Time-Resolved Fluorescence Investigations of the Interaction of the Voltage-Sensitive Probe RH421 with Lipid Membranes and Proteins†

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ABSTRACT: Fluorescence lifetimes and fluorescence anisotropy decays of the voltage-sensitive styryl-pyridinium dye RH421 have been measured in the presence of dimyristoylphosphatidylcholine vesicles, the water soluble enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco), and Na^+,K^+-ATPase-containing membrane fragments. The effect of an intramembrane electric field on the photophysical properties of the dye was investigated by the binding of the hydrophobic ion tetraphenylborate (TPB) to the membrane. TPB was found to significantly increase the average fluorescence lifetime and the order of membrane-bound dye. The increase in fluorescence lifetime is consistent with reorientation of the dye further into the membrane interior. The increase in order may be attributed to an electric field-induced alignment of the dye molecules. From the values of the rotational diffusion constant experimentally determined, the expected response time of the dye to an applied electric field can be calculated for a reorientational mechanism to be on the order of tens of nanoseconds. Experiments with rubisco showed that the dye interacts strongly with the protein. In this case, the dye is so tightly bound that it has almost no independent motion and rotates virtually solely with the protein. The rate of rotational motion of the dye in the presence of Na^+,K^+-ATPase-containing membrane fragments is similar to that in pure lipid membranes. The order parameter of the dye in the Na^+,K^+-ATPase membrane fragments is close to the maximum value. This is most probably due to the high density of protein molecules, which restricts the range of motion of the dye.

The voltage-sensitive styryl dye RH421 (see Figure 1) and dyes of related structure are presently being widely employed for the visualization of voltage transients in membrane preparations (Smith, 1990; Grinvald et al., 1988; Loew, 1988). The origin of the fluorescent response of the dyes is often assumed to be electrochromism, whereby the absorbance spectrum of the dye is shifted as a result of a change in membrane potential (Haugland, 1992). No direct proof of an electrochromic mechanism has yet been found, however, and recently the importance of solvatochromism for the fluorescent response has been stressed by several authors (Clarke et al., 1995; Fromherz & Schenk, 1994). A solvatochromic mechanism involves an electric field-induced reorientation of dye within the membrane so that it experiences a change in polarity of its environment.

In biological membrane systems, however, it is still not clear whether the dye is in truth responding to the membrane potential. In biological membranes, changes in membrane potential are produced by the action of membrane proteins. Therefore, the question must be asked: is the dye responding to the change in membrane potential or the change in conformation of the membrane protein. The latter could be the case if a direct interaction of dye with the protein is occurring rather than merely with the lipid matrix. Direct interactions of the styryl dye RH160 with the Na^+,K^+-ATPase (Klodos & Forbush, 1988) and with the H^+-ATPase of *Neurospora* (Nagel et al., 1991) have been suggested.

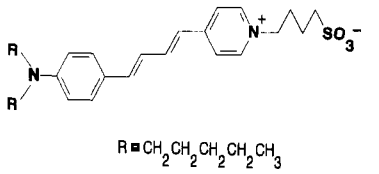

FIGURE 1: Structure of RH421.

The purpose of the present paper is, thus, twofold. Firstly, the effect of an intramembrane electric field on the fluorescence lifetime and anisotropy of membrane-bound RH421 has been investigated in dimyristoylphosphatidylcholine (DMPC) vesicles, whereby the intramembrane electric field was induced by the binding of the negatively charged hydrophobic ion tetraphenylborate (Clarke et al., 1995; Bühler et al., 1991). Bühler et al. (1991) have also carried out experiments in which a field of opposite polarity has been induced by the binding of the positively charged hydrophobic ion tetraphenylphosphonium. This is an indirect but simple method of inducing an intramembrane electric field. A direct external application of a field using electrodes

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would be preferable, but this would require significant technical development in order to combine time-resolved single photon counting fluorescence detection with the high electric field generation necessary for such experiments. The results obtained allow for an examination of which mechanism, electrochromic or solvatochromic, best explains the fluorescent response in pure lipid membranes. Secondly, the lifetime and anisotropy of dye in the presence of a globular enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco), and a membrane protein, the Na^+,K^+-ATPase, have been measured. The purpose of these experiments was to find whether evidence for a direct dye−protein interaction could be found.

MATERIALS AND METHODS

N-(4-Sulfobutyl)-4-[4-[*p*-(dipentylamino)phenyl]butadienyl]-pyridinium inner salt (RH421) was obtained from Molecular Probes (Eugene, OR) and was used without further purification. An ethanolic solution was checked for the presence of fluorescent impurities by recording the fluorescence emission spectrum at varying excitation wavelengths. A single peak was observed with a maximum at 695 nm, which was independent of the excitation wavelength, indicating a single dye species (Zouni et al., 1994).

Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL). DMPC unilamellar vesicles were prepared by the ethanol injection method described in detail elsewhere (Zouni et al., 1993, 1994). The final vesicle suspension contained no detectable trace of ethanol, i.e., [ethanol] $\leq$ 10 μM, according to an NADH/alcohol dehydrogenase enzymatic assay (Boehringer, Mannheim). Dialysis tubing was purchased from Medicell International (London, U.K.). The phospholipid content of the vesicle suspensions was determined by the phospholipid B test from Wako (Neuss, Germany).

A 0.01 M stock solution of sodium tetraphenylborate (TPB) was prepared in ethanol. For measurements with vesicles, 5 μL of the stock solution was added to 1 mL of vesicle suspension in buffer to give a final TPB concentration in the cuvette of 50 μM.

All measurements with the vesicles were performed in a buffer containing 30 mM Tris, 1 mM EDTA, and 150 mM NaCl. For the measurements with Na^+,K^+-ATPase-containing membrane fragments, the buffer contained 30 mM Tris, 1 mM EDTA, 50 mM NaCl, and 5 mM $MgCl_2 \cdot 6H_2O$. The pH of both buffers 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: tris-[(hydroxymethyl)amino]methane (99.9%, Sigma), EDTA (99%, Sigma), NaCl (analytical grade, Merck), HCl (0.1 M Titrisol solution, Merck), sodium tetraphenylborate (analytical grade, Merck), and dithioerythritol (>99%, Biomol).

Ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco; EC 4.1.1.39) was purified from spinach according to the method of Paulsen and Lane (1966) with the exception of the last purification step, for which gel chromatography was applied (Vater et al., 1983). The rubisco concentration of the preparation fractions was between 4 and 7 mg/mL. The protein concentration was determined from the absorbance at 280 nm (Paulsen & Lane, 1966). The activity of activated enzyme (Lorimer et al., 1976) was determined by a radiometric assay as described by Paulsen and Lane (1966). The specific activity was approximately 1.5 μmol of CO_2 per minute per milligram of protein at 25 °C. The purity of the enzyme was estimated from sodium dodecyl sulfate−polyacrylamide gels stained with coomassie blue (Laemmli, 1970; King & Laemmli, 1971) to be greater than 95%. The buffer used for the measurements with rubisco contained 20 mM Tris, 0.1 mM EDTA, and 1 mM dithioerythritol. The pH of the buffer was adjusted to pH 8 with HCl.

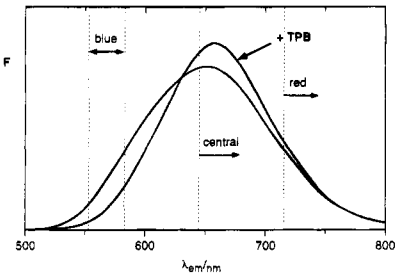

FIGURE 2: Fluorescence emission spectra of 9.2 μM RH421 in 543 μM DMPC vesicles in the presence and absence of 50 μM tetraphenylborate (TPB). λ_{ex} = 460 nm, and T = 30 °C.

The preparation and purification of the membrane fragments containing the Na^+,K^+-ATPase has been described elsewhere (Bühler et al., 1991). 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 2.6 mg/mL. The specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay (Schwartz et al., 1971). The specific activity in the presence of Na^+ and K^+ ions was 2200 μmol Pi per hour per milligram of protein at 37 °C. The phospholipid content was 0.82 mg per milligram of protein. The assay procedures have been described previously (Bühler et al., 1991).

Polarized time-resolved fluorescence decay curves of RH421 in the presence of membranes or proteins were acquired using a picosecond laser system in combination with time-correlated single photon counting as described in detail elsewhere (van Hoek & Visser, 1992; Pap et al., 1993; Visser et al., 1994). The excitation wavelength was 460 nm, and the fluorescence was detected in three regions of the fluorescence spectrum (see Figure 2). For detection at the blue edge of the emission, a 568.5 nm interference filter (bandwidth typically about 15 nm, Schott, Mainz, Germany) in combination with an OG515 long wavelength pass glass filter (Schott) was used. As a reference compound for these emission wavelengths, erythrosin B in water (τ = 80 ps) was used (Bastiaens et al., 1992). For detection at the main emission band and at longer wavelengths, the fluorescence was selected with OG515 and RG645 (Schott) long wavelength pass glass filters. Red-edge detection was accomplished with the aid of RG715 and OG515 (Schott) long wavelength pass glass filters. For the central and red detection regions, the instrumental response function was taken from the fluorescence of pinacyanol in ethanolic solution having an absorbance of 0.05 at 460 nm. The ultrashort decay time of this compound is about 10 ps (Zouni et al., 1993). The channel width of the multichannel analyzer amounted to 10 ps per channel, and each decay curve was collected in 1024 channels.

Data analysis was performed by the second generation global analysis software program described by Beechem et al. (1992) and obtained from Globals Unlimited (Urbana,

IL). The trial total fluorescence decay function was

$$S(t) = \sum_{i=1}^{n} \alpha_i e^{-t/\tau_i} \quad (1)$$

in which α_i is the pre-exponential factor belonging to the lifetime component τ_i and n is the minimum number of lifetimes to have an acceptable fit to the data ($\chi^2 \approx 1.0-1.5$ and randomly scattered weighted residuals between experimental and fitted decay curves). In all cases, it was found that four components were required to have an acceptable fit to the experimental data. The second order average lifetime of dye fluorescence, $\langle\tau\rangle$, was calculated from the fitted values of α_i and τ_i according to the following equation (Lakowicz, 1983):

$$\langle\tau\rangle = \frac{\sum_{i=1}^{n} \alpha_i \tau_i^2}{\sum_{i=1}^{n} \alpha_i \tau_i} \quad (2)$$

A detailed error analysis at the 66% confidence level was carried out in order to retrieve the lower and upper bounds of the average lifetime.

For experiments with vesicles and Na^+,K^+-ATPase-containing membrane fragments, the anisotropy decay could be fitted to the following function:

$$r(t) = \beta_1 e^{-t/\phi} + \beta_2 \quad (3)$$

in which ϕ is the correlation time for restricted rotation, $\beta_1 + \beta_2 = r_0$, the initial anisotropy at $t = 0$, and $\beta_2 = r_\infty$, the anisotropy at infinite time. The anisotropy data were weighted using the weighting factors as derived by Wahl (1979). Also, the error in the parameters was obtained as previously described (Wahl, 1979). From the retrieved amplitudes, the order parameter S could be obtained as follows (Heyn, 1989):

$$S = \left(\frac{\beta_2}{\beta_1 + \beta_2}\right)^{1/2} \quad (4)$$

If one assumes that the rotation of the chromophores can be described by the wobbling in a cone model, the cone angle, θ_c, over which the dye can move is obtained from (Kinosita et al., 1977)

$$S = \frac{(\cos\theta_c)(1 + \cos\theta_c)}{2} \quad (5)$$

From the correlation time of restricted rotation, one obtains the rotational diffusion coefficient for rotation perpendicular to the long axis of the chromophore, $D_\perp$, from (Szabo, 1984; Kinosita et al., 1984; Ameloot et al., 1984; Bastiaens et al., 1989)

$$D_\perp = \frac{1 - S^2}{6\phi} \quad (6)$$

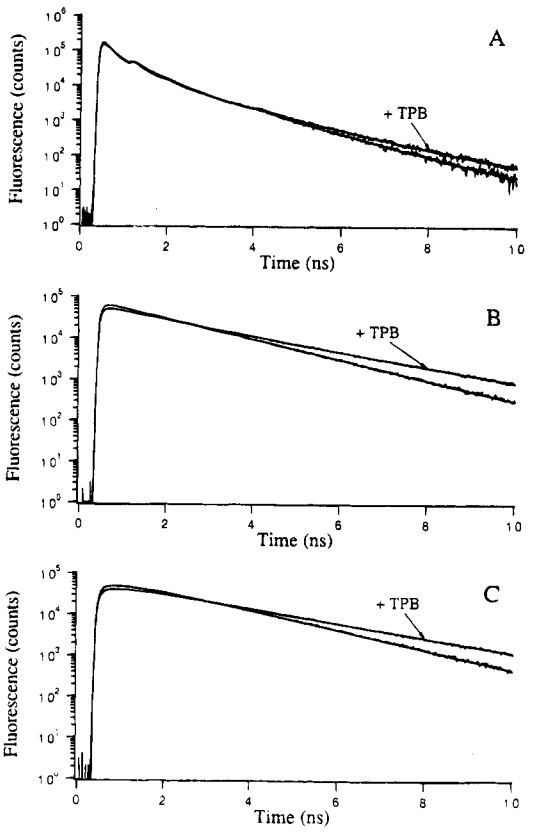

FIGURE 3: Experimental and fitted fluorescence decays of 9.2 μM RH421 in 543 μM DMPC vesicles in the presence and absence of 50 μM tetraphenylborate (TPB). The curves refer to blue (A), central (B), and red (C) emission. $T = 30$ °C.

In the case of experiments with rubisco, it was sufficient to fit the anisotropy decay to a single exponential function with $\beta_2(r_\infty) = 0$.

RESULTS

Polarized Fluorescence Decay Characteristics of RH421 in DMPC Vesicles. Experimental and calculated fluorescence decays of RH421 bound to DMPC vesicles in the presence and absence of TPB are shown in Figure 3 for the blue, central, and red portions of the fluorescence emission. The effect of TPB on the steady state fluorescence of bound dye is shown in Figure 2. The fluorescence decays were found to be very heterogeneous, and four exponential time functions were necessary to obtain an adequate fit to the data. Because the individual values of α_i and τ_i calculated are highly correlated, very different parameter sets may lead to equally good fit qualities. Therefore, we have decided to calculate the average lifetime, $\langle\tau\rangle$, according to eq 2 under Materials and Methods because this constitutes a reliable value which can be used for comparison under varying experimental conditions.

In the absence of TPB, the calculated values of $\langle\tau\rangle$ for the blue, central, and red emissions are given in Table 1. On addition of TPB, there was a significant increase in the measured average lifetime for the central and red emission (see Figure 3 and Table 1).

The central and red emission decay curves required, irrespective of whether TPB was present, a component with a negative pre-exponential factor in order to fit the data. This indicates a precursor–successor mechanism in which a

Table 1: Second Order Average Fluorescence Lifetimes, $\langle\tau\rangle$, for RH421 in the Presence of Membrane Preparations and Rubisco at Different Emission Wavelengths[a]

	wavelength region		
	blue	central	red
DMPC	0.608 (±0.002)	1.660 (±0.005)	1.88 (±0.10)
DMPC + TPB	0.61 (0.58−0.62)	2.150 (±0.005)	2.505 (± 0.005)
Na^+,K^+-ATPase membrane fragments	1.88 (±0.02)	2.52 (2.50−2.60)	2.56 (2.55−2.60)
Na^+,K^+-ATPase membrane fragments + ATP	1.79 (±0.01)	2.70 (2.69−2.72)	2.82 (2.79−2.88)
rubisco	1.73 (±0.24)	2.450 (±0.005)	2.500 (±0.005)

[a] Emission wavelength regions were selected by the following filters: blue, 568.5 nm interference filter; central (+red), RG645; and red, RG715. The errors are given at the 66% confidence level.

Table 2: Fluorescence Anisotropy, Order Parameters, Rotational Correlation Times, Rotational Diffusion Coefficients, and Cone Angles for RH421 in Membrane Preparations at Different Emission Wavelengths

wavelength	r_0	r_∞	S	ϕ (ns)	$D_\perp(10^7\ s^{-1})$	θ_c (deg)
			DMPC			
central	0.335 (±0.010)	0.107 (±0.005)	0.565 (±0.043)	3.50 (±0.27)	3.24 (±0.74)	47.6 (±2.8)
red	0.330 (±0.008)	0.093 (±0.003)	0.530 (±0.030)	3.30 (±0.18)	3.64 (±0.61)	49.9 (±2.0)
			DMPC + TPB			
central	0.313 (±0.009)	0.143 (±0.003)	0.677 (±0.034)	3.19 (±0.25)	2.83 (±0.50)	40.0 (±2.4)
red	0.306 (±0.006)	0.142 (±0.002)	0.680 (±0.011)	2.83 (±0.16)	3.17 (±0.28)	39.7 (±0.8)
			Na^+,K^+-ATPase Membrane Fragments			
central	0.361 (±0.006)	0.280 (±0.002)	0.880 (±0.010)	2.67 (±0.32)	1.41 (±0.20)	23.4 (±1.0)
red	0.354 (±0.006)	0.265 (±0.003)	0.866 (±0.012)	2.56 (±0.28)	1.63 (±0.22)	24.8 (±1.2)
			Na^+,K^+-ATPase Membrane Fragments + ATP			
central	0.359 (±0.006)	0.283 (±0.002)	0.888 (±0.011)	2.62 (±0.30)	1.35 (±0.19)	22.6 (±1.2)
red	0.354 (±0.005)	0.276 (±0.002)	0.882 (±0.006)	2.49 (±0.24)	1.49 (±0.17)	23.2 (±0.6)

second species is created in an excited state reaction involving a primary excited precursor (Kungl et al., 1992). This phenomenon has already been reported by us in earlier publications (Zouni et al., 1993; Visser et al., 1994). It has been found that the excited state reaction also occurs in various organic solvents and when the dye is bound to detergent micelles (Visser et al., 1994). The reaction, therefore, is not specific to the membrane environment; rather, it is a property of the dye itself. The reaction has been attributed to the formation of a fluorescent twisted intramolecular charge transfer (TICT) state, occurring simultaneously with the relaxation of the surrounding solvent cage. An additional factor which may contribute to the multiexponential nature of the fluorescence decay is ground state heterogeneity, i.e., different dye sites within the membrane.

Experimental and calculated fluorescence anisotropy decays of RH421 bound to DMPC vesicles in the presence and absence of TPB are shown in Figure 4 for the central and red portions of the fluorescence emission. It was not possible to accurately fit the blue emission because the decay of the fluorescence intensity is too rapid. The values of the initial anisotropy, r_0, the infinite time anisotropy, r_∞, the order parameter, S, the rotational correlation time, ϕ, the rotational diffusion coefficient, $D_\perp$, and the cone angle, θ_c, derived from the fits are given in Table 2. It can be seen that the rate of rotational motion, as reflected in the values of ϕ and $D_\perp$, is not greatly affected by the addition of TPB. There is, however, a significant increase in the order of the dye molecules in the membrane after the addition TPB. This can be seen from the order parameter, which increases from a value of around 0.55 in the absence of TPB to a value of 0.68 in the presence of TPB. The increase in order is similarly reflected in the calculated values of the cone angle: approximately 48° without TPB and 40° with TPB.

The reason for the lower values of r_0 for the red emission in comparison to those for central emission is the precursor−successor mechanism in the excited state causing a twist of the molecule, which results in a change in direction of the emission transition moment with respect to the absorption transition moment (Visser et al., 1994).

Polarized Fluorescence Decay Characteristics of RH421 with Rubisco. Experimental and calculated fluorescence and anisotropy decays of RH421 in the presence of the enzyme rubisco are shown in Figure 5 for the red emission. The average lifetimes, $\langle\tau\rangle$, for the blue, central, and red emission are given in Table 1. It was found that the anisotropy decay could be satisfactorily fitted by a single exponential function. The calculated rotational correlation times for the blue, central, and red emissions were 159 (±27) ns, 140 (±17) ns, and 131 (±13) ns, respectively. The corresponding values of the initial anisotropy, r_0, were 0.384 (±0.002), 0.372 (±0.002), and 0.370 (±0.002).

The fact that only a single slow correlation time was observed suggests that the dye is tightly bound to the protein, so that there is no internal probe motion and the correlation time measured corresponds to rotation of the protein as a whole. The high value of r_0 and the fact that there is only a small decrease on going from blue to red emission indicates that the excited state twist of the dye is hindered by the tight binding to the protein.

Polarized Fluorescence Decay Characteristics of RH421 with Na^+,K^+-ATPase Membrane Fragments. Fitted fluorescence decays of RH421 in the presence of Na^+,K^+-ATPase-containing membrane fragments are shown in Figure 6 for the red emission. The average lifetimes, $\langle\tau\rangle$, for the blue, central, and red emissions are given in Table 1. Upon the addition of ATP, there was an increase in the measured average lifetime for the central and red emission (see Figure 6 and Table 1). The increase in lifetime on addition of ATP

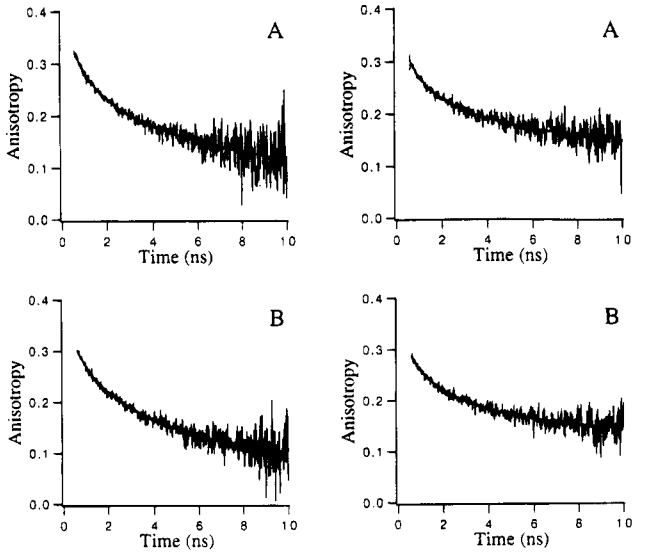

FIGURE 4: Experimental and fitted fluorescence anisotropy decays of 9.2 μM RH421 in 543 μM DMPC vesicles in the presence and absence of 50 μM TPB. The curves refer to central (A) and red (B) emission. The curves at the left refer to the absence of TPB and the curves at the right to the presence of TPB. The curves start at the fluorescence maximum. T = 30 °C.

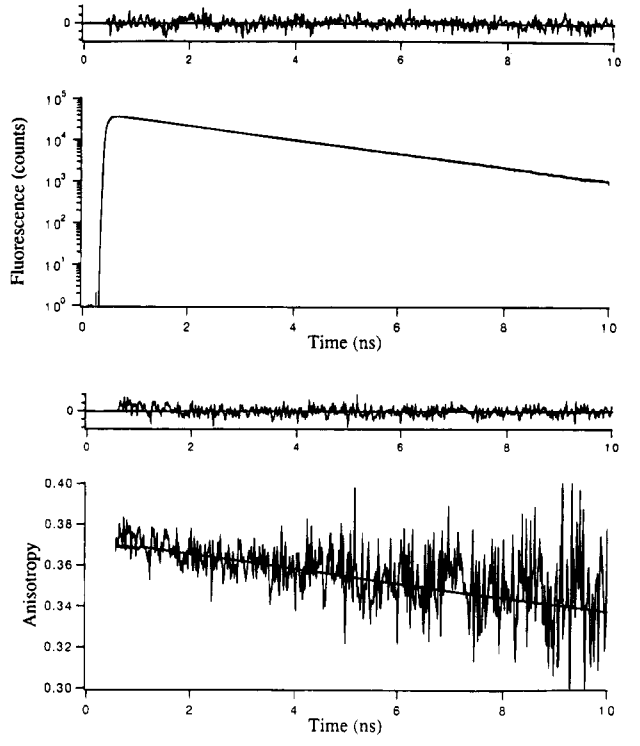

FIGURE 5: Experimental and fitted fluorescence decay (top) and fluorescence anisotropy decay (bottom) of 0.47 μM RH421 in the presence of 5.4 μM rubisco. λ_{ex} = 460 nm, and λ_{em} > 715 nm. Weighted residuals between experimental and fitted curves are shown as well. T = 21 °C.

can possibly be explained by phosphorylation of the enzyme, which is accompanied by a protein conformational change and the release of bound Na^+ ions to the surrounding solution (Bühler et al., 1991; Clarke et al., 1992). In order to confirm this, however, further experiments are necessary. The lifetime increase is consistent with the increase in quantum yield caused by ATP observed previously (Clarke et al., 1992).

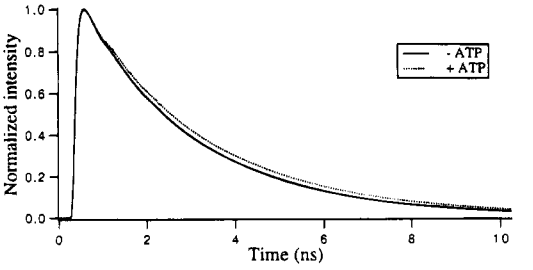

FIGURE 6: Fitted fluorescence decay of 0.74 μM RH421 in the presence of 40 μg/mL of Na^+,K^+-ATPase in the form of membrane fragments either in the absence of ATP (solid line) or in the presence of 0.5 mM ATP (dotted line). λ_{ex} = 460 nm, and λ_{em} > 715 nm. Because the relative changes are small, only fitted curves are presented. T = 20 °C.

Values of r_0, r_∞, S, ϕ, $D_\perp$, and θ_c determined from the fits of the anisotropy decays are given in Table 2. At least at the dye concentration used here, ATP does not appear to have any significant effect on the order or the mobility of the bound dye molecules.

DISCUSSION

In order to explain the spectral changes of membrane-bound RH421 on application of an electric field, two feasible mechanisms exist: (1) an electrochromic mechanism and (2) a reorientation/solvatochromic mechanism. An electrochromic mechanism involves a direct effect of the electric field on the electronic distribution caused by light absorption. As a consequence, the energies of the ground and excited states are stabilized or destabilized to varying degrees, resulting in a frequency shift of the absorbance spectrum (Waggoner & Grinvald, 1977). A reorientation/solvatochromic mechanism, on the other hand, acts by an electric field-induced reorientation of the whole dye molecule within the membrane. Due to the reorientation, the dye experiences a change in its local polarity, which can cause changes in the position and intensity of the absorbance band (Liptay, 1969).

In a previous publication (Clarke et al., 1995), it was shown that the absorbance changes caused by the TPB-induced electric field could be explained by a reorientation/solvatochromic mechanism. Now let us consider the fluorescence changes reported here. Firstly, it has been found that the order of the membrane-bound dye molecules is significantly increased by the addition of TPB. In the absence of TPB, the order parameter, S, calculated from the red emission was 0.53, whereas in the presence of TPB, S increased to 0.68. This is evidence favoring a reorientation/solvatochromic mechanism. One can imagine that the intramembrane electric field induced by TPB would tend to align the dye molecules according to the direction of their dipole moments (Clarke et al., 1995), leading to the observed increase in order. An electrochromic mechanism, in which only electronic displacement without nuclear displacement occurs, is not able to explain the observed changes in fluorescence anisotropy.

If one assumes the "wobbling in a cone" model of Kinosita et al. (1984), the changes in order parameter induced by TPB can be related to the cone angles, θ_c, for the wobbling motion. This results in values of approximately 48° in the absence and 40° in the presence of TPB. Is it possible that the TPB-induced electric field could cause a realignment of about 8° in the membrane? This depends on the magnitude of the

reorientational energy caused by the electric field relative to the thermal energy. For an ensemble of dipoles restricted in a conic segment of the membrane, it can be shown that, if one assumes that the dipoles have no preferred orientation within the cone, their orientational distribution in the absence of an electric field is given by

$$\frac{\Delta N}{N_{tot}} = \frac{\cos\theta - \cos(\theta + \Delta\theta)}{1 - \cos\theta_c} \tag{7}$$

where ΔN represents the number of dipoles within a cone bordered by the angles θ and $\theta + \Delta\theta$ and N_{tot} is the total number of dipoles. In the presence of an electric field, the distribution is modified to

$$\frac{\Delta N}{N_{tot}} = \frac{e^{x\cos\theta} - e^{x\cos(\theta+\Delta\theta)}}{e^{x} - e^{x\cos\theta_c}} \tag{8}$$

where

$$x = \frac{\mu E}{kT} \tag{9}$$

μ and E are the dipole moment of the dye and the electric field strength, respectively. Equations 7–9 are based on the theory of field-induced dipole orientation in an isotropic medium given elsewhere (Tanford, 1961; Atkins, 1983). The equations have been modified to take into account the anisotropic membrane environment. It has been shown previously (Clarke et al., 1995) that, at saturating concentrations of TPB, the reorientational energy caused by the electric field is for RH421 approximately 2.3 times greater than the thermal energy, i.e., $x = 2.3$. At such a value of x, it can be shown using eqs 7 and 8 that a significant realignment of dye in the membrane by the field would be expected. A major limitation of the above theoretical treatment, however, is the assumption in eq 7 of random orientation of dye within the conical membrane segment. In fact, it is more likely that even within the cone some dye orientations are prefered over others, due to interactions with the surrounding lipid molecules. A more exact theoretical treatment would, therefore, have to take this point into consideration. Furthermore, it cannot be excluded at this stage that TPB may have, in addition to its electrical effect, a steric influence on bound dye restricting its range of motion.

It has also been found that TPB causes a significant increase in the fluorescence lifetime of membrane-bound dye. In the absence of TPB, the average lifetime of the red emission was found to be 1.88 ns, whereas in the presence of TPB, the lifetime increased to 2.505 ns, i.e., a 33% increase. Such a change in lifetime is consistent with the increase in quantum yield induced by TPB, which has been reported previously (Clarke et al., 1995). It has been shown elsewhere that the fluorescence lifetimes of RH421 and related dyes are very sensitive to the polarity of their environment (Visser et al., 1994; Ephardt & Fromherz, 1989). The lifetime is enhanced by a decrease in polarity. The TPB-induced increase in lifetime observed here can, therefore, be well-explained within the framework of a reorientation/solvatochromic mechanism. Reorientation into the membrane interior would be expected to decrease the polarity of the dye's local environment, which would result in an increased lifetime. It should be noted that reorientation here refers to a rotation of the long axis of the dye molecule relative to the membrane surface. No direct translation of the dye into the membrane is likely to occur, because the hydrophilic sulfonate group would anchor one end of the molecule at the membrane surface. The direction of dye reorientation has been inferred from the lifetime changes alone. The change in order reported above gives no information about the direction of reorientation because of the lack of a point of reference.

Is it possible that an electrochromic mechanism could also cause an increase in fluorescence lifetime? A way in which this could occur would be if the magnitude of the transition moment, $|\mu|$, was altered by the electric field. According to Liptay (1969), the transition moment of a molecule in an electric field is given by the transition moment in the absence of the field, μ_0, plus an additional field-induced component, αE, where α is the transition polarizability and E is the electric field strength at the position of the molecule. For molecules in which αE is significant in comparison to μ_0, an electric field could cause changes in the transition moment which would be reflected in a change in the intensity of the absorbance band and a change in the radiative lifetime, because the probability of both absorption and emission are altered (Parker, 1968). Previous absorbance measurements, however, have shown that TPB induces an overall increase in the intensity of the absorbance band (Clarke et al., 1995). According to an electrochromic mechanism, therefore, a decrease in the radiative lifetime would be expected. The observed increase in the actual lifetime is, thus, inconsistent with an electrochromic mechanism. It appears, therefore, that all of the TPB-induced spectral changes of membrane-bound RH421, both absorbance and fluorescence, can only be explained by a reorientation/solvatochromic mechanism.

On the basis of a reorientation/solvatochromic mechanism, it is of interest to calculate the expected response time of the dye to an applied electric field. The response time can in this case be quantified by the rotational relaxation time, τ, which is related to the rotational diffusion coefficient by (Grant et al., 1978)

$$\tau = \frac{1}{2D_\perp} \tag{10}$$

Using the values of $D_\perp$ given in Table 2, τ can be calculated to be approximately 16 ns in DMPC vesicles and approximately 34 ns in Na^+,K^+-ATPase membrane fragments. This is certainly a sufficiently fast response for most kinetic measurements of voltage transients in membrane preparations.

The order parameters reported here for RH421 in DMPC membranes above the main phase transition temperature can be compared with values reported for the fluidity probe diphenyl-1,3,5-hexatriene (DPH) in the same lipid. From the data of Kinosita et al. (1984), at 35 °C, one can calculate a value of 0.34. Pap et al. (1994) have reported the same value at 28 °C. The values found here for RH421 are significantly higher. If one accepts that the order parameters of the probes reflect the order of the lipid, then the higher order parameter of RH421 can be interpreted as being due to the localization of the probe closer to the surface of the membrane than in the case of DPH. It has been shown from both nuclear magnetic resonance and fluorescence depolarization studies (van der Meer, 1993) that the order of the

lipid chains increases as one proceeds from the hydrophobic core toward the head group.

So far, we have discussed the voltage sensitivity of RH421 in pure lipid membranes. In the presence of proteins, however, the origin of observed spectral changes could be different. There are many probe molecules which interact directly with proteins and respond to protein conformational changes. A classic example is the probe toluidinylnaphthalene sulfonate (TNS) which binds to hydrophobic regions of various proteins and responds to changes in its local polarity caused by substrate binding (McClure & Edelman, 1966, 1967; Stryer, 1968). Here we have shown that the rotational correlation time of RH421 is considerably lengthened in the presence of the enzyme rubisco. In ethanolic solution, for example, the dye has a rotational correlation time for the red emission of 0.54 ns (Visser et al., 1994), whereas the corresponding value in the presence of rubisco is 131 ns. The much slower rotational motion must be due to a direct dye−protein interaction.

For globular proteins, the rotational correlation time is approximately related to the molecular weight, M, by the following equation (Lakowicz, 1983):

$$\phi = \frac{\eta M}{RT}(\bar{\nu} + h) \qquad (11)$$

where η is the solution viscosity, R is the gas constant, and T is the absolute temperature. $\bar{\nu}$ represents the specific volume of the protein, and h is the additional contribution to the volume of the hydrated protein from its surrounding solvating water molecules. It is known that spinach rubisco has a molecular weight of around 535 000 g mol^{-1} (Kapp et al., 1990). Assuming typical values for $\bar{\nu}$ and h of 0.74 cm^3 g^{-1} (Thornber et al., 1965) and 0.2 cm^3 g^{-1} (Lakowicz, 1983) and a viscosity of the aqueous solution of 0.978 cP (Weast, 1987) at 293.9 K, the expected rotational correlation time of rubisco can be calculated from eq 11 to be 201 ns. This value can be compared with the experimental rotational correlation times of the dye when bound to rubisco, which vary between 159 ns for blue emission and 131 ns for red emission. The fact that the experimental and theoretical values are of the same order of magnitude indicates that the binding of dye to the protein must be very tight. Note further that the average fluorescence lifetime is much shorter than the correlation time and that the correlation time has been retrieved from the initial part (first 10 ns) of the fluorescence anisotropy decay. It appears therefore that the dye has almost no independent motion and rotates together with the whole protein.

Experiments with Na^+,K^+-ATPase-containing membrane fragments have shown that the fluorescence lifetime is significantly longer than in pure DMPC lipid membranes. For red emission, for example, $\langle\tau\rangle$ has a value of 1.88 ns in DMPC vesicles, whereas in Na^+,K^+-ATPase membrane fragments, the value is 2.56 ns. The order parameter of dye bound to Na^+,K^+-ATPase-containing membrane fragments is also significantly higher than that found in pure DMPC vesicles. For red emission, S has a value of 0.53 in DMPC vesicles, whereas in Na^+,K^+-ATPase membrane fragments, the value is 0.87. These results indicate that the environment experienced by the dye when Na^+,K^+-ATPase is present is significantly different from pure lipid membranes. The longer fluorescence lifetime suggests that the polarity of the dye's surroundings is lower when the Na^+,K^+-ATPase is present. The higher order parameter indicates a significantly greater restriction on the range of rotational motion of the dye caused by the Na^+,K^+-ATPase. This can also be seen in the calculated values of the cone angle: 50° for DMPC vesicles and 25° for Na^+,K^+-ATPase membrane fragments. Very high order parameters for DPH in biological membranes containing a high concentration of protein have also been reported (Kinosita et al., 1984; van der Meer, 1993). In the case of the purple membrane of *Halobacterium halobium*, in which the protein bacteriorhodopsin forms a rigid crystalline lattice, S has a value of 0.80 at 35 °C (Kinosita et al., 1984). According to Kinosita et al., in the neighborhood of protein, lipid chains as well as probe molecules wobble in narrow "pseudo-potential wells" formed by the protein surface and surrounding lipid molecules. The narrower cone angle of rotation would result in a different time-averaged local environment of the probe, e.g., a changed local polarity. This could possibly account for the larger fluorescence lifetime of RH421 in the Na^+,K^+-ATPase membrane fragments.

The values of the rotational correlation time and the rotational diffusion coefficient are in the same order of magnitude for DMPC vesicles and Na^+,K^+-ATPase membrane fragments. This indicates that in both cases the measured fluorescence anisotropy decays are due to dye molecules in the lipid phase. Dye molecules bound strongly to the Na^+,K^+-ATPase would have a very slow anisotropy decay. The rotational correlation times of membrane proteins are typically in the range of tens to hundreds of microseconds (Henis, 1993). Such a slow anisotropy decay cannot be detected by our measurements, because the fluorescence lifetime of RH421 is too short. Therefore, although the anisotropy data reported here refer to dye in the lipid phase, it cannot be ruled out that some dye molecules may be bound directly to the Na^+,K^+-ATPase as in the case of rubisco.

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