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Spectroscopic investigations of the potential-sensitive membrane probe RH421

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The absorbance spectra, fluorescence emission and excitation spectra, and fluorescence anisotropy of the potential-sensitive styryl dye RH421 have been investigated in aqueous solution and bound to the lipid membrane. The potential-sensitive response of the dye has been studied using a preparation of membrane fragments containing a high density of Na^+,K^+-ATPase molecules. In aqueous solution the dye is sensitive both to changes in pH and ionic strength. Evidence has been found that the dye readily aggregates in aqueous solution. Aggregation is enhanced by an increase in ionic strength. The aggregates formed display a low fluorescence intensity. At high pH values (above approx. 8) changes in the dye's fluorescence spectra are observed, which may be due to a reaction of the dye with hydroxide ions. When bound to the membrane the dye also exhibits concentration-dependent fluorescence changes. The potential-sensitive response of the dye in Na^+,K^+-ATPase membrane fragments after addition of MgATP in the presence of Na^+ ions cannot be explained by a purely electrochromic mechanism. The results are consistent with either a potential-dependent equilibrium between membrane-bound dye monomers and membrane-bound dimers, similar to that previously proposed for the dye merocyanine 540, or with a field-induced structural change of the membrane.

Introduction

In recent years numerous potential-sensitive dyes have been developed in order to optically measure electrical activity in cells, cell organelles and synthetic membrane preparations [1–7]. These dyes have been found to respond to changes in membrane potential via a variety of different mechanisms, with response times ranging from microseconds to seconds. In some cases it has been found that the same dye displays different voltage sensitivity in different types of membrane preparation. In the hope of finding a dye with a rapid response which is independent of the specific membrane environment Loew and co-workers [7–12] and Grinvald and co-workers [13–16] have been involved in the synthesis of fluorescent styryl dyes, which it has been suggested may respond to changes in membrane potential via an electrochromic mechanism. The basis of an electrochromic mechanism is that the dye undergoes a significant change in its electronic distribution upon absorption of a photon, so that the molecule has significantly different dipole moments in its ground and excited states. An electrical potential gradient across the membrane will result in an electric field, which, if the dye molecule has a fixed orientation within the membrane, will cause different degrees of stabilisation or destabilisation of the ground and excited states, resulting in a shift of the absorption spectrum similar to the effects often observed upon changing the solvent. An important advantage of an electrochromic mechanism would be its rapid response time, being limited only by the time necessary to absorb a photon. Thus, subnanosecond response times could be expected, which would allow rapid kinetic measurements of electrical activity due to ion pumps and channels to be performed.

Up to now fluorescent styryl dyes have been applied to the optical recording of cell membrane potential changes [16], cardiac action potential [17], action and synaptic potentials from neuronal cultures [18], the photogenerated membrane potential due to bacteriorhodopsin [19], electrical activity in goldfish optic tectum [20], and conformational changes and charge translocation of the Na^+,K^+-ATPase [21–23]. However, in spite of these applications of the dyes their physicochemical and photophysical properties are not completely understood and the mechanism of their potential-sensitive response is still unclear. In the pre-

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sent paper we have investigated the styryl dye RH421, which has previously been found to show a particularly large potential-sensitive response [12,15]. The structure of the dye is shown in Fig. 1. Bühler et al. [22] have suggested that in membrane fragments the dye responds primarily via an electrochromic mechanism, whereas Fluhler et al. [12] have stated that its response in neuroblastoma cells is "clearly too large to be attributable to electrochromism". Here we report a spectroscopic study of the dye in aqueous solution and bound to membrane fragments. Although these two environments would appear to be quite dissimilar, it has been found that the absorption spectrum of the dye when bound to the membrane is very similar to that observed in aqueous solution [24]. Much greater absorbance shifts are observed on transfer of the dye to ethanol or other organic solvents of lower dielectric constant. Thus, it is hoped that by investigation of the spectroscopic behaviour of the dye in aqueous solution and comparison with results obtained in the membrane, information may be gained concerning the possible mechanism of the dye's potential-sensitive response.

Materials and Methods

N-(4-Sulphobutyl)-4-(4-(*p*-dipentylaminophenyl)-butadienyl) pyridinium inner salt (RH421) was obtained from Molecular Probes (Eugene, OR) and was used without further purification. A series of stock solutions of the dye were prepared in ethanol. For spectral measurements 5 μl of an ethanolic dye solution was added by weight to a 0.5 cm pathlength quartz cuvette containing 1 ml of aqueous solvent. The final solutions measured thus contained a small and constant percentage of 0.5% ethanol. Values of the experimental errors of the concentrations have been calculated according to the accuracy of the balance.

Membrane fragments containing Na^+,K^+-ATPase were a gift of Dr. Hans-Jürgen Apell, Universität Konstanz. Their preparation and purification has been described elsewhere [22]. The open fragments have a radius of 0.2–1 μm and a high density (10^3–10^4 per μm^2) of oriented Na^+,K^+-ATPase molecules. The protein concentration in the membrane suspension was normally about 3 mg/ml. The specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay [25]. For all preparations used the specific activity in the presence of Na^+ and K^+ ions was in the range 1500–2200 μmol P_i/h per mg protein at 37°C. The phospholipid content was on average 0.82 mg per mg protein. The protein and lipid concentrations were determined in the laboratory of Dr. Apell. The assay procedures have been described previously [22]. The spectroscopic measurements were performed in the absence of K^+ ions. Under these conditions the enzyme has a very low dephosphorylation rate [26], so that on the timescale of the measurements there is negligible consumption of ATP.

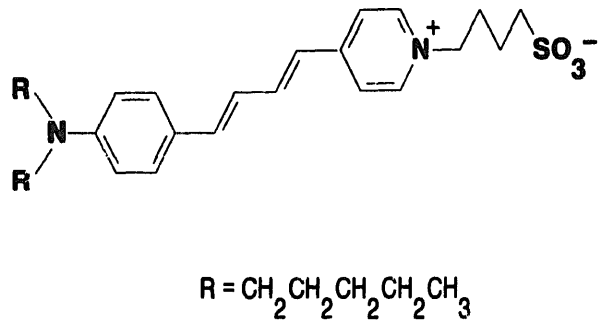

Fig. 1. Structure of RH421.

Dioleoylphosphatidylcholine (DOPC) was obtained from Avanti Polar Lipids (Alabaster, AL) and sodium cholate from Serva (Heidelberg). DOPC unilamellar vesicles were prepared according to a previously described detergent dialysis method [27,28], which produces vesicles with external diameters in the range 60–90 nm, as determined by cryoelectronmicroscopy. Dialysis tubing was purchased from Medicell International (London, UK). The phospholipid content of the vesicle suspensions was determined by the phospholipid B test from Wako (Neuss, Germany).

Measurements with the membrane fragments were performed in a buffer containing 30 mM imidazole, 5 mM $MgCl_2$ and 1 mM EDTA. The pH of the buffer was adjusted to pH 7.2 with HCl. All solutions were prepared using triply distilled water. The origins of the various reagents used were as follows: imidazole (99 + %, Sigma), $MgCl_2 \cdot 6H_2O$ (analytical grade, Merck), EDTA (99%, Sigma), NaCl (Suprapur, Merck), sodium acetate trihydrate (99 + %, Sigma), acetic acid (2.0 N solution, Sigma), ATP magnesium salt (approx. 95%, Sigma), NaOH (analytical grade, Merck), HCl (0.1 M Titrisol solution, Merck), choline chloride (3 × crystallized, Sigma) and ethanol (analytical grade, Merck).

Absorbance measurements were performed with a Shimadzu UV-2100 u.v.-visible recording spectrophotometer using a bandwidth of 5 nm. Fluorescence excitation and emission spectra as well as fluorescence anisotropy measurements were performed with a Shimadzu RF-5000 recording spectrofluorophotometer using bandwidths of 5 nm for both the excitation and emission monochromators. In order to minimise contributions from scattering of the exciting light and higher order wavelengths, glass cut-off filters from Schott (Mainz, Germany) were used in front of the excitation or emission monochromators where appropriate. For fluorescence anisotropy measurements visible linear film polarizers from Oriel (Darmstadt, Germany) were

employed. The anisotropy, r, was calculated according to the following equation [29],

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} is the fluorescence intensity (arbitrary units) when both excitation and emission polarizers are oriented so as to allow the passage of vertically polarized light, I_{VH} is the fluorescence intensity (arbitrary units) when the excitation and emission polarizers are oriented so as to allow the passage of vertically and horizontally polarized light, respectively, and G is a correction factor which is dependent on the emission wavelength and allows for the relative sensitivity of the emission monochromator to vertically and horizontally polarized light. The emission monochromator was calibrated and the G factor calculated over the wavelength range 560–820 nm by two different techniques. Firstly, different coloured light emitting diodes were placed in the cuvette holder and used as sources of unpolarized light. The light intensity was then measured with the emission polarizer in the vertical and horizontal positions, and the value of G was calculated from I_V/I_H. Secondly, the fluorescence of a solution of RH421 was measured using horizontally polarized exciting light and the emission polarizer in both vertical and horizontal orientations. The value of G was then calculated from I_{HV}/I_{HH}. Both methods gave identical values within experimental error. All spectroscopic measurements were recorded at a constant temperature of 20°C using thermostatically controlled cuvette holders.

Measurements of the pH dependence of the dye absorbance were carried out using a series of 0.1 M acetate buffers of varying pH [30]. For measurements in the presence of DOPC vesicles 1.0 M NaCl was added to the dialysis medium and the acetate buffers in order to prevent variations in the ionic strength on either side of the membrane. The determination of pK_a values was carried out by fitting the absorbance data obtained from the pH titrations to the Henderson-Hasselbalch equation [31] using the commercially available non-linear program ENZFITTER. The program was purchased from Biosoft (Cambridge, UK) and was run on a IBM-AT/386 compatible personal computer (mey-Soft, Berlin, Germany).

Theory

Let us consider the change in fluorescence which may be observed due to a change in electric field strength. If the solution is excited at a fixed wavelength, λ_{ex}, and the fluorescence emission is observed at λ_{em}, it can be shown [32,33] that the fluorescence intensities before, F_0, and after, $F_0 + \Delta F$, the change in electric field strength are given by

$$F_0 = f \cdot I_0 \cdot (1 - 10^{-A_0}) \cdot p_0 \cdot q_0 \quad (2)$$

$$F_0 + \Delta F = f \cdot I_0 \cdot (1 - 10^{-A_0 + \Delta A})(p_0 + \Delta p)(q_0 + \Delta q) \quad (3)$$

where f is the fraction of fluorescence light collected by the photomultiplier, A_0, p_0, and q_0 are the initial values of the absorbance, the fraction of the total emission occurring at λ_{em} and the quantum yield, respectively, and ΔA, Δp, and Δq are the corresponding changes after the electric field strength change. Dividing Eqn. 3 by Eqn. 2 and rearranging yields that the relative fluorescence change is given by

$$\frac{\Delta F}{F_0} = \frac{1 - 10^{-\Delta A}}{10^{-A_0} - 1} + \frac{\Delta p}{p_0}\left(\frac{10^{-A_0} - 10^{-\Delta A}}{10^{-A_0} - 1}\right) + \frac{\Delta q}{q_0}\left(\frac{10^{-A_0} - 10^{-\Delta A}}{10^{-A_0} - 1}\right) + \frac{\Delta p}{p_0} \cdot \frac{\Delta q}{q_0}\left(\frac{10^{-A_0} - 10^{-\Delta A}}{10^{-A_0} - 1}\right) \quad (4)$$

This equation allows one to estimate which photophysical process may be largely responsible for the observed potential-sensitive response. For example, if the observed fluorescence change is totally due to a shift in the absorbance spectrum, as would be expected for a purely electrochromic mechanism, then Δp and Δq would equal zero, and Eqn. 4 would reduce to

$$\frac{\Delta F}{F_0} = \frac{1 - 10^{-\Delta A}}{10^{-A_0} - 1} \quad (5)$$

If one ignores possible changes in the quantity p, it can be shown that for small absorbance values Eqn. 4 reduces to an equation previously derived by Bühler et al. (see Eqn. 5 in Ref. 22).

Results

Acid-base properties of RH421

On decreasing the pH of an aqueous solution of RH421 there is a dramatic decrease in absorbance of the band centred at approx. 480 nm and a new peak appears at approx. 370 nm (see Fig. 2). These changes are presumably due to protonation of either the anilino or the amino nitrogen of the dye. The variation of the molar absorptivity of the dye at 500 nm with pH is shown in Fig. 3. The data have been fitted to the Henderson-Hasselbalch equation as described in the Materials and Methods section and a pK_a value of 4.9 (±0.1) has been obtained.

A similar titration has been performed in the presence of 32 μg/ml ([lipid] = 41 μM) of unilamellar DOPC vesicles (see Fig. 3). Fitting the data as in the case of the aqueous dye solution, results in an apparent pK_a value of 4.1 (±0.1). The lower value of the pK_a obtained in the presence of vesicles can be explained

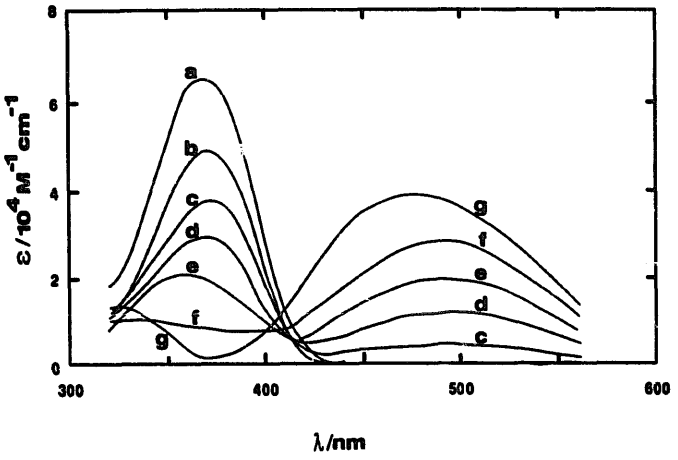

Fig. 2. Variation of the RH421 absorbance spectrum in aqueous solution as a function of pH; [RH421] = 650 nM, bandwidth = 5 nm, T = 20°C. The spectra were recorded in 0.1 M acetic acid/sodium acetate buffers of varying pH. The curves refer to the following pH values: (a) 1.73, (b) 3.05, (c) 4.01, (d) 4.63, (e) 5.02, (f) 5.61 and (g) 6.57.

by binding of the dye to the lipid membrane, which is likely to hinder contact of the dye with H^+ ions in the aqueous solution. A similar value of the apparent pK_a has also been found for the related styryl dye RH237 when bound to lipid vesicles [34].

At high pH values (above approx. 8) in aqueous solution the dye undergoes a slow reaction over minutes to hours, which is characterised by a large increase in fluorescence intensity. As the pH is increased the rate of the reaction increases. A likely explanation is that this is due to the attack of hydroxide ions on the pyridinium moiety of the dye, resulting initially in the formation of a pseudo-base [35]. A variety of subsequent reaction paths are possible.

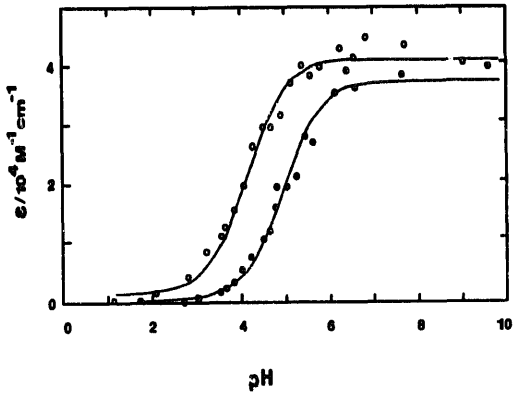

Fig. 3. Variation of the molar absorptivity, ϵ, of RH421 at 500 nm with pH; [RH421] = 650 nM, bandwidth = 5 nm, T = 20°C. The filled data points (●) refer to a titration in 0.1 M acetic acid/sodium acetate buffer solutions. The open data points (○) refer to a similar titration but with the addition of 41 μM of dioleoylphosphatidylcholine in the form of unilamellar vesicles and 1.0 M NaCl. The solid curves represent non-linear fits of the data to the Henderson-Hasselbalch equation.

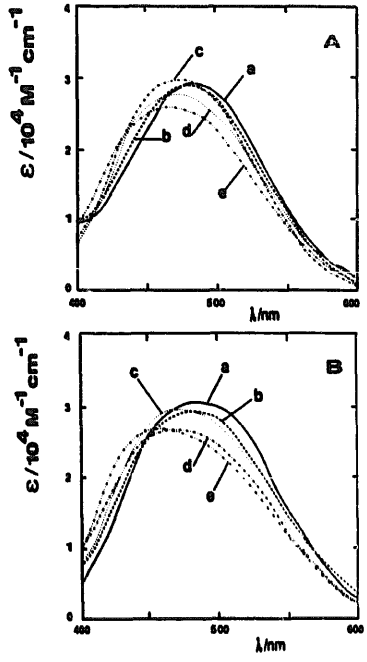

Fig. 4. (A) Variation of the RH421 absorbance spectrum in aqueous solution as a function of the NaCl concentration; [RH421] = 620 nM, bandwidth = 5 nm, T = 20°C. The curves refer to the following NaCl concentrations: (a) in water alone, (b) 0.25 M, (c) 0.75 M, (d) 1.25 M and (e) 2.0 M. (B) Variation of the RH421 absorbance spectrum in aqueous solution as a function of the dye concentration; [NaCl] = 2.0 M, bandwidth = 5 nm, T = 20°C. The curves refer to the following RH421 concentrations: (a) 74 nM, (b) 148 nM, (c) 247 nM, (d) 401 nM and (e) 722 nM.

Ionic strength and concentration effects on the spectral properties of RH421 in aqueous solution

If the salt concentration is increased at constant dye concentration the dye undergoes a blue shift of its absorbance maximum from approx. 495 nm in water to approx. 470 nm in 2.0 M NaCl solution (see Fig. 4A). At the same time there is an overall decrease in absorbance. Very similar effects are observed on increasing the dye concentration at constant salt concentration (see Fig. 4B). Thus, the spectral changes can be confidently attributed to dye aggregation. The lack of a true isosbestic point suggests that the aggregation proceeds to higher aggregates than just a dimer. The effect of salt can be easily explained by a facilitation of the aggregation due to a screening of the electrostatic repulsion between the charges of the dye molecules. Similar effects due to aggregation have been previously reported [36–39] for cyanine dyes, which have a related structure.

The effects of aggregation on dye fluorescence are much more dramatic than in the case of absorbance. The effect of varying the dye concentration on the fluorescence emission spectrum in water is shown in Fig. 5. The position of the fluorescence maximum shifts from approx. 655 nm at 10.7 nM to approx. 700 nm at 599 nM and there is a large decrease in fluorescence

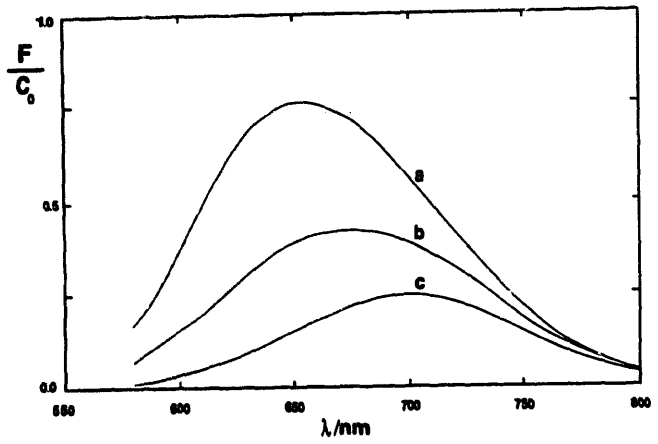

Fig. 5. Variation of the RH421 fluorescence emission spectrum in aqueous solution as a function of the dye concentration; $\lambda_{ex} = 510$ nm (+GG475 cut-off filter), bandwidths = 5 nm, $T = 20°C$. F/c_0 represents the molar fluorescence in arbitrary units M^{-1}. The curves refer to the following RH421 concentrations: (a) 11 nM, (b) 100 nM and (c) 599 nM.

intensity. As would be expected, an increase in salt concentration also produces a decrease in fluorescence intensity. In Fig. 6 the effects of transferring dye from a 2 M NaCl solution into water on the absorbance and fluorescence excitation spectra are shown. Very similar effects are observed if NaCl is replaced by KCl, CsCl or NaBr. It can be seen that the observed fluorescence change cannot be accounted for by the change in absorbance alone. In fact at low excitation wavelengths

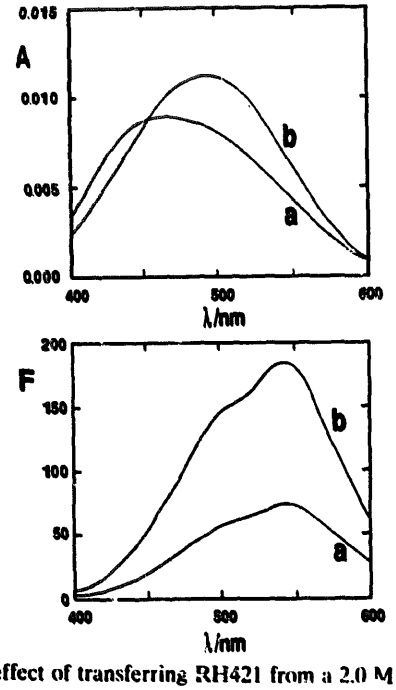

Fig. 6. The effect of transferring RH421 from a 2.0 M NaCl solution (curve a) to water (curve b) on the absorbance spectrum (above) and on the fluorescence excitation spectrum (below); [RH421] = 670 nM, bandwidths = 5 nm, $T = 20°C$. The fluorescence, F, (arbitrary units) was recorded at an emission wavelength of 680 nm (+RG665 cut-off filter).

the changes are in opposite directions. Thus, either a change in the degree of relaxation or a change in quantum yield must also make a significant contribution to the fluorescence change. It has been found, however, that at this dye concentration no significant change in the position of the fluorescence emission maximum is caused by the change in salt concentration. Thus, it appears that the major effect must be a change in quantum yield. The change is most dramatic at short wavelengths, where the contribution to the absorbance from dye aggregates is largest. It is also interesting to note in Fig. 6 that the maxima of the absorbance and fluorescence excitation spectra do not coincide. For example, in the case of dye in water the absorbance maximum occurs at approx. 490 nm, whereas the fluorescence excitation spectrum shows a maximum at 545 nm with a shoulder at 500 nm. Significant absorption occurs in the range 400–450 nm, although excitation in this range results in very little fluorescence intensity. The above observations indicate that the dye aggregates, whose absorbance spectrum is blue-shifted relative to that of dye monomers, have a much smaller quantum yield than that of the monomers. A greatly reduced quantum yield on aggregation has also been previously reported for certain cyanine dyes [36,37] in aqueous solution.

In order to estimate the dimerisation constant of RH421 the molar fluorescence of dye at 650 nm and an excitation wavelength of 510 nm has been measured as a function of the dye concentration (see Fig. 7). The absorbance at the emission wavelength of 650 nm never exceeded 0.002 over the entire concentration range measured. A decrease in fluorescence due to an inner filter effect can thus be excluded [40]. In the absence of higher aggregates the association constant between two dye molecules is equal to the reciprocal of the total dye concentration at which half the dye molecules are present in the monomer form and half in the dimer

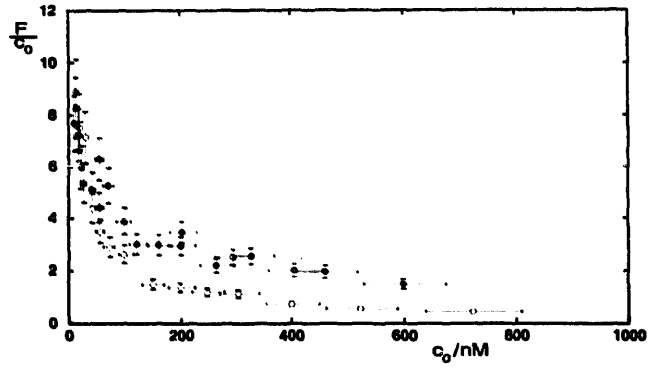

Fig. 7. Concentration dependence of the molar fluorescence, F/c_0, of RH421 in water (●) and in 2.0 M NaCl solution (○). The fluorescence (arbitrary units) was recorded at an emission wavelength of 650 nm and at an excitation wavelength of 510 nm (+GG475 cut-off filter); bandwidths = 5 nm, $T = 20°C$.

form. The results shown in Fig. 7 thus allow an estimate of the order of magnitude of the association constant from the dye concentration at which the molar fluorescence has decreased to half its infinite dilution value, i.e., 25–100 nM. This corresponds to a dye-dye association constant, K, of $1 \cdot 10^7$–$4 \cdot 10^7$ M^{-1}. The possibility that the decrease in fluorescence may be due to dynamic quenching via the collision between excited and ground state molecules should be considered. Assuming a fluorescence lifetime, τ_0, of 1 ns, it can be shown [41] from the definition of the Stern-Volmer quenching constant, $K = k_q \cdot \tau_0$, that the collision rate constant, k_q, would have to exceed the diffusion-controlled limit by a factor of approx. 10^6. Accordingly, the concentration dependent decrease in dye fluorescence can only be explained by static quenching via the association of dye molecules in the ground state. In the presence of 2 M NaCl the decrease in molar fluorescence appears to begin at lower dye concentrations, suggesting an enhanced aggregation, presumably due to the screening of charge repulsion at the higher ionic strength.

Fluorescence and fluorescence anisotropy of RH421 in aqueous solution and bound to the lipid membrane

In order to determine whether or not the fluorescence of RH421 is due to a single electronic band, measurements of fluorescence anisotropy have been performed across the entire fluorescence excitation spectrum. The fluorescence anisotropy, r, of a 736 nM solution of RH421 in water was measured at an emission wavelength of 680 nm (+RG665 cut-off filter). A constant value of r of 0.235 (±0.010) was found across the range of excitation wavelengths 420–600 nm. It has also been found that the wavelength of the fluorescence emission maximum in aqueous solution is independent of the excitation wavelength. These results together suggest that a single electronic band is responsible for the dye's fluorescence in aqueous solution. The shoulder observed in the fluorescence excitation spectrum (see Fig. 6) is thus likely to be due to vibrational structure. At dye concentrations in the range 20–120 nM, however, a significant increase in the value of r is observed at higher excitation wavelengths. This could possibly be explained by the coexistence in this concentration range of significant proportions of dye monomer as well as aggregates, which are excited to varying degrees at different excitation wavelengths and have different fluorescence anisotropies.

The fluorescence anisotropy of dye in the presence of Na^+,K^+-ATPase-containing membrane fragments was measured at an emission wavelength of 650 nm (see Fig. 8A). It has been found that there is an increase in fluorescence anisotropy as one proceeds to longer excitation wavelengths. The wavelength of the fluorescence emission maximum has also been found to

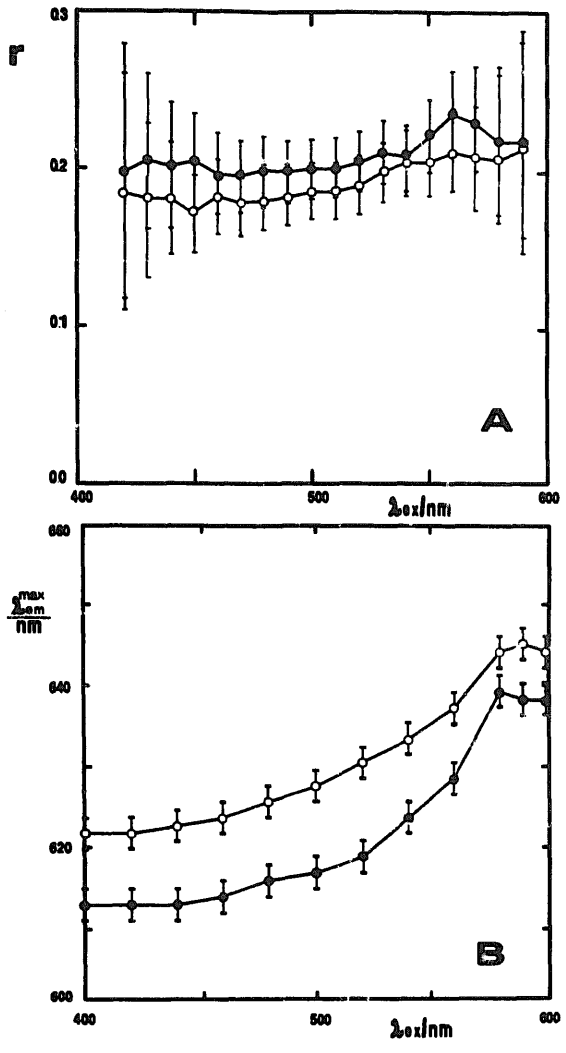

Fig. 8. (A) Fluorescence anisotropy, r, as a function of the excitation wavelength of a 637 nM solution of RH421 in the presence of 40 μg/ml of Na^+,K^+-ATPase in the form of membrane fragments; (●) alone and (○) after the addition of 0.5 mM of MgATP. The fluorescence (arbitrary units) was recorded at an emission wavelength of 650 nm (+RG645 cut-off filter); bandwidths = 5 nm, [NaCl] = 50 mM, T = 20°C. (B) Variation of the wavelength of the RH421 fluorescence emission maximum, λ_{em}^{max}, as a function of the excitation wavelength, λ_{ex}, in the presence of 40 μg/ml of Na^+,K^+-ATPase in the form of membrane fragments; (●) alone and (○) after the addition of 0.5 mM of MgATP; [RH421] = 637 nM, [NaCl] = 50 mM, bandwidths = 5 nm, T = 20°C.

depend on the excitation wavelength (see Fig. 8B). Excitation at the blue edge of the fluorescence excitation spectrum results in a maximum emission wavelength of 612 nm, whereas excitation at the red edge of the excitation spectrum causes a shift to 638 nm. Bühler et al. [22] have previously reported the same effect, which they suggested may be due to a red-edge-excitation red-shift of the emission spectrum of individual dye molecules due to a semi-rigid environment of bound dye. Such an effect has been reported by Demchenko and Shcherbatska [42] for the fluorescent probes ANS and TNS in glycerol solution at low temperatures, but

when bound to phosphatidylcholine vesicles at 20°C the effect disappeared. An alternative explanation for the excitation wavelength dependence of the emission maximum as well as the variation in the fluorescence anisotropy is that the dye exists in two different environments within the membrane. If both dye species have significant quantum yields and different fluorescence polarisations or lifetimes, both effects would be expected, since variation of the excitation wavelength would cause preferential excitation of one species or the other. Two different fluorescent dye species could come about by a distribution between different sites or orientations within the membrane or by an equilibrium between bound dye monomers and bound aggregates.

In order to investigate the possibility of dye aggregation within the lipid membrane the fluorescence excitation spectrum of RH421 in Na^+,K^+-ATPase-containing membrane fragments has been recorded over a range of different dye concentrations (see Fig. 9). Significant changes in the shape of the spectrum are observed on varying the dye concentration. At high dye concentrations a maximum occurs at approx. 500 nm with a shoulder at approx. 540 nm. As the dye concentration decreases the peak at 500 nm decreases in intensity and the shoulder at 540 nm grows. At the lowest dye concentration the spectrum approaches that observed for dye monomers in aqueous solution (cf. Fig. 6). The changes in the excitation spectrum on varying the dye concentration suggest the presence of fluorescent dye aggregates bound to the membrane. As the dye concentration is decreased the monomer/dimer equilibrium would shift towards the monomer, resulting in increased fluorescence at longer excitation wavelengths. It is also interesting to note that the discrepancy between the positions of the maxima of the absorbance and fluorescence excitation spectra at the same dye concentration is much smaller when the dye is bound to the membrane than when it is in aqueous solution (see Fig. 10). This could be explained by a reduced tendency towards higher aggregation in the membrane as compared to aqueous solution or by a difference in the structure of the aggregates formed in the two environments, so that those formed in the membrane have a higher quantum yield. The formation of membrane-bound dye aggregates has previously been reported for potential-sensitive cyanine [37–39,43,44] and merocyanine [4,45–49] dyes. For these dyes it has generally been found [4,37,43–49] that the membrane-bound dye aggregates have a very low quantum yield. In the case of RH421 the fact that concentration-dependent wavelength shifts of the excitation spectrum are observed means that, if they can be attributed to aggregation, the membrane-bound aggregates would have to be capable of fluorescing, although with a reduced intensity.

An alternative explanation for the results shown in Fig. 9, not involving aggregation, is that the dye causes a structural change in the membrane. As the dye concentration increases the membrane deformation

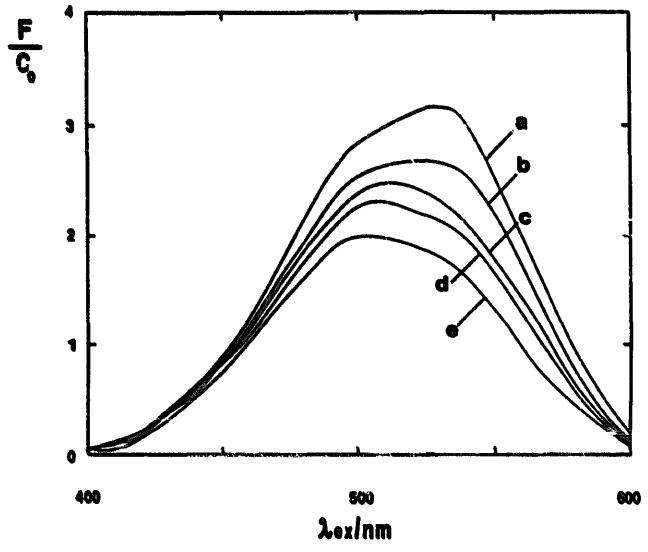

Fig. 9. Variation of the RH421 fluorescence excitation spectrum as a function of the dye concentration in the presence of 13.4 μg/ml of Na^+,K^+-ATPase in the form of membrane fragments; [NaCl] = 50 mM, λ_{em} = 650 nm (+RG645 cut-off filter), bandwidths = 5 nm, T = 20°C. The curves refer to the following RH421 concentrations: (a) 28 nM, (b) 73 nM, (c) 310 nM, (d) 561 nM and (e) 714 nM.

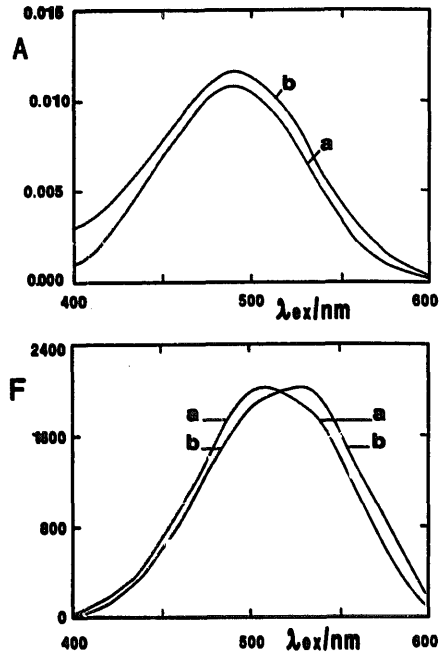

Fig. 10. The effect of adding 0.5 mM of MgATP to a solution of RH421 in the presence of 40 μg/ml of Na^+,K^+-ATPase in the form of membrane fragments on the absorbance spectrum (above) and the fluorescence excitation spectrum (below). In each case curve a represents the spectrum prior to the addition of MgATP and curve b represents the spectrum afterwards; [RH421] = 735 nM, [NaCl] = 50 mM, bandwidths = 5 nm, T = 20°C. The fluorescence (arbitrary units) was recorded at an emission wavelength of 650 nm (+RG645 cut-off filter).

may be such that the fluorescent properties of the bound dye are altered.

ATP-induced spectral changes of RH421 bound to Na^+,K^+-ATPase-containing membrane fragments

In order to study the potential-sensitive response of RH421 the absorbance and fluorescence changes on adding MgATP to a suspension of Na^+,K^+-ATPase-containing membrane fragments in the presence of 50 mM NaCl and 735 nM dye have been measured (see Fig. 10). The ionic strength was maintained at a constant value of 1.0 M by the addition of 950 mM of choline chloride. Prior to the addition of ATP and in the absence of K^+ ions the enzyme is in a conformation which has a high affinity for Na^+ ions [50–52]. Several authors [53–56] have reported apparent dissociation constants of the enzyme in this conformation for Na^+ ions in the range 5–8 mM. Thus, at a NaCl concentration of 50 mM a high proportion of the Na^+-binding sites of the enzyme would be occupied, which it has been suggested [22,23] produces a local electric field within the lipid matrix of the membrane adjacent to the enzyme. On addition of ATP the enzyme becomes phosphorylated and undergoes a conformational change during which the bound Na^+ ions are transported across the membrane [22,23,50–52]. In this new conformation the enzyme has a much lower affinity for Na^+ ions [50–52]. Widely varying values for the apparent dissociation constant of phosphorylated enzyme for Na^+ ions have been reported [55]. However, recent measurements of the ATP-induced fluorescence change of RH421 as a function of the Na^+ concentration suggest an apparent dissociation constant of the order of a few hundred millimolar [23], which is in agreement with kinetic measurements of Taniguchi and Post [57]. Therefore, at a Na^+ concentration of 50 mM the addition of ATP to the enzyme-containing membrane fragments would cause the dissociation of Na^+ ions from the enzyme. No changes in the overall transmembrane electrical potential difference can occur in these experiments since the open membrane fragments are short-circuited by the surrounding electrolyte solution. Therefore, it seems likely that the release of Na^+ ions from the enzyme can only cause a reduction in the electric field strength within the membrane. The absorbance and fluorescence responses of membrane bound dye molecules to these electrical events are shown in Fig. 10. Similar spectral changes have previously been observed for the same membrane preparation by Bühler et al. [22].

In order to determine whether an electrochromic mechanism can account for the fluorescence response, the absorbance and fluorescence changes have been compared using Eqn. 4 of the theory section (see Fig. 11). $\Delta F/F_0$ and $(1-10^{-\Delta A})/(10^{A_0}-1)$ have been plotted against the excitation wavelength. F_0 and A_0

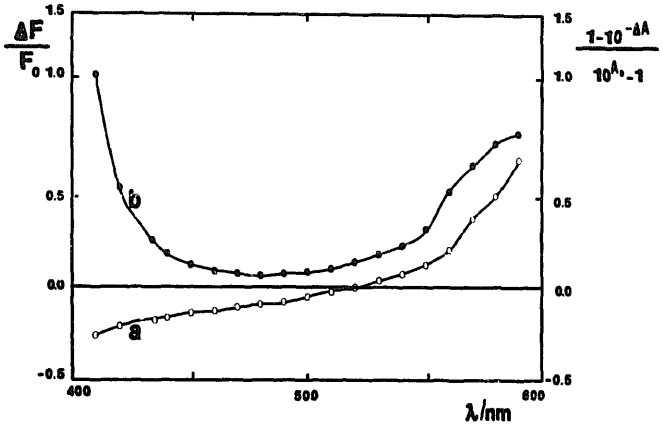

Fig. 11. Variation of (a) $\Delta F/F_0$ and (b) $(1-10^{-\Delta A})/(10^{A_0}-1)$ as a function of the excitation wavelength for RH421 in the presence of 40 μg/ml of Na^+,K^+-ATPase in the form of membrane fragments; [RH421] = 735 nM, [NaCl] = 50 mM, bandwidths = 5 nm, T = 20°C. The fluorescence (arbitrary units) was recorded at an emission wavelength of 650 nm (+RG645 cut-off filter). F_0 and A_0 refer to the values prior to the addition of 0.5 mM of MgATP, and ΔF and ΔA refer to the changes after the addition of MgATP.

refer to the values prior to the addition of ATP, and ΔF and ΔA refer to the changes after addition of ATP. It can be seen that the observed fluorescence change cannot be accounted for by the change in absorbance alone. At certain wavelengths the fluorescence change is positive and at other wavelengths it is negative, whereas the absorbance change is positive over the entire wavelength region. Therefore, the results are not consistent with a purely electrochromic mechanism. Significant changes in the quantum yield and/or the degree of relaxation must accompany the absorbance change. The fact that the fluorescence excitation spectrum shows a change in shape (see Fig. 10) is also by itself evidence against a purely electrochromic mechanism, since this would only predict a shift of the spectrum.

The addition of ATP also causes a decrease in the fluorescence anisotropy (see Fig. 9A) and a shift of the wavelength of the fluorescence emission maximum to longer wavelengths (see Fig. 9B). Both of these effects could be explained either by an increase in the fluorescence lifetime of the dye or by an increase in its rotational freedom within the membrane due to the decrease in electric field strength. However, in order to distinguish between these possibilities time-resolved fluorescence measurements are necessary.

If one compares the change in the fluorescence excitation spectrum of RH421 on addition of ATP (Fig. 10) with the changes caused by decreasing the dye concentration (Fig. 9), it can be seen that similar changes in the spectral shape are observed. In both cases there is an increase in the relative fluorescence intensity on excitation at 530 nm compared to excitation at 500 nm. If the spectral changes in Fig. 9 are

attributed to dye aggregation, it appears that the addition of ATP is consistent with the disaggregation of membrane-bound dimers and a consequent increase in the proportion of bound monomers. Such an effect of changes in electric field strength has previously been suggested [4,45–49,58] for the voltage-sensitive dye merocyanine 540. Alternatively, if the concentration-dependent fluorescence changes of the dye are due to a modification of the membrane structure, it may be that the electric field strength change also results in a structural change which is sensed by the dye. Field-induced changes in membrane viscosity have been reported by several authors [59,60].

Discussion

Experimental evidence based on absorbance and fluorescence titrations has been presented which suggest that the voltage-sensitive styryl dye RH421 readily aggregates in aqueous solution. The aggregates appear to have a low fluorescence intensity. In the membrane it has also been found that the observed fluorescence excitation spectrum is dependent on the dye concentration. Two possible explanations have been offered: (a) dye aggregation within the membrane, (b) dye-induced structural changes of the membrane. It is also possible that both might occur simultaneously. For example, the formation of a membrane-bound dimer by association of two membrane-bound monomers is likely to require a significant reorganisation of the lipid molecules as well.

By comparing the absorbance and fluorescence responses of the dye upon addition of ATP to Na^+,K^+-ATPase-containing membrane fragments it has been found that the voltage sensitivity of the dye cannot be explained by a purely electrochromic mechanism. Significant changes in the quantum yield and/or the degree of relaxation of the excited molecules must also occur. The spectral changes observed are not in complete agreement with those reported by Bühler et al. [22]. However, this could be explained by the different dye concentrations used, which, as shown in Fig. 9, would affect the initial fluorescent state of the dye. The observed changes in the fluorescence excitation spectrum are consistent with either the perturbation of a membrane-bound monomer/aggregate equilibrium by the change in electric field strength within the membrane or with a field-induced structural change of the membrane. Both mechanisms could easily account for the disagreement between the absorbance and fluorescence responses. Monomers and aggregates are likely to have differing quantum yields and relax to differing degrees prior to emission, and a structural change is likely to significantly alter the ease of quenching of the excited state as well as the extent of relaxation.

A response mechanism involving dye aggregation has previously been suggested [4,45–49,58] for the potential-sensitive dye merocyanine 540. There it is thought that the dye can bind to the membrane in two orientations; with its long axis perpendicular or parallel to the surface of the membrane. Molecules which are orientated parallel are able to readily undergo aggregation. The effect of a change in the electric field strength is thought to cause a reorientation of dye molecules in the membrane, because of their large dipole moment, and hence a shift of the monomer/ aggregate equilibrium, which results in the spectral response. According to temperature-jump measurements of Verkman and Frosch [58] the reorientation of the dye molecules appears to be the rate-determining step, with relaxation times in the range 0.6–1.4 ms depending on the dye concentration. In the case of a mechanism involving a structural change of the membrane it is more difficult to estimate an expected response time. The rate-determining step could be the rearrangement of the lipid molecules rather than the dye itself. Lipid reorganization during the gel-liquid crystal phase transition of pure phospholipid bilayers has been shown [61] to occur in a series of steps over the time range of microseconds to milliseconds.

An electrochromic response mechanism would be expected to have a response time in the subnanosecond range. Up to now the only study of the rate of response of RH421 is that of Müller et al. [17], who used the dye as a probe of cardiac action potential. They found that the onset of the action potential was detected by the dye within 0.5 ms. For a dye of very similar structure Chien and Pine [18] have reported a submillisecond response time. Response times of this order are not inconsistent with a mechanism involving an electric field dependent reorientation of dye or lipid molecules [4,58].

An additional effect of an electric field on the fluorescence properties of styryl dyes has been proposed by Ephardt and Fromherz [62]. Based on measurements of the fluorescence spectra, quantum yield and fluorescence lifetime of a styryl dye in a variety of solvents of different polarities and viscosities they have proposed that the styryl dyes can undergo a horizontal transition in the excited state to a twisted internal charge transfer (TICT) state. The transition to the TICT state is supposed to involve the transfer of positive charge from the pyridino group to the anilino group and a rotation about the alkene-aniline bond. In the TICT state the efficiency of internal conversion is supposed to be increased, so that the fluorescence is decreased. Ephardt and Fromherz have then proposed two possible mechanisms for the voltage sensitivity of the styryl dyes which could account for changes in the quantum yield. In the first mechanism, termed a direct yield mechanism, the electric field interacts with the

redistribution of charge in the molecule, so that changes in the electric field strength alter the activation energy of the twist and hence the rate constant for transition to the TICT state. Since fluorescence and internal conversion via the TICT state are competing processes, a change in the rate of transition to the TICT state would result in a different quantum yield. In the second mechanism, termed an indirect yield mechanism, the change in electric field strength causes a reorientation of dye within the lipid membrane either towards or away from the aqueous phase, thus causing a change in the environment of the dye molecules. Ephardt and Fromherz have suggested that the transition to the TICT state is enhanced by media of high polarity and low viscosity, because of the charge movement and the twist involved in the transition, respectively. Thus, any change to the polarity or viscosity in the dye's environment would cause a change in the rate of transition to the TICT state and hence a change in the quantum yield.

Although the data presented here are consistent with a mechanism involving an electric field dependent shift in a membrane-bound monomer/aggregate equilibrium or with a field-induced structural change, the mechanisms proposed by Ephardt and Fromherz can also not be ruled out. It is feasible that a combination of effects contribute to the voltage sensitivity of the dye. It does not appear justified, however, to assume that the styryl dyes respond by a purely electrochromic mechanism. This has important consequences for rapid kinetic measurements of electrical events in membrane preparations. For such measurements it is necessary that the probe response time be much shorter than the timescale of the process being measured. Although an electrochromic mechanism would be expected to have a response time in the subnanosecond range, a reorientation/dimerisation mechanism would be likely to require times in the order of microseconds to milliseconds.

A further important point is that the voltage sensitivity of RH421 appears to vary from one membrane preparation to another [12,15,22]. In the case of an electrochromic mechanism this would not be expected [12,63], but a reorientation/dimerisation mechanism might be able to provide an explanation. Bühler et al. [22] have found that the voltage sensitivity of RH421 is significantly less in lipid vesicles compared to membrane fragments. A possible explanation is that the different membrane curvatures of these two preparations and their consequent differences in the packing of the lipid molecules affect the ability of the dye molecules to reorientate and form aggregates, particularly if, as has been suggested [48,49] for merocyanine 540, the aggregates are orientated with their long axis parallel to the membrane surface. Lipid packing may be a critical parameter in any mechanism in which molecular motion within the membrane is involved. The above observations stress the importance of evaluating the voltage sensitivity of the styryl dyes in each type of membrane preparation studied.

In order to further elucidate the mechanism of the voltage sensitivity of RH421 and to characterise its fluorescent properties in aqueous solution and in the membrane, it is intended to carry out time-resolved fluorescence measurements.

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