc_o}[\exp(-zy') + \exp(-zy'')] + s_p \tag{A7}$$

$$y' - y'' = \frac{c}{zc_o}(1+2\alpha)[\exp(-zy') - \exp(-zy'')] + (1-2\rho)s_p \tag{A8}$$

$$c_o \equiv \frac{kTC}{z^2e_o^2\beta}; \qquad s_p \equiv \frac{e_o\sigma_p}{kTC}. \tag{A9}$$

If n is the number of dye molecules per unit area bound to each half of the lipid bilayer and A the total bilayer area in the membrane suspension, the fluorescence intensity F may be represented by

$$F = nA(f' + f''). \tag{A10}$$

f' and f'' are the (field-dependent) fluorescence contributions per dye molecule in the left-hand and right-hand interface. Assuming that the dependence of fluorescence intensity on electric field-strength results from a linear electrochromic effect (Waggoner & Grinvald, 1977; Loew, 1982), the quantities f' and f'' are given by

$$f' = f^o(1 + pE'); \qquad f'' = f^o(1 + pE''). \tag{A11}$$

E' and E'' are the field strengths in the membrane in the left-hand and right-hand interface, p is a wavelength-dependent coefficient and f^o the field-independent component of f' and f''. Equation (A11) hold for $|pE| \ll 1$. The coefficient p also accounts for geometrical factors such as the location and orientation of the transition moment of the dye with respect to the field. With $E' = -\psi'_a/a$, $E'' = \psi''_a/a$ and $y = \psi_a e_o/kT$, Eq. (A10) assumes the form

$$F = \frac{nAf^o pkT}{e_o a}(y' + y'') + 2nAf^o. \tag{A12}$$

If F_o and F_x are the fluorescence intensities before and after the change of field strength, the relative fluorescence change $\Delta F/F_o \equiv (F_x - F_o)/F_o$ is obtained as

$$\frac{\Delta F}{F_o} = H\frac{y'_x + y''_x - (y'_o + y''_o)}{2 + H(y'_o + y''_o)} \tag{A13}$$

$$H \equiv \frac{pkT}{e_o a} = \frac{pkTC}{e_o \varepsilon_o \varepsilon_a}. \tag{A14}$$

The quantities of y'_o, y''_o, y'_x, y''_x, which are defined in an analogous way as F_o and F_x, have to be evaluated numerically from the implicit equations (A7) and (A8). In the following, we apply Eq. (A13) to three special cases.

i) We consider binding of lipophilic ions to membrane fragments without pump activation, assuming that charges in the protein can be neglected ($s_p = 0$). Under this condition, according to assumption i, the system is symmetrical, so that the relations $y'_o = y''_o = 0$ (prior to addition of lipophilic ions), and $y'_x = y''_x \equiv y$ (after binding of lipophilic ions) hold. Equation (A7) and (A13) then reduce to

$$y = \frac{c}{zc_o}\exp(-zy) \tag{A15}$$

$$\frac{\Delta F}{F_o} = Hy. \tag{A16}$$

These relations are identical with Eqs. (6) and (7) of the text. The application of Eqs. (A15) and (A16) to experiments with lipophilic ions represents an approximation, since binding of lipophilic ions leads to large fluorescence changes which are outside the range of validity of Eq. (A11). Under this condition the quantity p has to be considered as a phenomenological parameter which may contain additional contributions other than the electrochromic effect.

ii) We consider an initially symmetric charge distribution in the membrane ($\rho = 1/2$). In this case, the relations $y'_o = y''_o \equiv y_o$ and $y'_x = y''_x \equiv y_x$ hold, so that Eqs. (A7), and (A13) assume the form (with $s_p(t = 0) \equiv s_p^o$ and $s_p(t \to \infty) \equiv s_p^x$)

$$\frac{\Delta F}{F_o} = H\frac{y_x - y_o}{1 + Hy_o} \tag{A17}$$

$$y_o = \frac{c}{zc_o}\exp(-zy_o) + s_p^o/2 \tag{A18}$$

$$y_x = \frac{c}{zc_o}\exp(-zy_x) + s_p^x/2. \tag{A19}$$

By numerical evaluation of y_o and y_x from Eqs. (A18) and (A19) it can be shown that for a fixed value of $s_p^o/2$, the difference $y_x - y_o$ approaches zero for $c/c_o \to \infty$. This means that the fluorescence change $\Delta F/F_o$ (Eq. A17) should vanish at large concentrations of the lipophilic ion. This expectation is borne out by the experimental findings (Fig. 12).

iii) In the absence of lipophilic ions ($c = 0$), the relations $y'_o + y''_o = s_p^o \equiv s_p(t = 0)$, $y'_x + y''_x = s_p^x \equiv s_p(t \to \infty)$ hold, so that the relative fluorescence change (Eq. A13) is predicted to be

$$\frac{\Delta F}{F_o} = p\frac{\sigma_p^x - \sigma_p^o}{2\varepsilon_o\varepsilon_a + p\sigma_p^o}. \tag{A20}$$

Thus, $\Delta F/F_o$ is independent of ρ, meaning that translocation of charge without release from the membrane ($s_p^o = s_p^x$) does not lead to a fluorescence change. This is a consequence of the assumption introduced above that the membrane is symmetrical with respect to dye binding.