Fluorescent Styryl Dyes as Probes for Na,K-ATPase Reaction Mechanism: Significance of the Charge of the Hydrophilic Moiety of RH Dyes†

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Received August 31, 1995; Revised Manuscript Received October 23, 1995®

ABSTRACT: The fluorescence responses of a series of potential-sensitive styryl-based dyes (either zwitterionic RH160, RH421, di-4-ANEPPS, or positively charged RH795, RH414, RH461) to phosphorylation of Na,K-ATPase from ATP or inorganic phosphate, and ouabain binding to phospho- or dephosphoforms, have been characterized and compared in broken membrane preparations of the enzyme. Zwitterionic dyes were more sensitive to molecular events in the Na,K-ATPase reaction cycle than positively charged dyes, but the net charge did not affect the sensitivity of the dyes to a transmembrane electric field. The major part of the response of the zwitterionic dyes to formation of phosphoenzymes was due to a change in the quantum yield of fluorescence. Computer modeling of dyes with identical chromophore structure, and experimental characterization of their optical properties in bulk solvents, revealed two general trends: (1) the absorption maximum of the zwitterionic dye was blue-shifted with respect to the positively charged dye; (2) the quantum yield of the zwitterionic dye was higher and the fluorescence lifetime was longer than that for the positively charged dye. Spectral properties of the dyes in the membrane depended on the presence of Na,K-ATPase. We suggest, that (1) electrostatic interactions between the enzyme and the hydrophilic headgroup of the dye by changing the charge of hydrophilic moiety and thus modifying the net charge of the dye molecule cause both the spectral shifts and the changes in the quantum yield, and (2) interactions between the styryl dyes and the Na,K-ATPase depend on the conformational state of the enzyme.

Amphiphilic styryl-based dyes were introduced to investigations of the reaction mechanism of cation-transporting ATPases by Klodos and Forbush (1988), who reported on changes in the fluorescence of RH160 bound to Na,K-ATPase enriched broken membranes in response to an altered distribution of enzyme states. The fluorescence responses of these dyes are induced by transitions otherwise known to be electrogenic like Na^+ binding to E_1[1] or Na^+ release upon formation of E_2P but not by electroneutral transitions like the $E_1 \leftrightarrows E_2$ conversion or occlusion and deocclusion of K^+ [for references to fluorescence responses, see Klodos and Forbush (1988), Bühler et al. (1991), Forbush and Klodos (1991), Läuger (1991), Stürmer et al. (1991), and Klodos (1994); for references to electrogeneity of Na,K-ATPase transitions, see Rephaeli et al. (1986a,b), Rakowski et al. (1991), Gadsby et al. (1993), and Hilgemann (1994)]. Since styryl-based dyes are potential sensitive (Grinvald et al., 1982; Loew, 1982), it has been proposed that they monitor charge movements within the membrane dielectric associated with the pumping cycle of Na,K-ATPase (Bühler et al., 1991; Stürmer et al., 1991). Effects of lipophilic ions, partitioning into lipid membrane and thus changing the local electric field, on the fluorescence were taken as support for this hypothesis (Bühler et al., 1991). According to these authors a change in the local electric field induces a shift of the absorbance spectrum of the dye, the so-called electrochromic shift (Läuger, 1991). Bühler et al. (1991) conclude that changes in the absorbance and excitation spectra are "consistent with the notion that at least part of the spectral change results from an electrochromic effect ...".

It is clear, however, that the fluorescence responses to the reactions of Na,K-ATPase significantly deviate from those expected for a pure electrochromic mechanism. Bühler et al. (1991) hypothesize that the deviation could be because "the electrochromic band shift is superimposed by a change of quantum yield, which becomes prominent at long wavelengths. Such a change of quantum yield may result from a field effect on the rate of radiationless desactivation...". Clarke et al. (1992) ascribe the observed deviations from the electrochromic mechanism to "either a potential-dependent equilibrium between membrane-bound dye monomers and membrane-bound dimers ..., or to a field-induced structural change of the membrane". In other words both groups ascribe the fluorescence responses to electric field effects and refer the observed deviations from expected electrochromic behavior to additional nonelectrochromic mechanisms of response of styryl dyes to changes in the electric field. As a consequence of this assumption some partial reactions like binding of ouabain to dephosphoforms of Na,K-ATPase, not otherwise shown to be electrogenic, are now described as being associated with proton binding to the enzyme (Schwappach et al., 1994).

† This work was supported in part by The Danish Research Academy (fellowship to N.U.F.), The Danish Medical Research Council, and The Danish Biomembrane Research Centre, University of Aarhus.

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® Abstract published in *Advance ACS Abstracts,* December 1, 1995.

[1] Abbreviations: E_1, Na,K-ATPase form with high affinity toward ATP and Na^+; E_2, Na,K-ATPase form with high affinity toward K^+ and low affinity to ATP; EP, phosphoenzyme; E_1P, ADP-sensitive phosphoenzyme; E_2P, K-sensitive phosphoenzyme; E_2−O, dephosphoenzyme−ouabain complex; E_2P−O, phosphoenzyme−ouabain complex; P_i, inorganic phosphate; LUMO, lowest unoccupied molecular orbital; HOMO, highest occupied molecular orbital; INDO, intermediate neglect of differential overlap.

0006-2960/95/0434-16806$09.00/0

One may ask, however, whether the electric field change is the only event reported by these dyes? Can it be that the dyes in these membrane systems respond also to nonelectric events and that this is the cause for the deviation from purely electrochromic behavior?

To investigate this possibility and to further understand the fluorescence response of RH dyes in relation to the Na,K-ATPase, we have chosen a number of reactions where such a response has been observed, namely, the combined step of Na^+-transport and phosphorylation, phosphorylation from inorganic phosphate and also ouabain binding. We have characterized and compared the fluorescence responses of a number of styryl-based dyes to those reactions in an attempt to correlate the structure of the probe with its response. We searched for other effects of the structural details on the spectral characteristics of the dyes by computer modeling of the dyes structure and characterization of the optical properties of the dyes in the bulk solvents. The results provided some clues about both the possible mechanisms of the dyes response to Na,K-ATPase reaction and also molecular events in the Na,K-ATPase reaction cycle.

EXPERIMENTAL PROCEDURES

Enzyme Preparation. Na,K-ATPase (EC 3.6.1.37) enriched membrane fragments from shark rectal glands were purified as previously described (Skou & Esmann, 1979). Na,K-ATPase from pig kidney was prepared according to Jørgensen (1974) as modified by Jensen et al. (1984). The activity, measured at 37 °C and under standard conditions [according to Ottolenghi (1975), but without bovine serum albumin], was 25 units/mg of protein.

The protein amount was determined according to Lowry et al. (1951), as described by Jensen and Ottolenghi (1983), using bovine serum albumin as standard.

Materials. ATP, purchased as sodium salt from Boehringer Mannheim, Germany, was converted to Tris salt by chromatography on a Dowex 1 column (from Sigma Chemicals Co., St. Louis, MO). RH160, RH414, RH421, RH461, RH795, di-4-ANEPPS, valinomycin, oxonol VI, and rhodamine B were purchased from Molecular Probes, Inc., Eugene, OR. All RH dyes and di-4-ANEPPS were dissolved in dimethyl sulfoxide. Valinomycin, oxonol VI, and rhodamine B were dissolved in ethanol. Lipids were purchased from Avanti Polar Lipids, Alabaster, AL. *N*-2-Hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES), 2-*N*-morpholinoethanesulfonic acid (MES), and *N*-methyl-D-glucamine were purchased from Sigma. All other reagents were reagent grade.

Fluorescence Measurements. The fluorescence of styryl probes in micromolar concentrations was measured with 50 μg/mL protein in 10 mM HEPES and 10 mM MES, adjusted with *N*-methyl-D-glucamine to pH 7.5. Measurements of fluorescence were performed with a SPEX Fluorolog fluorometer at 20 °C in a cuvette (1 cm light path) with continuous stirring. In all experiments the excitation bandpass was 1 nm and emission was measured with a 630 nm cut-off filter on the emission side. In time-resolved experiments the excitation wavelength was 580 nm.

Steady-state level of phosphoenzyme was induced either through the physiological route (Albers et al., 1963; Post et al., 1965) in the presence of 15−30 μM ATP and 16 mM NaCl, or through the direct route (Albers et al., 1968; Lindenmayer et al., 1968) with 4 mM P_i in the absence of NaCl (all concentrations are given as final concentrations). For each dye the fluorescence excitation spectra in the presence of Mg^{2+} and Na^+ (or Mg^{2+} alone) were compared with those under steady-state phosphorylation.

Ouabain was bound to the dephosphoenzyme in the presence of Mg^{2+} alone or to the enzyme phosphorylated either from P_i in the absence of Na^+, or from ATP after addition of 16 mM Na^+.

Effects of transmembrane potential on the RH dye fluorescence were evaluated using phosphatidylcholine/cholesterol (60/40 mol %) liposomes. The internal buffer contained either 100 mM NaCl, or 1 mM NaCl and 198 mM sucrose. Transmembrane potentials of opposite polarity were induced by addition of valinomycin to these vesicles suspended in buffers to give a Na^+ gradient of either 100:1 or 1:100. In the absence of K^+ valinomycin acts as an ionophore for Na^+. The presence of stable Na^+-diffusion potentials was controlled by oxonol VI fluorescence, as previously described (Cornelius, 1989), and no transmembrane potential was observed in the absence of a Na^+ gradient (1 mM Na^+ present on both sides of vesicle membrane). The general procedure for preparation of liposomes and proteoliposomes with reconstituted Na,K-ATPase (protein/lipid weight ratio of 1:5 or 1:20) was described previously (Cornelius, 1988). The RH dye was bound either to the inner leaflet by adding the dye during vesicle formation followed by washing, or to the outer leaflet by adding the dye after vesicle formation.

Fluorescence lifetime and quantum yield measurements were performed with a SLM 4800C AMINCO subnanosecond spectrofluorometer at 20 °C. Lifetimes were measured by the phase-modulation method, using modulation frequency 30 MHz. Quantum yields of the RH dyes were determined by a comparative procedure using a solution of rhodamine B in ethanol with a quantum yield Q=0.97 as a standard. For the absorbance measurements a Perkin Elmer UV/Vis spectrometer Lambda 11/Bio was used.

Phosphorylation. Phosphorylation of the enzyme from $^{32}P_i$ (4 mM) or [γ-^{32}P]ATP (15−30 μM) was performed under conditions identical to those used in the fluorescence measurements and was measured at 20 °C according to Cornelius (1995).

Calculations of Molecular Orbital Structures. Calculations of the molecular orbital structures of the styryl dyes were performed with the use of HyperChem software (Hypercube, Inc.). The geometry optimization of both RH160 and RH795 molecules was first performed in the MM+ molecular mechanic force field, and then the dye molecules were subjected to the quantum mechanic calculations. The methods used differed in the level of approximation to the Schrödinger equation and in the way of parameterization. The simplest and fastest all-valence electron method (the extended Hückel molecular orbital method) has no explicit treatment of electron−electron interactions. The semiempirical NDO method (INDO) considers electron repulsions and to some extent nuclear repulsions but neglects the effects of density overlap between atomic orbitals. Its important advantage is the possibility to use mixed mode calculations: application of the semiempirical method to a selected part of the molecule, e.g., the chromophore, allowing at the same time correction for the influence of the rest of the atoms as a set of classical point charges. A third method applied

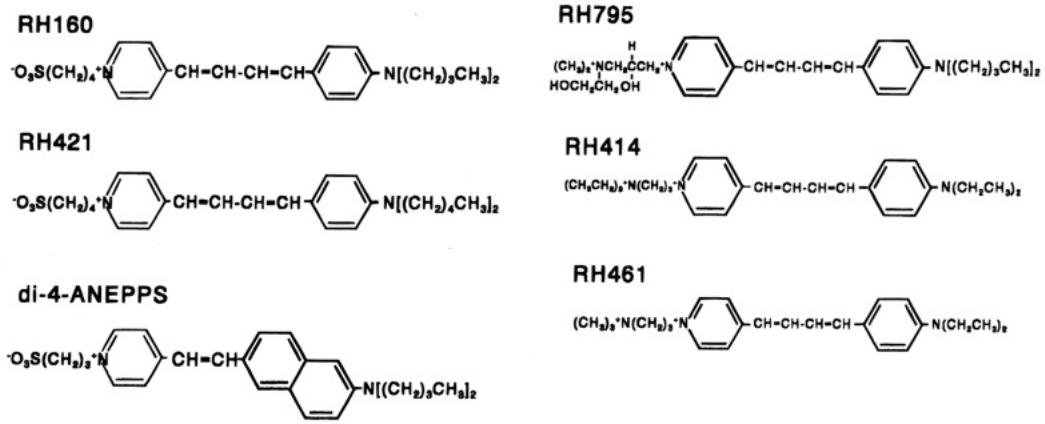

FIGURE 1: Chemical structure of styryl dyes. (Left panel) Zwitterionic dyes. (Right panel) Positively charged dyes.

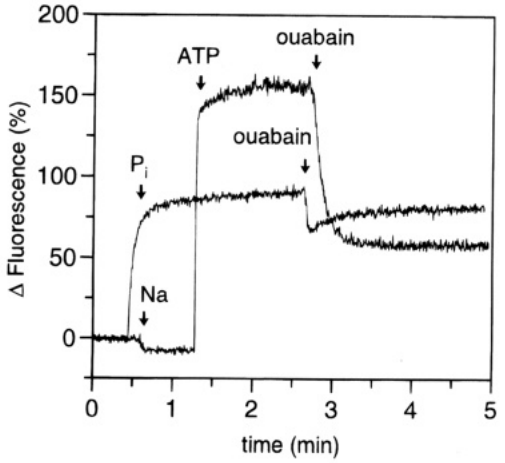

FIGURE 2: Typical recordings of RH421 fluorescence changes caused by enzyme phosphorylation from either P_i or ATP and following ouabain binding to the phosphorylated enzyme. One trace demonstrating ATP-phosphorylation shows responses to consecutive additions of 16 mM NaCl, 30 μM ATP, and 20 μM ouabain. Another trace demonstrating P_i-phosphorylation shows responses to addition of 4 mM P_i and 20 μM ouabain. The total emission >630 nm upon excitation at 580 nm was measured in the presence of 0.4 μM RH421 (final concentration). The fluorescence changes are expressed as percentage of the initial level in the presence of 4 mM $MgCl_2$.

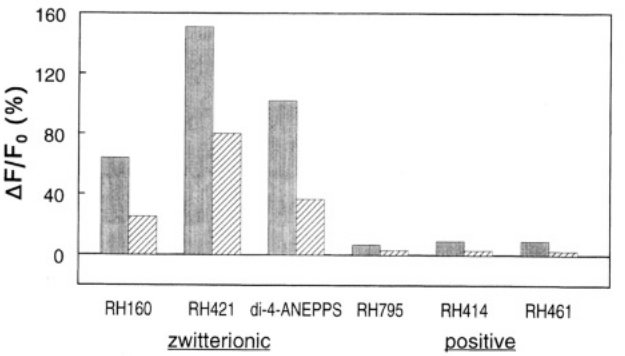

FIGURE 3: Relative change in the fluorescence of different RH dyes due to phosphorylation of shark Na,K-ATPase from ATP (shaded bars) or P_i (hatched bars). The responses were recorded as shown in Figure 2, either in the presence of 4 mM $MgCl_2$, 16 mM NaCl, and 30 μM ATP or with 4 mM $MgCl_2$ and 4 mM P_i. Fluorescence responses were measured in the presence of 0.2 μg of dyes/mL corresponding to final concentrations of 0.3−0.4 μM.

(AM1) takes into account an additional class of electron interactions. The calculations provide a qualitative description of the electronic structure for molecules as large as RH dyes.

RESULTS

Styryl Dyes as Probes for Na,K-ATPase Reaction. A series of styryl-based fluorescent dyes have been screened for the responses to phosphorylation of Na,K-ATPase and to ouabain binding. The range of dyes used includes those depicted in Figure 1 differing in charge polarity of their hydrophilic moiety, in the length of the aliphatic chains of the hydrophobic segment, and in the size of the chromophore. A typical experiment demonstrating the changes in fluorescence of RH421 associated with these reactions is shown in Figure 2. The enzyme was phosphorylated in the presence of either Mg^{2+}, Na^+ and ATP, or Mg^{2+} and P_i inducing preferentially E_2P (Cornelius, 1995) followed by ouabain addition to the phosphoenzyme.

Maximal responses of the various dyes to phosphorylation of Na,K-ATPase from either P_i or ATP, measured as the relative change of the fluorescence $\Delta F/F_o$ (percent), were observed at an excitation wavelength of 580 nm and summarized in Figure 3. All dyes responded to phosphorylation with an increase in the fluorescence, and for each individual dye the increase in response to P_i-phosphorylation was consistently smaller than in response to ATP-phosphorylation. For shark enzyme this was paralleled by a significantly lower steady-state level of phosphoenzyme reached by P_i-phosphorylation than by ATP-phosphorylation measured chemically at identical conditions. The steady-state phosphorylation level was $\approx$ 1.5 nmol of $E^{32}P$/mg of protein in the presence of 4 mM P_i and 4 mM $MgCl_2$ and about 2.5 nmol of $E^{32}P$/mg of protein with 16 mM NaCl, 4 mM $MgCl_2$, and 25 μM ATP.

The response of the dyes to the phosphorylation of Na,K-ATPase correlated with the net charge of the dye molecule (Figure 3); zwitterionic dyes (RH160, RH421, and di-4−ANEPPS) being more sensitive to phosphorylation than dyes carrying a net positive charge (RH795, RH414, and RH461).

The final fluorescence level recorded in the presence of enzyme−ouabain complex was also much higher for zwitterionic than for the positively charged dyes, as exemplified by the experiments with RH160 and RH795 in Figures 4 and 5. It is furthermore obvious that there were essential quantitative and qualitative differences in the response of the two groups of dyes to ouabain binding. Ouabain addition to the dephosphoenzyme (Figure 4) was characterized by (1) a large, slow increase in fluorescence of the zwitterionic

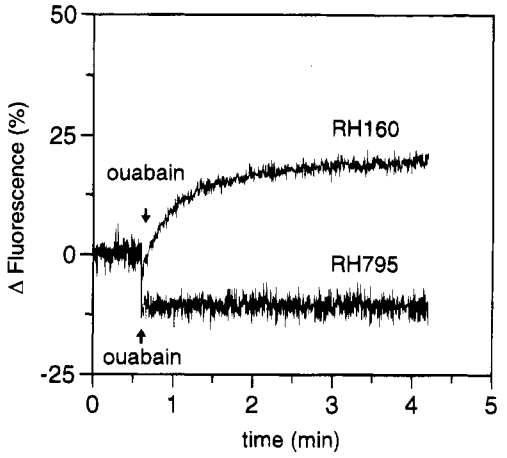

FIGURE 4: Ouabain induced changes in the fluorescence of RH160 and RH795 bound to the Na,K-ATPase membrane preparation. Addition of ouabain to a final concentration of 0.5 mM is shown by the arrows. The fluorescence was measured as in Figure 2, and fluorescence change is expressed as percentage of the initial level in the presence of 4 mM $MgCl_2$.

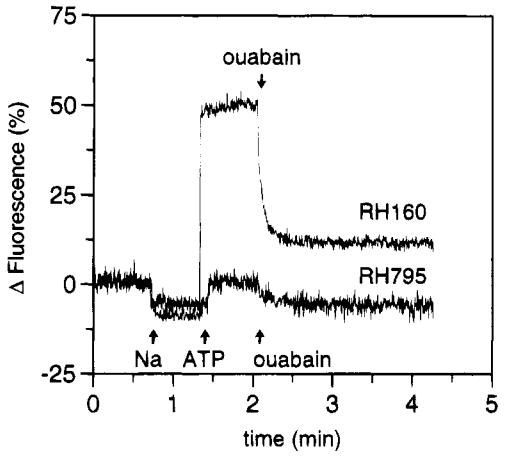

FIGURE 5: Different levels of RH160 and RH795 fluorescence caused by enzyme phosphorylation and the response to ouabain binding to the phosphorylated enzyme. Consecutive additions of 16 mM NaCl, 150 μM ATP, and 20 μM ouabain are shown by the arrows. The fluorescence was measured as in Figure 2, and fluorescence changes are expressed as percentage of the initial level in the presence of 4 mM $MgCl_2$.

RH160, which was shown to develop in parallel with the ouabain binding to the enzyme (Forbush and Klodos, unpublished) but (2) a small, instantaneous decrease in fluorescence of the positively charged RH795. Ouabain binding to enzyme phosphorylated in the presence of ATP, Na^+, and Mg^{2+} elicited a decrease in the fluorescence for both zwitterionic and positively charged dyes, compared with the fluorescence level of phosphorylated enzyme (Figure 5). For the positively charged RH795 the final level of fluorescence after ouabain addition, $F_{EP\text{-}O}$, was close to the steady-state level of fluorescence of the Na-bound dephosphoform, $F_{E\text{-}Na}$ (Figure 5), and higher than the level achieved after ouabain addition in the presence of Mg^{2+} alone, $F_{E\text{-}O}$ (Figure 4): $F_{EP\text{-}O} \approx F_{E\text{-}Na} > F_{E\text{-}O}$. For the zwitterionic RH160 the fluorescence level after ouabain binding to the phosphoform was slightly lower than that observed for the dephosphoenzyme−ouabain complex, both levels being significantly higher than the fluorescence level of Na-bound dephosphoform (compare Figures 4 and 5): $F_{E\text{-}O} \geq F_{EP\text{-}O} > F_{E\text{-}Na}$. The similarity of the final fluorescence levels of the dephosphoenzyme−ouabain and the phosphoenzyme−ouabain complexes observed with RH160 upon excitation at 580 nm is only coincidental as shown by the excitation spectra analysis described below.

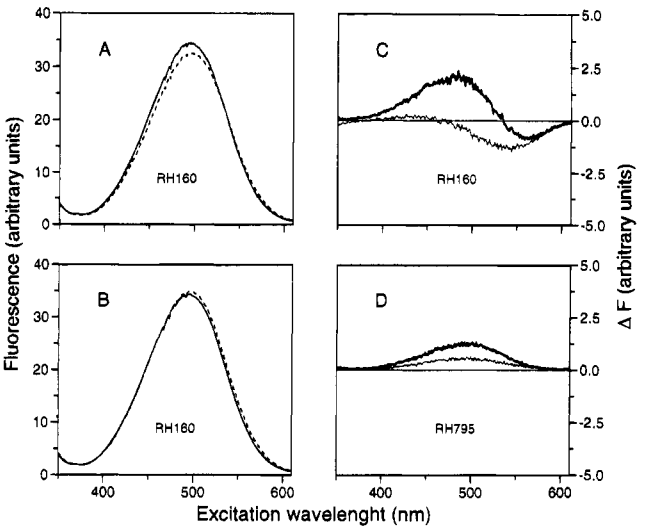

FIGURE 6: Fluorescence response to ouabain addition to dephosphoenzyme and to the enzyme phosphorylated from ATP. (Panels A and B) Corrected excitation spectra for RH160. Continuous lines show spectra in the presence of 4 mM $MgCl_2$, $F_0(\lambda)$. Broken lines show $F_1(\lambda)$, spectra after addition of either 0.5 mM ouabain alone (panel A) or 20 μM ouabain in the presence of 16 mM NaCl and 150 μM ATP (panel B). (Panels C and D) Corrected excitation response spectra $\Delta F(\lambda) = F_0(\lambda) - F_1(\lambda)$ for RH160 (panel C) and RH795 (panel D). Thick lines, spectra in the presence 4 mM $MgCl_2$ minus spectra in the presence of 4 mM $MgCl_2$ + 0.5 mM ouabain. Thin lines, spectra in the presence 4 mM $MgCl_2$ minus spectra in the presence of 20 μM ouabain, 16 mM NaCl + 150 μM ATP. Total emission >630 nm was recorded and experimental conditions were as described in the legends to Figures 4 and 5.

Spectral Characteristics of the Responses. Results of the spectral analysis, performed for the dephosphoenzyme−ouabain complex and the phosphoenzyme-ouabain complex formed after phosphorylation from ATP, are shown in Figure 6. The excitation response spectra $\Delta F(\lambda) = F_0(\lambda) - F_{E\text{-}O}(\lambda)$ or $\Delta F(\lambda) = F_0(\lambda) - F_{EP\text{-}O}(\lambda)$ [$F_0(\lambda)$ being the fluorescence in the presence of Mg^{2+} alone] for RH160 showed qualitative and quantitative differences between responses to ouabain binding to dephospho- and phosphoenzyme. The positively charged RH795 responded to the formation of both complexes with an overall decrease of the fluorescence intensity, the decrease being significantly larger for the dephosphoenzyme−ouabain complex.

The response of RH dyes to the phosphorylation of the enzyme was previously described as a response to a change in the electrostatic potential profile in the membrane dielectric. As discussed by Loew (1982), membrane-incorporated styryl dyes respond to the transmembrane electric field (due to their optimal orientation and relative weakness of the field) according to the so-called linear electrochromic mechanism. An important criterion for such an electrochromic response is that the corrected excitation response spectrum, i.e., the fluorescence change elicited by an applied electric field $F_0(\lambda) - F_1(\lambda)$ and the first derivative of the spectrum in the absence of an electric field $dF_0(\lambda)/d\lambda$ should match each other (Liptay, 1969). To investigate if this was the case for enzyme phosphorylation, the first derivative of the fluorescence excitation spectra before enzyme phosphorylation ($dF_0(\lambda)/d\lambda$) was compared with the

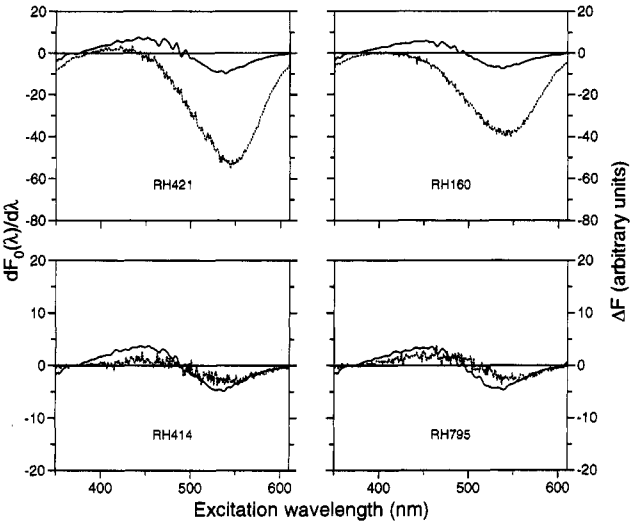

FIGURE 7: Fluorescence response to phosphorylation of Na,K-ATPase. Comparison of the first derivative of the resting excitation spectra, $dF_0(\lambda)/d\lambda$ (continuous lines) and the excitation response spectra after the phosphorylation, $\Delta F(\lambda) = F_0(\lambda) - F_1(\lambda)$ (dotted lines). $F_0(\lambda)$ is the excitation spectrum recorded in the presence of 4 mM $MgCl_2$ and 16 mM NaCl. $F_1(\lambda)$ is the excitation spectrum recorded after phosphorylation in the presence of 30 μM ATP, 4 mM $MgCl_2$, and 16 mM NaCl. Total emission >630 nm was recorded.

fluorescence change in excitation spectra after enzyme phosphorylation $[F_0(\lambda) - F_1(\lambda)]$. For the zwitterionic dyes (RH160 and RH421) the excitation response spectra deviated significantly from the first derivatives of the excitation spectra before the enzyme phosphorylation (Figure 7), the largest deviation being observed at long excitation wavelengths. This fact indicates a change in the shape of the spectra upon enzyme phosphorylation, most pronounced in the red-edge part of the spectra. With the positively charged dyes (RH414 and RH795), as seen from the two lower panels in Figure 7, the difference between the response spectra and the first derivatives of the resting excitation spectra was less pronounced.

The binding constant of a styryl dye and the depth of its insertion into the membrane is expected to increase both with the number of carbons between the chromophore and the charged hydrophilic headgroup and with the length of the hydrocarbon chains on the aniline nitrogen (Fluhler et al., 1985). As the sensitivity and mode of operation of RH dyes could be affected by their location in the bilayer, we chose RH160 and RH795 for further comparison. These two dyes have identical chromophore, hydrophobic moiety, and similar distance between the charge of the hydrophilic head and the chromophore, and thus similar binding properties and the position of the chromophore in the membrane. In spite of that, the zwitterionic RH160 was significantly more sensitive to phosphorylation of shark Na,K-ATPase than the double charged, positive RH795 (Figure 3). This leads to the question whether the negative charge of the hydrophilic group made the zwitterionic styryl dye susceptible to an additional mechanism of voltage sensitivity different from that of electrochromism, or if the zwitterionic dyes responded to additional events not directly connected to a charge redistribution in the membrane dielectric?

Voltage Sensitivity and the Net Charge of the Dye Molecule. In order to compare the response of the RH dyes caused by the electric field and those induced by phosphorylation of the Na,K-ATPase, pure lipid vesicles (liposomes) with incorporated styryl dyes were used, in which transmembrane potentials of chosen polarity could be induced (see Experimental Procedures). The potential sensitivity of the dyes, located either in the inner leaflet or in the outer leaflet, was compared. For both dye locations, the relative responses to a transmembrane potential were qualitatively independent of the potential polarity, but since the sensitivity was higher for the inside-bound dyes (not shown), further experiments were performed exclusively with the dyes incorporated to the inner bilayer leaflet. The results of these experiments with RH160 and RH795 are summarized in Figure 8. As seen, opposite electric gradients caused spectral shifts in opposite directions. The essential independence of the responses on the charge of the dye argues in favor of electrochromism as the molecular mechanism of response to the transmembrane potentials. In accordance with previous reports (Loew & Simpson, 1981; Grinvald et al., 1982), our experiments demonstrate that the charge of the hydrophilic headgroup is not crucial for the electric field sensitivity.

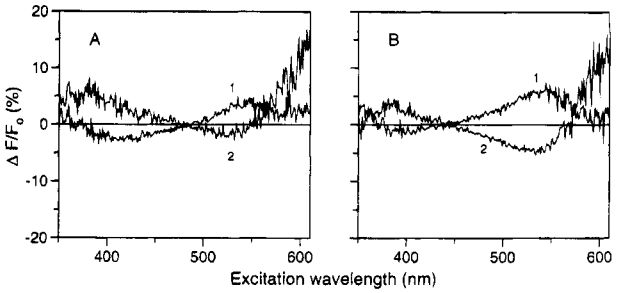

FIGURE 8: Relative fluorescence excitation response spectra of RH795 (A) and RH160 (B) induced by the formation of a transmembrane potential in liposomes. Total emission >630 nm was recorded. Stable transmembrane potentials of approximately +116 mV (1) or −116 mV (2), created by opposite Na^+ gradients in the presence of 5×10^{-8} M valinomycin (final) was reported by the dye bound to the inner leaflet of the vesicles.

Spectroscopic Properties and the Net Charge of the Molecules. To understand why the responses of the two groups of dyes to the same reaction step differed, we searched for other manifestations of the net charge of the dyes on their individual spectroscopic characteristics. The absorbance spectra of RH160 and RH795 in bulk solvents showed that the molar extinction of the two dyes was about the same. The only difference was in the position of the absorption maximum which for RH795 was slightly red-shifted relative to RH160 both in water and in ethanol (Figure 9).

The difference between the two dyes became more obvious when the excitation spectra were compared (Figure 9). The quantum yield of RH160 was found to be considerably higher than that of RH795 (Figure 10). The fluorescence quantum yield of a molecule can be modified by any factor which affects either the emission rate constant or the rate constant of radiationless decay. Direct measurements of the fluorescence lifetimes and quantum yields (Table 1) revealed that the charge of the hydrophilic moiety of the dye affected the properties of the chromophore. The fluorescence lifetime for RH421 found in the present study is in accordance with that previously measured by Visser et al. (1994) when the difference in temperature of the measurement is taken into account. Both lifetime and quantum yield were increased for the zwitterionic dyes compared to the positively charged dyes, although all four dyes had identical chromophore

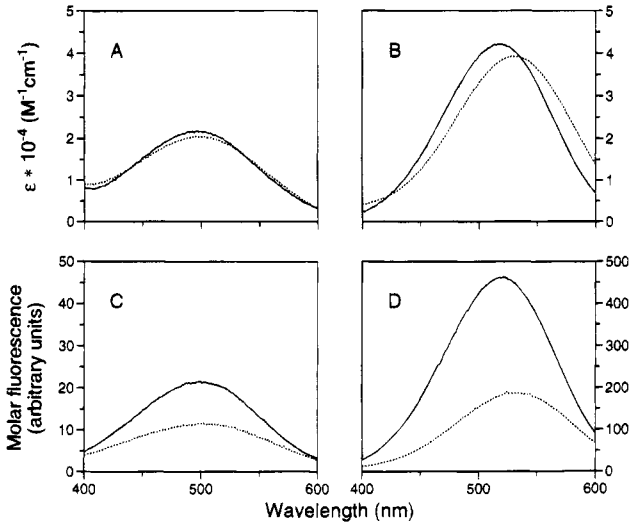

FIGURE 9: Absorbance (panels A and B) and fluorescence excitation (panels C and D) spectra of RH160 (continuous line) and RH795 (dotted line) in different solvents: (A, C) in water; (B, D) in ethanol. In panels C and D total emission >630 nm was recorded.

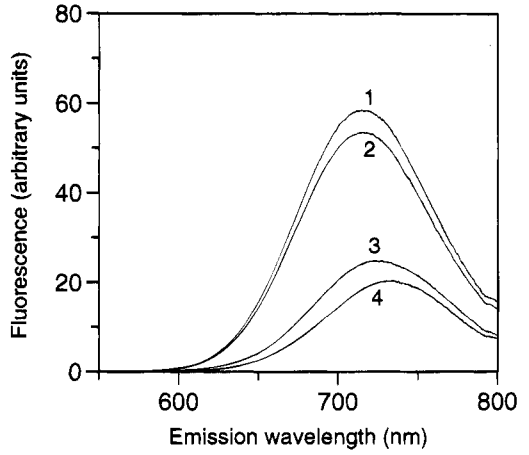

FIGURE 10: Corrected fluorescence emission spectra of RH421 (1), RH160 (2), RH795 (3), and RH414 (4) in ethanol. The dyes fluorescence was excited at wavelengths of their respective absorption maxima: 515 nm for RH421, 518 nm for RH160, 530 nm for RH795, and 531 nm for RH414.

Table 1: Fluorescence Lifetimes, τ, and Quantum Yields, Q, of RH Dyes in Ethanol: Calculated Parameters for the Depopulation of the Excited State of Chromophore, $k_{emission} = Q/\tau$ and $k_{decay} = 1/\tau - k_{emission}$

dye	excitation wavelength (nm)	τ (ns)[a]	Q	$k_{emission}$ (ns^{-1})	k_{decay} (ns^{-1})
RH421	515	0.406 ± 0.050	0.32	0.788	1.68
RH160	518	0.466 ± 0.054	0.29	0.622	1.52
RH795	530	0.193 ± 0.070	0.13	0.674	4.51
RH414	531	0.251 ± 0.064	0.11	0.438	3.55

[a] Emission wavelengths were 704 nm for RH421, 705 nm for RH160, 712 nm for RH795, and 714 nm for RH414.

structure. In addition, the data for RH160 and RH795 indicated that the charge of the dye molecule affected the rate of radiationless decay. Thus, in summary, the net charge of RH dyes affected both the position of the electronic absorption band and modified the quantum yield of fluorescence.

Acid–Base Properties. Analysis of the acid–base properties of the dyes confirmed that the absorbance in the measured area of the spectrum was due to the presence of the lone-pair electrons on the aniline nitrogen since a protonation of the aniline nitrogen with pK = 4.9 [see also Clarke et al. (1992)] led to the disappearance of the characteristic absorption peak for both RH160 and RH795 (not shown).

Theoretical Model Calculations. A model analysis of the various RH dyes provided further insight into the electron distribution and molecular orbital structure of the chosen dyes. Through such an analysis and by varying the net charge of the molecule, we could predict the influence of the charge of the hydrophilic moiety on the electronic structure of the chromophore.

The properties of the chromophores in RH160 and RH795 revealed by the extended Hückel molecular orbital method were similar to those, described by Loew et al. (1978) for the di-5-ASP chromophore. Since this method neglects electron–electron interactions, thus making the charge of the hydrophilic moiety unimportant, no differences between RH795 and RH160 were obtained (Table 2).

The more complex mixed mode INDO calculations revealed, however, differences between positive and zwitterionic dyes: the energy separation of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) in the chromophore was smaller for the positive dye than for the zwitterionic dye. Since this energy gap between electronic levels determines the position of the absorption band in the spectrum, the calculations predicted a slight red-shift of the absorbance maximum for RH795 compared to the absorption maximum of RH160, in agreement with the experimental results in Figure 9. Simulating a change in the net charge of both dyes to +1 by changing the charge of their hydrophilic moiety, in RH160 by protonation of the SO_3^- group, and in RH795 by changing the quaternary ammonium to an unprotonated tertiary amine (by removing one of methyl groups, see Figure 1), diminished the difference in their absorbance spectra. For RH160 a change to one positive net charge reduced the energy of electronic excitation (Table 2), i.e., red-shifted its absorbance spectrum. Removal of one positive charge in RH795 led to an increase in the energy of excitation (Table 2), blue-shifting the absorbance spectrum. The AM1 method gave essentially the same results (not shown).

The conclusion drawn from the model calculations is that the charge of the hydrophilic headgroup by contributing to the total charge of the molecule affects the electronic structure of the chromophore and, therefore, the energy required for the excitation.

Protein–Dye Interaction in the Membrane. As shown above, the charge of the hydrophilic group affected the position of the absorption band in agreement with the observation that both in the bulk solvents (Figure 9), and in pure liposomes (Figure 11A) the excitation spectrum of RH795 was slightly red-shifted with respect to RH160. However, in lipid vesicles with reconstituted Na,K-ATPase the difference between the two spectra became less evident as the protein content increased (Figure 11B,C). In other words, the increase in the content of Na,K-ATPase diminished the influence of the charged hydrophilic headgroup on the optical properties of the chromophore causing the spectra to move closer to each other.

Table 2: Electronic Properties of the RH160 and RH795 Obtained by Quantum Mechanical Calculations

method	dye	net charge of the dye	charge on the pyridinium nitrogen	charge on the aniline nitrogen	energy of HOMO (eV)	energy of LUMO (eV)	ΔE (eV)
extended	RH795	+2	0.063	−0.636	−11.579	−9.970	1.609
Hückel	RH160	0	0.059	−0.636	−11.578	−9.970	1.608
INDO	RH795	+2	0.037	−0.131	−11.145	−3.977	7.168
	RH795[a]	+1	0.098	−0.126	−12.848	−4.164	8.684
	RH160	0	0.068	−0.127	−12.121	−3.184	8.937
	RH160[b]	+1	0.046	−0.126	−13.108	−4.689	8.419

[a] RH795 headgroup charge modified by substitution of quaternary ammonium with tertiary amine (by removal of methyl group). [b] RH160 headgroup charge modified by protonation of sulfonate group.

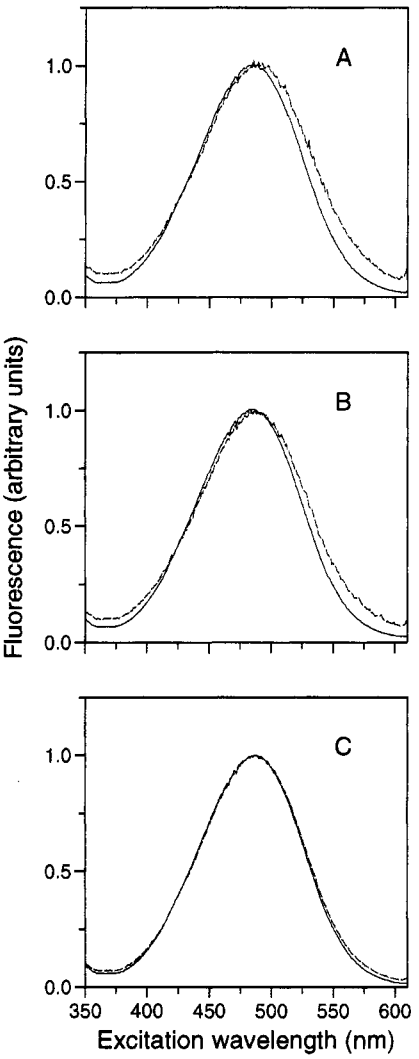

FIGURE 11: Normalized corrected excitation spectra of RH160 (continuous line) and RH795 (dotted line) in liposomes (A), in proteoliposomes with a low protein content, 1:20 protein/lipid ratio (B), in proteoliposomes with a high protein content, 1:5 protein/lipid ratio (C). The spectra were recorded with the dyes bound to the outer leaflet of the liposomes. Total emission >630 nm was recorded. Both external and internal buffer contained 30 mM histidine, pH 7.0, 1 mM NaCl, and 258 mM sucrose.

DISCUSSION

Styryl-based probes of RH-type were originally developed for fast optical recording of transmembrane potentials (Loew & Simpson, 1981; Grinvald et al., 1982, 1983; Loew, 1982). In broken, Na,K-ATPase enriched membranes, i.e., in the absence of transmembrane potential, the dyes respond to some molecular events in the Na,K-ATPase reaction cycle (Klodos & Forbush, 1988; Bühler et al., 1991; Läuger, 1991; Stürmer et al., 1991; Stürmer & Apell, 1992; Pratap & Robinson, 1993; Klodos, 1994). Since several of the molecular events reported by the dyes were assumed to be electrogenic, the response of the dyes has been interpreted always to result from changes in the local electric field in the membrane, e.g., associated with the binding and release of cations transported by the Na,K-ATPase.

Our present data show, however, that the fluorescence response to phosphorylation of Na,K-ATPase depends strongly on the structure of the probe. Within the group of zwitterionic dyes changes in the length of aliphatic chains (one additional methylene-group) caused an almost 2-fold increase in the size of the response (compare RH421 and RH160 in Figures 1 and 3). The major factor determining both the sensitivity and the spectral characteristics of the dyes response to phosphorylation appears, however, to be the net charge of the dye molecule. A significant nonelectrochromic component was only seen for the zwitterionic dyes, i.e., the dyes with negatively charged hydrophilic moiety. Zwitterionic dyes, like RH160, RH421, and di-4-ANEPPS, are several times more sensitive to phosphorylation of Na,K-ATPase than positively charged dyes, RH795, RH414, and RH461.

In contrast, no net charge-dependent alterations in voltage sensitivity was observed in the RH dyes response to transmembrane potential in model membranes or in experiments with various cell types (Loew & Simpson, 1981; Grinvald et al., 1982; present work). It appears therefore necessary to consider whether the observed increased sensitivity of the zwitterionic dyes to the enzyme phosphorylation could be due to some other phenomena than a putative charge reorganization in the membrane dielectric.

Both the characterization of the individual dyes in bulk solvents and the theoretical model calculations indicate that changes of the net charge of the molecule can affect the quantum yield of fluorescence and change the position of the absorption maximum. The charge of the hydrophilic moiety even when bound to the same chromophore, as in RH160 and RH795, affects the ratio between the emission rate and the rate of radiationless decay thus changing the fraction of fluorophores which decay through emission, i.e., it affects the quantum yield (Table 1). The position of the absorption maximum is also affected by the net charge of the dyes as shown experimentally both with the dye in different bulk solvents (Figure 9), in liposomes (Figure 11A), and through theoretical model calculations (Table 2). According to the model calculations the charge of the hydrophilic headgroup affects the energy gap between the highest occupied (HOMO) and the lowest unoccupied (LUMO) molecular orbitals, i.e., the position of the absorption maximum. Although there is no absolute agreement between

the experimentally determined positions of the absorption maxima and those obtained from model calculations, probably because the model calculations are performed for an isolated molecule *in vacuo*, the general tendency is the same: the absorbance spectrum of the positively charged RH795 is red-shifted compared to the zwitterionic RH160. The difference in the position of the excitation maxima of RH795 and RH160 observed with liposomes or bulk solvents disappears, however, when the dyes are bound to Na,K-ATPase membranes or proteoliposomes with a high content of incorporated Na,K-ATPase (Figure 11B,C). This effect of Na,K-ATPase on the spectral characteristics of RH dyes indicates that the charge of the hydrophilic headgroup could either be involved in some interaction with the protein or be screened by the protein-containing membrane environment.

The fluorescence emission spectra of RH dyes bound to Na,K-ATPase enriched membranes show dependence on the excitation wavelength (not shown) in agreement with Bühler et al. (1991) and Clarke et al. (1992). Such a dependence implies the existence of different populations of the dye. Due to a heterogeneity of fluorophore−environment interactions, existing in the time scale of the fluorescence lifetime, different dyes molecules are excited at different excitation wavelengths (Lakowicz & Keating-Nakamoto, 1984; Chattopadhyay & Mukherjee, 1993). The response of the zwitterionic dyes to phosphorylation of Na,K-ATPase, involving increase in the quantum yield, is most pronounced in the red-edge part of the excitation spectrum. In other words the phosphorylation appears to be reported preferentially by a fraction of dye molecules with a specific type of interaction with the surroundings. The simplest explanation for such a selective response is that the responding dye molecules interact with the enzyme in the membrane, i.e., they are localized in close proximity of the enzyme. A similar suggestion based on the spectral analysis and lifetime measurements of RH421 fluorescence was put forward by Schwappach et al. (1991). It is thus conceivable that (1) the hydrophilic moiety of the RH dyes participates in an enzyme−dye interaction, and (2) the interaction results in a change of the effective electric charge of the hydrophilic headgroup. Such a change in the effect of the charge of the hydrophilic headgroup could be a result of formation/dissociation of a dye−enzyme complex (R. J. Clarke, personal communication) or of a screening/exposure of the charge on the dye. Phosphorylation of the Na,K-ATPase and subsequent conformational transition $E_1P \leftrightarrows E_2P$ involves a significant reorganization of the protein structure (Jørgensen & Karlish, 1980) and may well change the properties of the immediate surrounding of the dye. As discussed above, a change of the charge of the hydrophilic moiety affects the fluorescence quantum yield and results in a shift of the absorption maximum.

All the above mentioned findings and assumptions suggest to us that a comprehensive theory for the mechanism of the RH dye response to the Na,K-ATPase reactions should include the following, illustrated here for the response of zwitterionic dyes to the phosphorylation: (1) the charge of hydrophilic headgroup of the dye is shielded when the Na,K-ATPase is unphosphorylated (the net charge of the dye approaches +1), and (2) phosphorylation leads to an exposure of the charge of the hydrophilic moiety (changing the net charge of the dye to about 0). In other words, the fluorescence response of zwitterionic dyes to the phosphorylation of the enzyme is caused mainly by an increase of the quantum yield resulting from an exposure of the charge of the hydrophilic moiety induced by a conformational change of the Na,K-ATPase.

Our suggestion, that the RH dyes may respond to conformational changes (cf. Nagel et al., 1991) in addition to responding to changes of the electrostatic potential in the membrane dielectric, simplifies the explanation for some of the RH responses to specific steps in the enzyme reaction as discussed above.

The fluorescence response to phosphorylation of Na,K-ATPase from P_i was always lower, about half of that elicited by phosphorylation from ATP. That is true for both shark and pig kidney Na,K-ATPase. For shark enzyme this difference in fluorescence responses was parallelled by differences in the levels of phosphoenzyme, $E^{32}P$, measured under identical conditions: the $E^{32}P$ level obtained in the presence of P_i was about half of that obtained from ATP. For kidney enzyme, however, the measured $E^{32}P$ levels were identical, amounting to 2.3 nmol/mg for both P_i- and ATP-phosphorylation, despite the difference in fluorescence responses. The simplest explanation in this case appears to be that different subconformations are formed by the two different routes of phosphorylation by the two enzymes.

When explaining ouabain binding experiments, Stürmer and Apell (1992) adhere to the hypothesis that changes in the electrostatic field are the main, if not the only, events reported by RH dyes. Accordingly, they interpret the decrease in fluorescence induced by ouabain binding after phosphorylation from ATP as a rebinding of two Na^+ to E_2P, i.e., an import of positive charges. In other words, they suggest, in agreement with the proposal of Lee and Fortes (1985), that ouabain binding to the phosphorylated enzyme stabilizes enzyme species with two Na^+ bound. Schwappach et al. (1994) observed the same fluorescence levels of membrane-bound RH421 when ouabain was bound to the enzyme phosphorylated from ATP in the presence of Na^+ as when it was bound to the enzyme phosphorylated from P_i in the absence of Na^+ or when it was bound to dephosphoenzyme (no Na^+). Applying the same hypothesis as Stürmer and Apell (1992) they ascribed the same charge to all three ouabain-enzyme complexes and suggested that all three complexes contain two bound cations: (1) an E_2P-(Na_2)−ouabain complex formed after the phosphorylation from ATP, (2) an $E_2P(H_2)$−ouabain complex formed after phosphorylation from P_i, and (3) an E_2H_2−ouabain complex formed after ouabain binding to dephosphoenzyme.

However, two observations reported in the present work contradict the above hypothesis and interpretation of the fluorescence signals. First of all, if the formation of ouabain−dephosphoenzyme complex was accompanied by an import of positive charges this charge should be reported by all RH dyes which is clearly not the case since only the zwitterionic RH160 responded (Figure 4). Secondly, even for the zwitterionic RH160, which is very similar to RH421 used by Schwappach et al. (1994), the spectral characteristics of two ouabain−enzyme complexes, E_2−O and E_2P−O, were different and the identical fluorescence level observed for these two complexes upon excitation at 580 nm is only coincidental (Figure 6).

The response of the styryl dyes to ouabain binding apparently cannot be explained exclusively by a change of

the electric field within the membrane dielectric and is most simply explained as resulting from a change in enzyme conformation induced by the ouabain binding itself. The differences in the fluorescence levels attained by the different ouabain bound forms could indicate different enzyme–ouabain complex subconformations (Askari et al., 1988).

To conclude, the present investigation does not rule out the possibility that fluorescence responses to some events in the Na,K-ATPase cycle are elicited by changes in the local electric field induced by cation binding or release but points to participation of additional mechanisms of the response. In case of Na,K-ATPase the responses to phosphorylation and ouabain binding depend on the charge of the hydrophilic headgroup of the dye. This could indicate that changes in the electrostatic interactions or in solvent screening caused by conformational transition of the enzyme could induce the fluorescence change and be the reason for the different sensitivity of the dyes to the same kinetic event in Na,K-ATPase reaction.

ACKNOWLEDGMENT

Hanne Zakarias, Angielina Damgaard, and Jesper M. Kielsgaard are gratefully acknowledged for their excellent technical assistance. We thank Dr. Bliss Forbush III, with whom the studies of RH dyes began, and Dr. C. Gutiérrez-Merino for the help with the measurements of fluorescence lifetimes and quantum yields. Stimulating discussions with Dr. J. G. Nørby are gratefully acknowledged.

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BI9520693