Interaction of the fluorescent probe RH421 with ribulose-1,5-bisphosphate carboxylase/oxygenase and with Na$^+$,K$^+$-ATPase membrane fragments

Joachim Frank [a], Athina Zouni [a,1], Arie van Hoek [b], Antonie J.W.G. Visser [b], Ronald J. Clarke [c,*]

[a] Department of Physical Chemistry, Fritz-Haber-Institut der Max-Planck-Gesellschaft, Faradayweg 4–6, D-14195 Berlin, Germany
[b] Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
[c] Department of Biophysical Chemistry, Max-Planck-Institut für Biophysik, Kennedyallee 70, D-60596 Frankfurt am Main, Germany

Abstract

Fluorescence titrations have shown that the voltage-sensitive probe RH421 interacts with the water-soluble protein ribulose-1,5-bis-phosphate carboxylase/oxygenase and with Na$^+$,K$^+$-ATPase membrane fragments. The probe exhibits significantly different fluorescence excitation spectra in pure lipid and pure protein environments. Experiments with a range of polyamino acids showed interactions of the probe with tyrosine, lysine and arginine residues. At saturating RH421 concentrations (≥ 5 μM) the probe quenches 60–75% of the total tryptophan fluorescence of the Na$^+$,K$^+$-ATPase preparation. Inhibition of the hydrolytic activity of the Na$^+$,K$^+$-ATPase occurs at RH421 concentrations in the micromolar range. This may be due to a probe-induced change in membrane fluidity. The sensitivity of the probe towards conformational changes of the Na$^+$,K$^+$-ATPase decreases hyperbolically as one increases the probe concentration. The decrease in sensitivity correlates well with association of the probe in the vicinity of membrane protein, as measured by tryptophan quenching. These results have important practical consequences for the application of RH421 as a voltage indicator in membrane preparations. Based on these and previously reported results, the fluorescent response of RH421 to the ATP-induced conformational change of the Na$^+$,K$^+$-ATPase is consistent with either a redistribution of dye from the liquid-crystalline lipid matrix into the vicinity of membrane protein or a reorganisation of the lipids surrounding the protein into a more rigid structure caused by the conformational change of the protein.

1. Introduction

Fluorescent styryl dyes are presently being widely employed for the visualization of voltage transients in membrane preparations [1–3]. The origin of the voltage changes is ion transport by protein channels and pumps embedded in the cell or organelle membrane, but the origin of the fluorescent response of the dyes is less clear. One possibility is that they act as true voltage indicators, responding directly to the change in membrane potential. Alterna-

tively, if the dyes interact with membrane proteins, a change in protein conformation may be sufficient to cause a fluorescence change of bound dye.

Klodos and Forbush [4] first showed that the styryl dye RH160 could be used to detect changes in the distribution of enzyme states of the Na$^+$,K$^+$-ATPase in membrane fragments. Since then styryl dyes have been used as probes of ion pump function in a number of laboratories [5–22]. In the experiments of Klodos and Forbush, no membrane potential was present because the fragments were open and, thus, short-circuited by the surrounding electrolyte. Therefore, they attributed the dye response either to local electric field effects within the membrane or to a direct interaction of the dye with the Na$^+$,K$^+$-ATPase. Since then Nagel et al. [5] have applied the same dye to the detection of substates of the H$^+$-ATPase of Neurospora,

* Corresponding author. Fax: +49 69 6303305; e-mail: clarker@kennedy.mpibp.uni-frankfurt.de.
[1] Present address: Max-Volmer-Institut, Technische Universität, Strasse des 17. Juni 135, D-10623 Berlin, Germany.

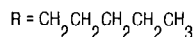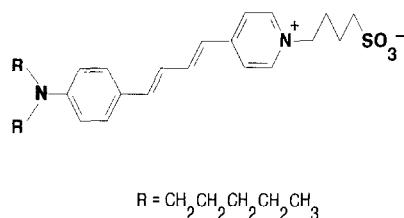R = CH$_2$CH$_2$CH$_2$CH$_2$CH$_3$

Fig. 1. Structure of RH421.

an analogue of the Na$^+$,K$^+$-ATPase. They concluded that the dye interacted directly with their protein. Similar conclusions were drawn by Schwappach et al. [6], who on the basis of fluorescence lifetime measurements suggested that the related styryl dye RH421 interacts with the Na$^+$,K$^+$-ATPase protein as well as with the lipid bilayer. On the other hand, Bühler et al. [7], who used RH421 to investigate the kinetics of the Na$^+$,K$^+$-ATPase, support the idea that the dye responds to local electric field strength changes within the membrane via an electrochromic mechanism.

In an attempt to clarify this situation we have carried out a detailed study of the spectral behaviour of the dye RH421 (see Fig. 1) in pure lipid membranes, in the presence of water-soluble globular proteins and in the presence of Na$^+$,K$^+$-ATPase-containing membrane fragments. The results of our experiments in pure lipid membranes have been published previously [14,23–25]. There it was found [25] that a major cause of the dye's response to field changes within the lipid matrix was solvatochromism, i.e., field-induced dye reorientation resulting in a change in polarity of the dye environment. Time-resolved fluorescence anisotropy measurements have indicated that the dye associates directly with the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco). In addition, it was found that in membrane fragments the order parameter of membrane-associated dye is increased in the presence of the Na$^+$,K$^+$-ATPase [26]. In the present paper we report on experiments aimed at further characterising the nature of the interaction with these two proteins and the effects of substrate binding on the dye fluorescence. Finally, the possible origin of the ATP-induced dye response in Na$^+$,K$^+$-ATPase preparations will be discussed in the light of the present and previous results.

2. 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. A series of stock solutions of the dye were prepared in ethanol. The solutions were 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 [24]. The exact dye concentration of each ethanolic solution was determined spectrophotometrically at 500 nm using a molar absorptivity of $(4.93 \pm 0.10) \cdot 10^4$ M^{-1} cm^{-1}. For spectral measurements in the presence of proteins, 5 μl of an ethanolic dye solution was added to a quartz cuvette containing 1 ml of aqueous solvent. The final solutions thus contained a small and constant percentage of 0.5% ethanol.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco; EC 4.1.1.39) was purified from spinach according to the method of Paulsen and Lane [27] with the exception of the last purification step, for which gel chromatography was applied [28]. 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 [27]. For the calculation of the molar concentration of rubisco a molecular weight of 535 000 g mol^{-1} has been assumed [29]. The activity of activated enzyme [30] was determined by a radiometric assay as described by Paulsen and Lane [27]. The specific activity was approx. 1.5 μmol CO$_2$/min per mg protein at 25°C. The purity of the enzyme was estimated from SDS-polyacrylamide gels stained with Coomassie blue [31,32] to be greater than 95%.

The preparation and purification of membrane fragments containing the Na$^+$,K$^+$-ATPase from the outer medulla of rabbit kidney has been carried out according to procedure C of Jørgensen [33,34]. The membrane fragments obtained by this method contain about 0.6 mg phospholipid and 0.25 mg cholesterol per mg protein [34,35]. This corresponds, assuming an average lipid molecular weight of 700 g mol^{-1}, to a molecular ratio of approx. 180 lipid molecules per $\alpha\beta$ unit of the Na$^+$,K$^+$-ATPase. 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, as determined by cryoelectronmicroscopy [36]. The protein concentration in the membrane suspension was determined to be 2.9 mg/ml using the Bradford method [37]. The specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay [38]. At 37°C the specific activity in the presence of Na$^+$ and K$^+$ ions was 700 μmol P$_i$/h per mg protein. At least 95% of the activity was ouabain-sensitive. For the calculation of the molar protein concentration of the preparation a molecular weight of an $\alpha\beta$ unit of the Na$^+$,K$^+$-ATPase of 147 000 g mol^{-1} has been assumed [39].

For comparison, experiments were also performed using a preparation of Na$^+$,K$^+$-ATPase membrane fragments obtained from the outer medulla of pig kidney. The preparation procedure was the same as that for the rabbit preparation. In this case the protein concentration in the membrane suspension was 2.1 mg/ml. The specific ATPase activity at 37°C was 2200 μmol P$_i$/h per mg protein.

All measurements with rubisco were performed in a buffer containing 20 mM Tris, 0.1 mM EDTA and 1 mM dithioerythritol. The pH of the buffer was adjusted to pH 8 with HCl. The measurements with Na^+,K^+-ATPase-containing membrane fragments were performed in a buffer containing 30 mM imidazole, 1 mM EDTA, 5 mM $MgCl_2 \cdot 6H_2O$ and 50 mM NaCl. 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: tris((hydroxymethyl)amino)methane (99.9%, Sigma), imidazole (99 + %, Sigma), EDTA (99%, Sigma), NaCl (analytical grade, Merck), $MgCl_2 \cdot 6H_2O$ (analytical grade, Merck), HCl (0.1 M Titrisol solution, Merck), dithioerythritol (> 99%, Biomol), ATP magnesium salt (approx. 95%, Sigma), ethanol (analytical grade, Merck), phospho*enol*pyruvate tricyclohexylammonium salt (98%, Boehringer-Mannheim), NADH disodium salt (ca. 100%, Boehringer-Mannheim) and pyruvate kinase/lactate dehydrogenase (suspension in ammonium sulfate solution, Boehringer Mannheim). 2-Carboxyarabinitol 1,5-bisphosphate was a gift of Prof. G. Lorimer, DuPont de Nemours and Co., Wilmington, USA. All polyamino acids were purchased from Sigma. The molecular weights of the various polyamino acids were between 14 000 and 25 000 g mol^{-1}.

Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL). DMPC unilamellar vesicles were prepared by the ethanol injection method described in detail elsewhere [23,24]. The final vesicle suspension contained no detectable trace of ethanol, i.e., [ethanol] ≤ 10 μM, according to a nicotinamide adenine dinucleotide/alcohol dehydrogenase enzymatic assay (Boehringer, Mannheim). Dialysis tubing was purchased from Medicell International (London, UK). The phospholipid content of the vesicle suspension was determined by the phospholipid B test from Wako (Neuss, Germany).

The absorbance measurements were performed with a Shimadzu UV-2100 UV-visible recording spectrophotometer. Steady state fluorescence measurements were recorded with a Shimadzu RF-5000 recording spectrofluorophotometer. To minimize contributions from scattering of the exciting light and higher order wavelengths, glass cut-off filters were used in front of the excitation and emission monochromators where appropriate. The temperature of the cuvette holders was thermostatically controlled.

Polarized time-resolved fluorescence decay curves of the tryptophan fluorescence of the pig kidney Na^+,K^+-ATPase membrane preparation in the presence and absence of RH421 were acquired using a picosecond laser system in combination with time-correlated single photon counting as described in detail elsewhere [40,41]. The excitation wavelength was 300 nm and the fluorescence was detected at 349 nm using a combination of a 348.8 nm interference filter (bandwidth 5.4 nm, Schott, Mainz, Germany) and a WG320 cut-off filter (Schott). The instrumental response function, corresponding to the laser pulse

convoluted with the detection response, was determined by measuring the fluorescence decay of p-terphenyl (BDH) in cyclohexane (spectroscopic grade, Merck) quenched by the addition of 30% (v/v) carbon tetrachloride resulting in a lifetime of (16 ± 3) picoseconds [42]. Polarized fluorescence intensity components $I_\parallel(t)$ and $I_\perp(t)$ were registered. From this the total fluorescence decay $S(t) = I_\parallel(t) + 2I_\perp(t)$ and the fluorescence anisotropy decay $r(t) = (I_\parallel(t) - I_\perp(t))/(I_\parallel(t) + 2I_\perp(t))$ were obtained.

The total fluorescence decay, $S(t)$, was fitted using the global analysis program described by Beechem et al. [43] (Global Unlimited TM, Urbana, IL USA) to the following equation:

$$S(t) = \sum_{i=1}^{m} \alpha_i e^{-t/\tau}$$ (1)

in which α_i is the pre-exponential factor belonging to the lifetime component τ_i and m 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 three components were required to have an acceptable fit to the experimental data. The first 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:

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

First order average lifetimes have been used to calculate the association constant of dye to membrane fragments because they are directly comparable to the fluorescence intensity from steady-state fluorescence experiments [44].

The calculation of the association constant, K, or the affinity, nK, of dye for the protein or membrane preparation [23] was performed using the commercially available least squares program ENZFITTER. The program was purchased from Biosoft (Cambridge, UK) and was run on an IBM-AT/386 compatible personal computer (mey-Soft, Berlin, Germany).

It should be noted that when dealing with membrane proteins embedded in a lipid matrix, the apparent affinity, nK, of a dye to the membrane can be defined in two different ways: (1) in terms of the molar protein concentration, or (2) in terms of the molar lipid concentration. In this paper all titrations in which membrane fragments are added have been expressed in terms of the protein concentration, so that, except where explicitly stated otherwise, we have used the first definition. The values can, however, be easily interconverted using the following equation:

$$nK_{Lipid} = nK_{Protein} \times \frac{(P)}{(L)} \times \frac{M_L}{M_P}$$ (3)

where $(P)/(L)$ is the protein/lipid weight ratio of the preparation and M_L and M_P are the molecular weights of lipid and protein, respectively.

3. Results

3.1. RH421 and rubisco

The addition of small volumes of a concentrated stock solution of rubisco (final concentration in the cuvette 1.5 μM) to an aqueous solution of RH421 (25 μM) causes a red shift of the dye's absorbance spectrum and an increase in the maximal absorbance of approx. 10%. Dye alone has a λ_{max} of 470 nm, whereas after the addition of rubisco λ_{max} shifts to 498 nm. These absorbance changes are accompanied by a large increase in fluorescence. Very similar spectral changes have previously been observed on disaggregation of dye in aqueous solution induced by changes in the dye concentration and in the ionic strength [14]. The spectral changes observed with rubisco can, therefore, be interpreted as being due to association of dye with the protein occurring simultaneously with dye disaggregation in the aqueous phase, whereby the absorbance and fluorescence changes arise partly because of the different environment within the protein but probably predominantly because of the change in aggregation state of the dye. A similar red shift of the absorbance spectrum was observed on addition of the protein lysozyme to RH421.

The fluorescence excitation spectrum of RH421 associated with rubisco is shown in Fig. 2. For comparison, the excitation spectrum of RH421 associated with dimyristoyl-phosphatidylcholine vesicles in the liquid crystalline state are also shown. It can be seen that significant shape changes in the excitation spectrum are observed on going from a pure protein environment to a pure lipid environment. When associated with rubisco the dye has a fluorescence excitation maximum at 550 nm and a shoulder at
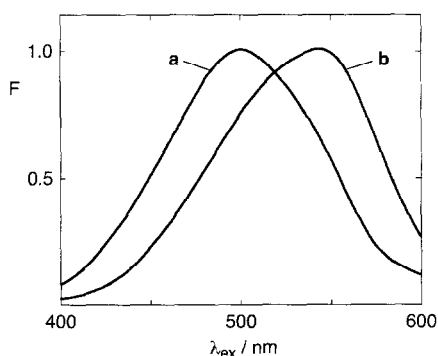
Fig. 2. Normalized fluorescence excitation spectra of RH421 in the presence of 500 μM of DMPC in the form of unilamellar vesicles (curve a) and in the presence of 9 μM of rubisco (curve b). The dye concentrations used were 6.4 μM and 1 μM for the measurements with DMPC and rubisco, respectively; $\lambda_{em} = 650$ nm (+RG645 cut-off filter), $T = 30°$C for DMPC and 25°C for rubisco.
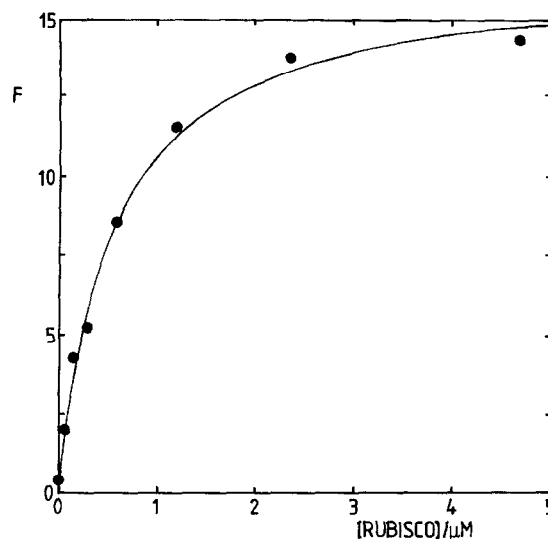
Fig. 3. Fluorescence, F, of RH421 in arbitrary units as a function of the rubisco concentration at a constant total dye concentration of 408 nM; $\lambda_{ex} = 500$ nm (+GG475 cut-off filter), $\lambda_{em} = 640$ nm, bandwidths = 3 nm, $T = 25°$C. The solid line represents a fit of the data to Eq. 4.

approximately 500 nm. When associated with DMPC vesicles the positions of the maximum and the shoulder are reversed, i.e., there is a maximum at 500 nm and a shoulder at approximately 550 nm.

The affinity, nK, of RH421 for rubisco was determined by fluorescence titration of the dye with protein (see Fig. 3) and fitting the fluorescence, F, to the total concentrations of dye, c_D^*, and protein, c_P^*, according to the following equation:

$$F = f_w c_D^* + (f_p - f_w) \frac{nK c_D^* c_P^*}{1 + nK c_P^*} \tag{4}$$

where f_w and f_p are the molar fluorescences (arbitrary units M^{-1}) of dye in the aqueous solution and associated with the protein, respectively. A derivation of Eq. 4, which is based on the assumption of a saturable number of association sites, is given in detail elsewhere [23]. The equation is mathematically equivalent to that previously derived by Bashford and Smith [45] for the association of dyes with lipid membranes. An equation of the same form, but using a different theoretical approach, has also been derived by Fromherz and Röcker [46]. Furthermore, under conditions where the fluorescence of dye in the aqueous solution can be neglected, Eq. 4 reduces to a form presented by Ehrenberg [47].

At 25°C, $nK = (1.8 \pm 0.3) \cdot 10^6$ M^{-1}. Upon association of the dye with rubisco, partial quenching of the protein's tryptophan fluorescence is observed. At saturating dye concentrations, i.e., greater than approx. 5 μM, the degree of quenching reaches a value of about 25%.

An estimate of the polarity of the dye association site can be obtained from the Stokes shift of associated dye. The fluorescence emission spectrum of bound dye has a maximum at 640 nm. This corresponds to a Stokes shift of

4455 cm^{-1}. Comparison with values measured in organic solvents [25] indicates a polarity similar to hexanol, i.e., relatively nonpolar.

Some fluorescence changes of associated RH421 can be induced by the addition of substrates of rubisco. The addition of a saturating concentration of $MgCl_2$ (20 mM) causes approximately a 1% decrease in fluorescence intensity across the whole emission spectrum. Subsequent addition of 2-carboxyarabinitol 1,5-bisphosphate (CABP), a transition state analogue of the substrate ribulose 1,5-bisphosphate, leads to a further 4% decrease in the fluorescence intensity. The addition of CABP in the absence of Mg^{2+} causes only a 1.5% decrease in the fluorescence intensity. The CABP-induced fluorescence changes are, therefore, significantly enhanced by the presence of Mg^{2+}. Mg^{2+} is a necessary catalytic cofactor in the CO_2 fixation reaction of rubisco [30]. Using the fluorescent probe 2-p-toluidinonaphthalene-6-sulfonate (TNS) Vater et al. [48] observed large ionic strength dependent changes in the fluorescence of TNS bound to rubisco on binding of Mg^{2+}. They interpreted these changes as arising from conformational changes of the enzyme-TNS complex. CABP binds to rubisco and in the presence of Mg^{2+} the binding is accompanied by a conformational change of the protein in the region of the active site [49]. The small CABP-induced fluorescence changes measured here using RH421 can, therefore, be attributed to substrate-induced conformational changes of the protein, leading either to a slight change in the molecular environment of the associated dye molecules or to a release of some dye molecules to the aqueous solution. No change in the absorbance spectrum of bound dye due to the substrates could be detected. The fluorescence decreases can therefore be attributed to a decrease in quantum yield rather than in the excitation efficiency.

3.2. RH421 and the Na$^+$,K$^+$-ATPase

The dye RH421 has been used in various laboratories to investigate the mechanism of the Na$^+$,K$^+$-ATPase [7–13,15–22]. 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 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. On the addition of ATP, the enzyme becomes phosphorylated and undergoes a conformational change during which the bound Na$^+$ ions are transported across the membrane [7,8,50–52]. In the presence of RH421 this activity of the Na$^+$,K$^+$-ATPase initiated by ATP is reflected in absorbance and fluorescence changes of associated dye. Up to now, however, little attention has been given to the question of whether the dye itself interferes with the enzyme activity or whether the magnitude of the ATP-induced spectral
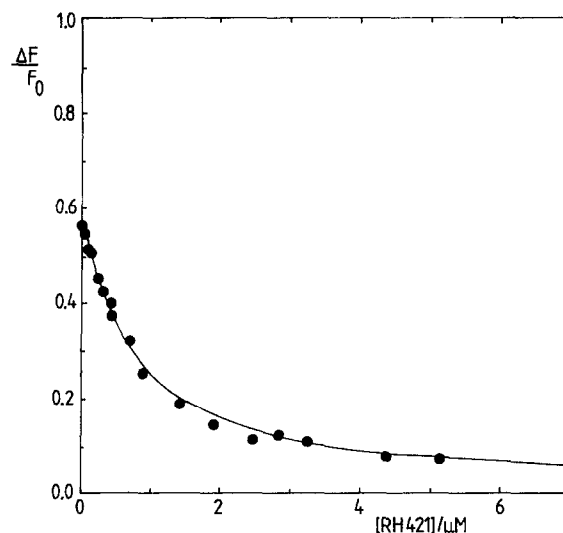

Fig. 4. Variation of the relative fluorescence change, $\Delta F/F_o$, of RH421 after the addition of 0.53 mM MgATP in the presence of Na$^+$,K$^+$-ATPase membrane fragments from rabbit kidney as a function of the dye concentration; [Protein] = 29 μg/ml, λ_{ex} = 570 nm (+OG550 cut-off filter), λ_{em} = 650 nm (+RG630 cut-off filter), bandwidths = 5 nm, T = 20°C. F_o refers to the fluorescence prior to the addition of ATP. The solid line represents a fit of the data to Eq. 11.

changes depends on the dye concentration. In the following section, therefore, the influence of RH421 on the Na$^+$,K$^+$-ATPase will be discussed.

It has been found that the magnitude of the ATP-induced fluorescence change of RH421 associated with Na$^+$,K$^+$-ATPase-containing membrane fragments is very dependent on the dye concentration used (see Fig. 4). The largest relative fluorescence changes, $\Delta F/F_o$, occur at low dye concentrations and as the dye concentration is increased there is a continual drop in $\Delta F/F_o$. This drop in $\Delta F/F_o$ could have two possible causes: (1) a dye-induced inhibition of the enzyme activity, or (2) a decrease in sensitivity of the dye to enzyme activity. In order to investigate the first possibility the enzyme activity has been determined by the pyruvate kinase/lactate dehydrogenase assay in the presence of increasing dye concentrations. For experiments in the absence of dye, 5 μl of ethanol was added in order to compensate for any effect of the solvent on the Na$^+$,K$^+$-ATPase activity or on that of the enzymatic assay system. At 20°C there is approximately a 30% reduction in the Na$^+$,K$^+$-ATPase activity on increasing the dye concentration from zero to 4 μM. Results of experiments carried out at 37°C are shown in Fig. 5. Control experiments in which the oxidation of NADH was measured after the addition of ADP showed no effect of RH421 on the activities of the enzymes pyruvate kinase and lactate dehydrogenase, indicating that the activity reduction is in fact due to the Na$^+$,K$^+$-ATPase. The decrease in enzymatic activity, however, is insufficient to explain the much larger dye concentration dependent drop in $\Delta F/F_o$ (cf. Fig. 4). At 4 μM dye $\Delta F/F_o$ has

already decreased by more than 80% from its maximum infinite dilution value. Therefore, it appears that the sensitivity of the dye must also be concentration dependent.

From measurements of tryptophan fluorescence quenching as a function of dye concentration and fitting of the data to Eq. A8 the apparent association constant, K, of RH421 with the Na^+,K^+-ATPase membrane fragments from rabbit kidney was determined to be $(1.5 \pm 0.3) \cdot 10^6$ M^{-1} (see Fig. 6B). At this stage, the origin of the tryptophan quenching should be considered. Time-resolved fluorescence measurements on Na^+,K^+-ATPase membrane fragments from pig kidney (see Figs. 7 and 8) have shown that RH421 reduces the first order average fluorescence lifetime of the tryptophan fluorescence from a value of 3.6 (± 0.1) ns in the absence of RH421 to 1.3 (± 0.2) ns in the presence of saturating concentrations of RH421. The corresponding values of the second order average lifetime [26,57] are 5.4 (± 0.1) ns for Na^+,K^+-ATPase membrane fragments alone, in agreement with the value of around 5 ns reported by Demchenko et al. [58], and 2.5 (± 0.2) ns in the presence of saturating concentrations of RH421. The quenching is, therefore, of a dynamic nature. Apart from its main absorbance band at around 500 nm, RH421 also has a less intense band in the ultraviolet region with an absorbance maximum at approximately 320 nm, which overlaps strongly with the fluorescence emission spectrum of tryptophan. The origin of the quenching is, therefore, most probably due to energy transfer in the excited state.

Energy transfer could occur either to dye molecules in the aqueous phase or to dye molecules associated with the membrane fragments (either in the lipid or the protein phase). First let us consider quenching from dye molecules in the aqueous phase. This would require diffusion of dye molecules from the aqueous phase into the vicinity of a
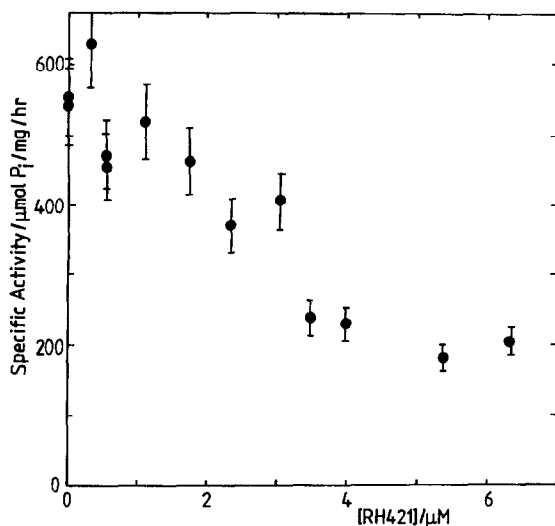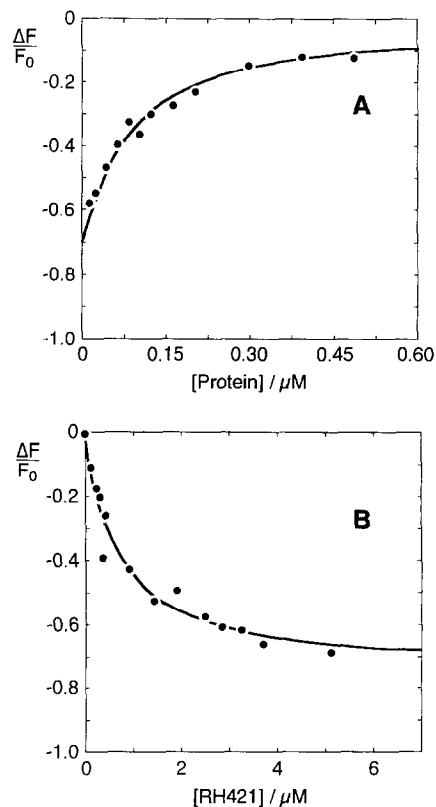

Fig. 6. Quenching of the tryptophan fluorescence of Na^+,K^+-ATPase membrane fragments from rabbit kidney by RH421. (A) Relative fluorescence change, $\Delta F/F_0$, of the Na^+,K^+-ATPase membrane fragments after the addition of RH421 as a function of the protein concentration; [RH421]= 510 nM, λ_{ex} = 280 nm (+WG230 cut-off filter), λ_{em} = 340 nm (+WG320 cut-off filter), bandwidths = 3 nm, T = 20°C. F_0 refers to the fluorescence prior to the addition of RH421. The solid line represents a fit of the data to Eq. A9. (B) Relative fluorescence change of Na^+,K^+-ATPase membrane fragments after the addition of RH421 as a function of the dye concentration; [Protein]= 29 $\mu g/ml$. The wavelengths, bandwidths and temperature were as in (A). The solid line represents a fit of the data to Eq. A8.

protein molecule. Using the second order average fluorescence lifetime, τ_0, of 5.4 ns and assuming a diffusion-controlled collision rate constant, k_q, of $1 \cdot 10^{10}$ M^{-1} s^{-1} [59] it can be shown from the definition of the Stern-Volmer dynamic quenching constant, $K = k_q \tau_0$, that a value of K of the order of 54 would be expected, i.e., far less than that experimentally observed. The quenching must, therefore, arise from interaction of tryptophan with dye molecules associated with the membrane fragments, i.e., either in the lipid phase or associated with protein.

Quenching of tryptophan by dye molecules in the membrane could arise from energy transfer to dye molecules already located prior to excitation in the direct vicinity of the tryptophan residues or to those diffusing to the protein during the excited state lifetime. According to Eftink [60], the diffusion-controlled quenching rate constant for the two-dimensional diffusion in membranes, k_q, can be approximated by

$$k_q \approx 1.58\,hDN_A \tag{5}$$

Fig. 5. Specific activity (μmol P_i/mg protein/h) of Na^+,K^+-ATPase from rabbit kidney in the form of membrane fragments as a function of the RH421 concentration; [Protein]= 1.8 $\mu g/ml$, T = 37°C. The specific activities were determined by the pyruvate kinase/lactate dehydrogenase assay as described under Section 2.
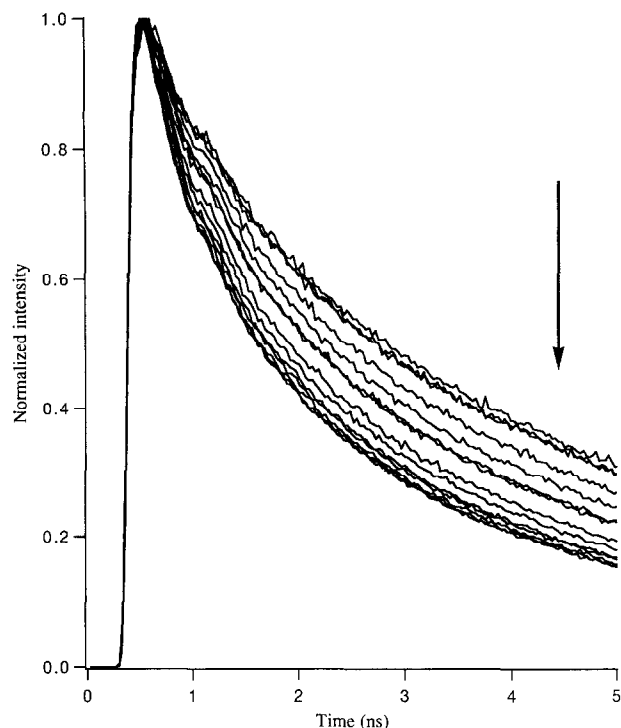
Fig. 7. Normalized fluorescence decays of the tryptophan fluorescence of Na^+,K^+-ATPase membrane fragments from pig kidney at increasing concentrations of RH421; [Protein] = 50 μg/ml, $\lambda_{ex} = 300$ nm, $\lambda_{em} = 349$ nm (+WG320 cut-off filter and a 348.8 nm interference filter), $T = 20°C$. The arrow indicates the direction of increasing dye concentration.

where h is the thickness of the membrane, D is the diffusion coefficient of the quencher and N_A is Avogadro's constant. Assuming a membrane thickness of 4 nm and a typical diffusion coefficient for a small molecule in a membrane of 10^{-8} cm^2 s^{-1} [61], it can be shown from Eq. 5 that k_q would have a value of the order of $4 \cdot 10^6$ M^{-1} s^{-1}. Multiplying this value by the fluorescence lifetime of tryptophan yields a value for the Stern-Volmer quenching
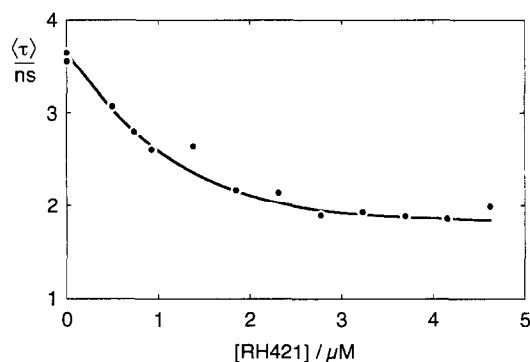

Fig. 8. Variation of the first order average fluorescence lifetime of the tryptophan fluorescence of Na^+,K^+-ATPase membrane fragments from pig kidney as a function of the RH421 concentration; [Protein] = 50 μg/ml, $\lambda_{ex} = 300$ nm, $\lambda_{em} = 349$ nm (+WG320 cut-off filter and a 348.8 nm interference filter), $T = 20°C$. The solid line represents a fit of the data to Eq. 9.

constant, K, of the order of 0.022 M^{-1}. The reciprocal of this value yields the concentration of quencher in the membrane required to quench half of the accessible tryptophans, i.e., approx. 46 M. It is not feasible that such a high concentration of dye is present in the membrane. The highest aqueous dye concentrations used here were in the micromolar range. It will be shown later that at non-saturating dye concentrations, the dye concentration in the membrane is approx. 10^5 times greater than that in the aqueous solution. Therefore, an aqueous dye concentration of 1 μM corresponds to a membrane dye concentration of around 0.1 M in a solution of highly diluted membranes. This is, however, still significantly less than the concentration of 46 M required for diffusional quenching of tryptophan fluorescence. Therefore, it seems that the quenching of the fluorescence of the Na^+,K^+-ATPase membrane fragments by RH421 can be confidently attributed to energy transfer from tryptophan residues to RH421 molecules located prior to excitation in the direct vicinity of the protein.

The proximity of the quenching dye molecules to the tryptophan residues of the protein can be estimated from the Förster theory [62,63] of resonance energy transfer. According to this theory, the critical transfer distance, R_0, (in nm) at which 50% of the excitation energy is transferred to the acceptor can be expressed by the following equation:

$$R_o = 9.78 \cdot 10^2 \left(\kappa^2 \cdot n^{-4} \cdot q \cdot J(\lambda) \right)^{1/6} \qquad (6)$$

where κ^2 is the orientation factor, n is the refractive index of the solvent (1.4), and q is the quantum yield of the tryptophan residues in the absence of dye. The spectral overlap integral, $J(\lambda)$, between tryptophan fluorescence and dye absorbance is given by,

$$J(\lambda) = \frac{\int F_d(\lambda)\, \epsilon_a(\lambda)\, \lambda^4 \, d\lambda}{\int F_d(\lambda)\, d\lambda} \qquad (7)$$

where $F_d(\lambda)$ is the fluorescence of tryptophan in arbitrary units, $\epsilon_a(\lambda)$ is the molar absorptivity of dye bound to the membrane fragments in M^{-1} cm^{-1} and λ is the wavelength. If we assume dynamic averaging of the tryptophan emission and dye absorption dipoles, the value of κ^2 can be approximated to be 0.67. The quantum yield of the tryptophans can be estimated by dividing the second order average lifetime of 5.4 ns quoted above by the radiative lifetime of tryptophan. Lifetime measurements of tryptophan residues of membrane proteins by Döring et al. [64], in which the tryptophans are located in an hydrophobic environment so that access to quenching agents is greatly restricted, have yielded a lifetime component of 30 ns. This value can be compared to the radiative lifetimes of the ^1L_a and ^1L_b transitions of tryptophan calculated by Henry and Hochstrasser [65] of 20 ns and 100 ns, respectively. Kirby and Steiner [66] determined the radiative lifetime of tryptophan in aqueous solution experimentally

to be 20 ns ($\pm 20\%$). Using the value of Döring et al. [64] as an estimate of the radiative lifetime of a tryptophan residue yields a quantum yield for the Na^+,K^+-ATPase of 0.18. The spectral overlap integral was evaluated according to Eq. 7 as $5.7 \cdot 10^{-15}$ cm^3 M^{-1}. Insertion of these values into Eq. 6 gives a value for R_0 of 2.3 nm.

The value of R_0 should be compared with the expected average distance between a protein and dye molecule in the membrane based on statistical reasons alone. For the measurements shown in Figs. 7 and 8 it is a simple matter to calculate the stoichiometric ratio of dye/protein from their relative concentrations. Thus, at the protein concentration used of 50 μg/ml ($\equiv 0.34$ μM) and a dye concentration of 1 μM there are approx. 3 dye molecules in the membrane for every protein molecule. The number of lipid molecules per dye is given by the number of lipid molecules per protein, i.e., 180 (see Section 2), divided by 3, which yields 60 lipids per dye. The membrane area occupied per protein molecule can be estimated from the reciprocal of the protein density (see Section 2). This yields a value of 10^2–10^3 nm^2 of membrane per protein molecule. The average distance between protein molecules is then given by the square root of this range, i.e., 10–32 nm. If one assumes that the protein molecules are evenly distributed on a hexagonal lattice across the membrane surface and that there is no preference for dye molecules to be located either close to or far from a protein molecule, then at the protein and dye concentrations stated above it can be shown by distributing the dye molecules evenly within the protein lattice (a centred rectangular lattice of dye gives the necessary stoichiometric ratio of 3:1), that the average distance between a dye molecule and a protein molecule is 6–20 nm, i.e., significantly greater than the R_0 value of 2.3 nm. The range 6–20 nm can be calculated theoretically [63] to yield expected quenching efficiencies of less than 1%. Fig. 8 shows, however, that at a dye concentration of 1 μM approximately 60% of the tryptophan fluorescence, which is accessible to quenching, has already been quenched. The degree of quenching is, therefore, much greater than that which would be expected from a merely statistical distribution of dye within the membrane. This suggests that the dye has the tendency to associate with the membrane preferentially in the neighbourhood of a protein molecule.

From the time-resolved fluorescence data of the pig kidney preparation it is also possible to calculate an apparent association constant of RH421 to the membrane fragments. Mathematically the decrease in the average fluorescence lifetime, $\langle \tau \rangle$, can be expressed as follows:

$$\langle \tau \rangle = \langle \tau \rangle_o - \frac{c_{DP}^*}{nc_P^*}(\langle \tau \rangle_o - \langle \tau \rangle_f) \qquad (8)$$

where $\langle \tau \rangle_o$ is the average lifetime of the tryptophan fluorescence in the absence of RH421 and $\langle \tau \rangle_f$ is the final average fluorescence lifetime when all quenchable trypto-
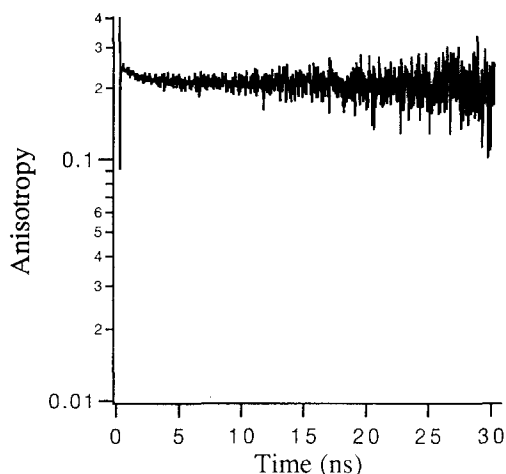

Fig. 9. Fluorescence anisotropy decay of the tryptophan fluorescence of Na^+,K^+-ATPase membrane fragments from pig kidney; [Protein] = 50 μg/ml, $\lambda_{ex} = 300$ nm, $\lambda_{em} = 349$ nm ($+$WG320 cut-off filter and a 348.8 nm interference filter), $T = 20°C$.

phan residues have been quenched. Substituting for c_{DP}^* from Eq. A3 for the condition of excess dye, this equation transforms to:

$$\langle \tau \rangle = \langle \tau \rangle_o - \frac{Kc_D^*}{1 + Kc_D^*}(\langle \tau \rangle_o - \langle \tau \rangle_f) \qquad (9)$$

Fitting the experimental data in Fig. 8 to Eq. 9 yields a value of $(8 \pm 2) \cdot 10^5$ M^{-1} for K. A corresponding fit of the second order average lifetime gives a slightly higher value of $(9 \pm 2) \cdot 10^5$ M^{-1}. Both values are of the same order of magnitude as that found for the rabbit kidney preparation, although the values for the pig kidney preparation are somewhat lower. This could perhaps be accounted for by differences in the protein content of the two preparations.

The anisotropy decay of the tryptophan fluorescence is also interesting to note (see Fig. 9). Except for a small initial drop in the anisotropy, which is possibly due to energy transfer between the tryptophan residues, the anisotropy hardly decays at all from a value of approx. 0.22. This indicates that the tryptophan residues are completely immobilized on the timescale of the measurement (0–30 ns), which is consistent with their location in the membrane fragments.

From previous measurements with lipid vesicles it is known that the dye associates strongly with lipid membranes [23,24]. The question therefore arises whether the dye is located preferentially in the lipid matrix of the membrane or in the vicinity of membrane protein. The tryptophan quenching experiments detect dye association in regions of close proximity to the tryptophan residues (within a distance of about 2 nm for quenching via resonance energy transfer). Fluorescence titrations in which the dye fluorescence was measured (see Fig. 10) yielded an apparent association constant, K, of $(6.0 \pm 0.7) \cdot 10^5$ M^{-1}. This value refers to association with the total membrane,
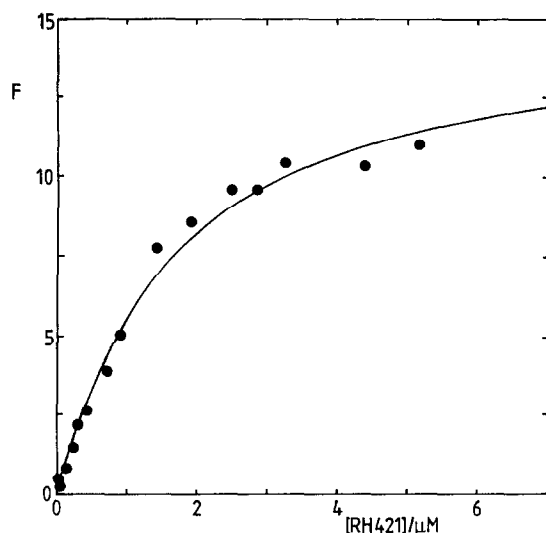
Fig. 10. Fluorescence, F, of RH421 in arbitrary units in the presence of Na^+,K^+-ATPase membrane fragments from rabbit kidney as a function of the dye concentration; [Protein] = 29 μg/ml, λ_{ex} = 570 nm (+OG550 cut-off filter), λ_{em} = 650 nm (+RG630 cut-off filter), bandwidths = 3 nm, T = 20°C. The solid line represents a fit of the data to Eq. A4.

i.e., lipid plus protein. The higher value obtained from tryptophan quenching indicates a slight preference of the dye for membrane areas close to a protein molecule.

The decrease in the ATP-induced fluorescence change correlates very well with the association of RH421 in the vicinity of membrane protein, as determined by tryptophan quenching (cf. Figs. 4 and 6B). Thus, it seems very likely that the decrease in $\Delta F/F_o$ at increasing dye concentrations is due to a decrease in dye sensitivity as a result of increased dye association either close or directly to the Na^+,K^+-ATPase. Assuming this to be the case, it is possible to construct a fit to the experimental data shown in Fig. 4. The decrease in $\Delta F/F_o$ is assumed to be proportional to the fraction of dye association sites occupied per protein molecule, c^*_{DP}/nc^*_P. Mathematically this can be expressed as follows:

$$\frac{\Delta F}{F_o} = \left(\frac{\Delta F}{F_o}\right)_o\left(1 - \frac{c^*_{DP}}{nc^*_P}\right) \tag{10}$$

where $(\Delta F/F_o)_o$ is the maximum value of $\Delta F/F_o$ at infinite dilution of dye. Substituting for c^*_{DP} from Eq. A3 for the condition of excess dye, this equation can be simplified to:

$$\frac{\Delta F}{F_o} = \left(\frac{\Delta F_o}{F_o}\right)_o\frac{1}{1 + Kc^*_D} \tag{11}$$

Fitting the experimental data for the rabbit kidney preparation in Fig. 4 to Eq. 11 yields a value of $(1.4 \pm 0.7) \cdot 10^6$ M^{-1} for K. This agrees very well with the association constant of $(1.5 \pm 0.3) \cdot 10^6$ M^{-1} calculated from tryptophan quenching. It, therefore, appears that the first few dye molecules which associate with the Na^+,K^+-ATPase membrane fragments give a strong response to the hydrolytic

activity of the protein, whereas further dye molecules give a reduced response.

From measurements of the steady-state tryptophan fluorescence quenching as a function of the protein concentration it is possible to estimate the affinity, nK, of dye for the membrane. Fitting the data to Eq. A9 yields a value of nK of $(1.23 \pm 0.12) \cdot 10^7$ M^{-1} (see Fig. 6A). This value can be compared with the partition coefficient, γ, of $2.5 \cdot 10^5$ reported by Bühler et al. [7] for the partitioning of RH421 between Na^+,K^+-ATPase-containing membrane fragments and aqueous solution. This partition coefficient corresponds to an affinity on the molar lipid concentration scale of approx. $1.9 \cdot 10^5$ M^{-1}[23]. This value can be converted to the molar protein concentration scale using Eq. 3 and the lipid/protein weight ratios given under Section 2. This yields a value of nK(protein) in the range 2–$4 \cdot 10^7$ M^{-1}, i.e., of the same order of magnitude as the value reported here of $1.23 \cdot 10^7$ M^{-1}. The differences in the two values could be accounted for by differences in the lipid constituents of the two preparations and in the lipid/protein weight ratios.

When the membrane fragments from rabbit kidney are saturated with dye, the quenching of the steady-state tryptophan fluorescence reaches a value of $(75 \pm 4)\%$. For comparison, the degree of quenching of the pig kidney preparation can be calculated from the ratio of the first order average fluorescence lifetimes in the absence of RH421 and in the presence of saturating concentrations. In this case the value is $(64 \pm 9)\%$. The two values are the same within experimental error. Both are significantly greater than that observed for rubisco. This could have a number of reasons. Firstly, rubisco has a much larger number of tryptophan residues contributing to its total fluorescence, i.e., 96 [29] in comparison to 16 for the Na^+,K^+-ATPase (12 in the α subunit [67] and 4 in the β subunit [68]). Secondly, the tryptophan residues of the Na^+,K^+-ATPase membrane fragments may be more accessible to quenching via energy transfer than those of rubisco. This could partly be accounted for by the smaller size of the Na^+,K^+-ATPase. Furthermore, one could imagine that dye molecules in the neighbouring lipid phase could quench tryptophan residues in transmembrane segments of the protein in the hydrophobic interior of the membrane, although according to Shull et al. [67,68] based on hydropathy calculations only two tryptophan residues in the α-subunit are located in transmembrane segments. In the case of rubisco, tryptophan residues in the hydrophobic core of the enzyme are likely to be inaccessible to quenching by the dye.

3.3. RH421 and polyamino acids

In order to identify the possible site of association with proteins, the interaction of the dye with a range of synthetic polyamino acids has been investigated. The absorbance spectrum was recorded for the dye alone in
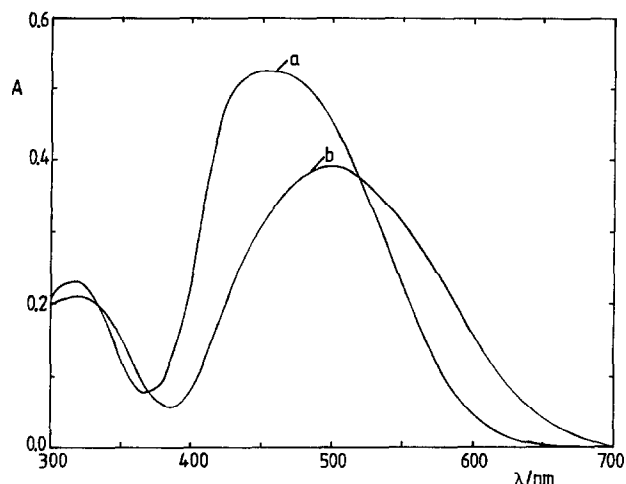
Fig. 11. Absorbance spectra of (a) RH421 alone and (b) in the presence of 1.7 μM poly(L-tyrosine) (molecular weight = 13 900 g mol^{-1}); [RH421] = 52 μM, bandwidth = 2 nm, pH 7.2, $T = 25°C$. After the addition of poly(L-tyrosine) 30 min equilibration time was allowed before recording the spectrum.

aqueous solution and after the addition of small volumes of concentrated stock solutions of the polyamino acids. Significant shifts in the absorbance spectrum were observed after the addition of poly(L-lysine), poly(L-arginine) and poly(L-tyrosine) (see Fig. 11). In all three cases a red shift of the spectrum was immediately apparent. Subsequently a slow decrease in absorbance occurred. After approximately 30 min no further spectral changes were observed. The time dependent absorbance decrease could be associated with the disaggregation of dye aggregates in the aqueous phase. Stopped-flow measurements [24] have shown this to be a slow process, occurring on the timescale of seconds–minutes. No spectral changes could be detected after the addition of poly(L-glutamic acid), poly(L-aspartic acid) or poly(L-serine).

4. Discussion

It has been found that the dye RH421 can interact strongly with proteins. Experiments with polyamino acids suggest that the sulfonate group may interact with positively charged side chains of lysine and arginine residues. A hydrophobic interaction between the aromatic chromophore and aromatic amino acids such as tyrosine is also likely to occur. Experiments with dimyristoylphosphatidyl-choline vesicles and with rubisco have shown that the fluorescence excitation spectrum of the dye is significantly different in pure lipid and pure protein environments (see Fig. 2). In biological membranes, any change in distribution of the dye between lipid and protein environments is, therefore, likely to induce a fluorescent response.

In systems in which both lipid and protein components are present, it cannot be assumed at this stage that the dye simply detects electric field changes in the lipid matrix

adjacent to ion translocating proteins. Interaction with the protein itself could produce fluorescence changes associated with protein conformational changes. Even when no significant charge movement occurs, a fluorescence change of associated dye might be possible, if the conformational change brings the dye into an environment of a different local polarity. This would result in solvatochromic changes in the dye's absorbance and fluorescence spectra. Support for such a mechanism has been found in pure lipid systems [25].

It is furthermore possible that the dye may be sensitive towards both conformational changes and electric field strength changes. Stürmer et al. [8] have shown that fluorescence changes of associated RH421 are observed on addition of Na$^+$ ions to Na$^+$,K$^+$-ATPase membrane fragments in the absence of ATP. Under these conditions the enzyme cannot undergo its major conformational change. Stürmer et al. [8], therefore, attributed the fluorescence changes to a small electric field strength change caused by Na$^+$ binding. Fendler et al. [69], on the other hand, have found evidence based on electrical measurements that Na$^+$ binding at the intracellular side of the membrane is an electrically neutral process. Therefore, the origin of these Na$^+$-induced RH421 signals, electric or conformational, is at present unclear.

Bühler et al. [7] have shown that the addition of ATP to Na$^+$,K$^+$-ATPase membrane fragments in the presence of K$^+$ ions and absence of Na$^+$ ions results in only minor changes in the fluorescence of associated dye, although under these conditions a significant conformational change of the enzyme is expected to occur. In this case it has been suggested [15] that the conformational change does not involve any significant electric field strength changes because of screening of the charge of the bound K$^+$ ions by negatively charged amino acid side chains. These results would seem to support the hypothesis that the dye is mainly detecting changes in electric field strength in the membrane interior. It cannot be concluded from this, however, that the dye is insensitive to all possible conformational changes of the protein.

Experiments on the sensitivity of RH421 in Na$^+$,K$^+$-ATPase-containing membrane fragments and its effect on enzyme activity have provided important practical information for the application of the dye in kinetic investigations of ion pump function. The sensitivity of RH421 towards the hydrolytic activity of the Na$^+$,K$^+$-ATPase has been found to be dye concentration dependent. In order to obtain the largest possible relative fluorescence changes in kinetic or equilibrium studies of the Na$^+$,K$^+$-ATPase mechanism, the dye concentration should be kept as low as possible, preferably well below 1 μM. The decrease in dye sensitivity is correlated to association of the dye in the vicinity of membrane protein. One can imagine two possible causes for the decrease in dye sensitivity. Firstly, it could be accounted for by the distance of the dye molecules from an active site of the enzyme. If the dye associates

preferentially close to an enzyme active site, then at low dye concentrations a large response would be expected. At higher dye concentrations, however, more dye would associate at a position further away from the active sites. This would produce a constant fluorescence background signal which could eventually swamp the fluorescence change. An active site is here defined as a part of the Na^+,K^+-ATPase molecule producing a signal which is detected by the dye. Since the mechanism of the dye response is not yet clear, it is not possible to specify the active site locations at this stage. Experiments of Schwappach et al. [18] showed, however, that after treatment of the membrane fragments with trypsin, which removes part of the enzyme containing the ATP binding site, Na^+-induced fluorescence changes of the dye were still apparent. Therefore, the ATP binding site is not a necessary requirement to obtain a dye response.

A second possible explanation for the drop in dye sensitivity is related to an electric screening effect of the dye. If the dye is in fact responding to changes in the electric field strength produced by charge movements of the enzyme, then the enzyme-induced electric field strength change would cause a polarisation of neighbouring dye molecules, creating an opposing electric field strength change. At high concentrations of associated dye only dye molecules located directly adjacent to an enzyme molecule would respond to the enzyme-induced electric field change. Dye molecules located further away would be screened from the field by the intervening dye molecules. As in the first explanation, the screened dye molecules would produce a constant background signal, eventually overwhelming the dye response.

At high dye concentrations (> 1 μM) it has also been found that the dye inhibits the activity of the Na^+,K^+-ATPase. The inhibition requires higher dye concentrations than that necessary to inhibit the fluorescent response of RH421 (cf. Figs. 5 and 4). Therefore, it cannot be simply attributed to dye association in the vicinity of membrane protein. Possibly it is due to association with the lipid matrix which may cause a stiffening of the membrane and consequently hinder the protein conformational change. Such an inhibition mechanism has been suggested for the inhibition of the Na^+,K^+-ATPase by cholesterol [70]. A dye-induced reduction in Na^+,K^+-ATPase activity may be able to partially explain a discrepancy reported by Forbush and Klodos [10] between dephosphorylation rates and the rates of transient signals monitored by RH421. There it was found that, above pH 7.5, the rate of the reaction monitored by RH421 was a factor two to four times slower than the dephosphorylation rate measured by ^{32}P release.

The addition of saturating concentrations of RH421 (≥ 5 μM) to Na^+,K^+-ATPase membrane fragments leads to 60–75% quenching of the tryptophan fluorescence. Similar experiments carried out by Tyson and Steinberg [71] using acrylamide as a quencher gave a maximal quenching of native protein fluorescence of 53%. The

larger degree of quenching obtained with RH421 indicates that more tryptophan residues are accessible to quenching. This can be explained by two possible effects. Firstly, RH421 can be separated from a tryptophan residue by a greater distance than acrylamide and still result in quenching. This is because of their different quenching mechanisms. Acrylamide is a collisional quencher [72], which requires direct contact with the fluorophore, whereas in the case of RH421 direct contact is not necessary because the quenching occurs via energy transfer. Secondly, because of its amphiphilic nature RH421 can penetrate further into the hydrophobic interior of the membrane and, therefore, may be able to quench tryptophans in transmembrane segments of the protein. According to Shull et al. [67,68] only two of the sixteen total tryptophan residues of the Na^+,K^+-ATPase are likely to be located in transmembrane segments. Due to their hydrophobic environment, however, they would be expected to have a significantly higher quantum yield than surface tryptophans, so that if quenching by RH421 is possible, a significant drop in fluorescence intensity could be expected.

The association of RH421 with proteins and in the vicinity of membrane protein is able to provide an explanation for experimental observations which were not previously understood. For example, Bühler et al. [7] reported that the sensitivity of styryl dyes in reconstituted vesicles was much lower than in Na^+,K^+-ATPase membranes. This effect could now be explained by the difference in the protein densities of the two preparations. In reconstituted vesicles the number of lipid molecules per protein molecule is much greater than in the membrane fragments. In the vesicles a large volume of lipid is, thus, available for the association of RH421. The lipid associated dye molecules which are located far from a protein molecule would produce a large constant background fluorescence, so that the relative fluorescence change arising from dye molecules associated with or in the close vicinity of the Na^+,K^+-ATPase would be significantly reduced. Klodos [16] has reported that the magnitude of the response of RH421 depends on the purity of the enzyme and on the enzyme source (pig or dog kidney, shark rectal gland, etc.). The effect of purity can be explained as above. The presence of membrane proteins other than the Na^+,K^+-ATPase to which RH421 can associate would, similar to lipid, produce a background fluorescence signal, which would decrease the dye response. The effect of enzyme source could be explained by differences in the association constant, number of association sites and the location of protein associated RH421 molecules, which could be reflected in a change in sensitivity.

Finally, the origin of ATP-induced fluorescence changes of RH421 in Na^+,K^+-ATPase membrane fragments should be discussed in the light of the present and previous results. In a previous publication it was shown that the shape of the fluorescence excitation spectrum of associated dye was dependent on the dye:membrane fragment ratio

and on the presence or absence of ATP (see Figs. 9 and 10 of [14]). At that time this was interpreted as being due to a shift in equilibrium between membrane-associated dye monomers and aggregates. Since then, however, it has been found [23] using pure lipid vesicles that the shape of the excitation spectrum is independent of the dye:lipid ratio and only depends on the phase of the membrane, i.e., different spectra are obtained in the gel and liquid-crystalline states. In a subsequent paper [25], it was, furthermore, found that the magnitude of voltage-induced fluorescence changes of RH421 in lipid vesicles was independent of the dye concentration. Since it is now known that the dye can interact with proteins, a more likely explanation for the changes in shape of the excitation spectrum reported previously [14] on changing the dye:membrane fragment ratio, is a shift in equilibrium between dye in the liquid-crystalline lipid matrix and dye in the vicinity of membrane protein. This is supported by a comparison of the excitation spectra of dye associated with Na^+,K^+-ATPase membrane fragments with that of dye associated with rubisco and with DMPC vesicles. At low dye/membrane fragment ratios the excitation spectrum of dye associated with Na^+,K^+-ATPase membrane fragments [14] resembles more closely that of rubisco(protein)-associated dye (see Fig. 2) or dye associated with gel-phase lipid membranes [23], whereas at high dye:membrane fragment ratios the excitation spectrum resembles more closely that of dye associated with liquid-crystalline lipid membranes (see Fig. 2). The ATP-induced fluorescence signals of RH421 are, therefore, consistent with either a redistribution of dye from the lipid matrix into the vicinity of membrane proteins, caused either by changes in the distribution of electric field lines within the membrane or due to the conformational change of the protein, or with a protein-induced conformational change of its surrounding lipids to a more rigid structure.
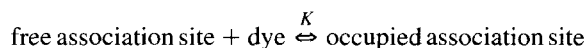
Acknowledgements

Appendix A

The association of a dye with a membrane can be most simply described by the following equilibrium:

$$\text{free association site} + \text{dye} \overset{K}{\Leftrightarrow} \text{occupied association site}$$

The intrinsic (microscopic) association constant, K, is defined by

$$K = \frac{c_{DP}^*}{(nc_P^* - c_{DP}^*)(c_D^* - c_{DP}^*)} \tag{A1}$$

where c_{DP}^* is the total concentration of associated dye, c_P^* is the total concentration of protein or lipid, c_D^* is the total concentration of dye (free plus associated) and n is the number of association sites per protein or lipid molecule. Here it is assumed that each association site can only accommodate one dye molecule and that all association sites have identical association constants. It should be noted that the term association site here does not imply a specific interaction as in the case of a substrate binding to the active site of an enzyme. Instead it is merely used as a mathematical convenience in order to set a limit to the number of dye molecules which can associate with a single protein or lipid molecule. The treatment is, therefore, similar to that described previously for the association of a dye with lipid vesicles [23]. Alternatively, the data could be treated in terms of a partition equilibrium of the dye between aqueous and membrane phases. The association and partition models are, however, mathematically equivalent and predict identical experimental behaviour [23,47], except at high dye concentrations where, in the case of the association model, saturation of the association sites would become apparent.

If one assumes that the dye can exist in only two distinct fluorescing states, then the fluorescence, F, is given according to Beer's law by

$$F = f_w c_D + f_P c_{DP}^* \tag{A2}$$

where f_w and f_p are the molar fluorescences (arbitrary units M^{-1}) in the aqueous solution and when associated with the membrane, respectively, and c_D is the concentration of free dye.

In order to determine spectroscopically the association constant of a dye to a membrane, a titration must be performed of dye at constant protein concentration. In the case of excess dye over occupied association sites ($c_D^* >> c_{DP}^*$) it can be shown from Eq. A1 that c_{DP}^* is given by

$$c_{DP}^* = \frac{nKc_D^* c_P^*}{1 + Kc_D^*} \tag{A3}$$

Substituting this expression into Eq. A2 for c_{DP}^* as well as $(c_D^* - c_{DP}^*)$ for c_D yields the following equation for the fluorescence under the condition of excess dye,

$$F = f_w c_D^* + n(f_p - f_w) \frac{Kc_D^* c_P^*}{1 + Kc_D^*} \tag{A4}$$

A nonlinear least squares fit of the observed fluorescence, F, at constant protein or lipid concentration to Eq. A4 as a function of the dye concentration allows the determination of the parameters f_w, $n(f_p - f_w)$ and K.

An alternative experimental method which can sometimes be used to determine the value of K is to measure the quenching of the tryptophan fluorescence of protein via energy transfer to the associated dye. This method is, however, only applicable when the separation distance between the associated dye and the tryptophan residues is sufficiently small so that significant quenching occurs. Assuming that in the fluorescence emission range of tryptophan there is no significant fluorescence from the dye itself, the fluorescence is given by

$$F = f_B(nc_P^* - c_{DP}^*) + f_c c_P^* \tag{A5}$$

where f_B is the molar fluorescence of tryptophan residues which can be quenched by the dye and f_C is the molar fluorescence of tryptophan residues which are incapable of being quenched. Prior to the addition of dye (quencher) the initial fluorescence, F_o, is

$$F_o = f_B nc_P^* + f_c c_P^* \tag{A6}$$

Subtracting F_o from F, the fluorescence change, ΔF, due to the dye is simply given by

$$\Delta F = -f_B c_{DP}^* \tag{A7}$$

Substituting for c_{DP}^* from Eq. A3, we have the following expression for the dependence of the relative fluorescence change, $\Delta F / F_o$, on the dye concentration,

$$\frac{\Delta F}{F_o} = \left(\frac{\Delta F}{F_o}\right)_{max} \frac{Kc_D^*}{1 + Kc_D^*} \tag{A8}$$

where $(\Delta F / F_o)_{max}$, the maximum fluorescence change when all the association sites are occupied, is equal to $-nf_B / (nf_B + f_C)$. The corresponding expression for titration with excess membrane, which can be used to determine the affinity, nK, is given by,

$$\frac{\Delta F}{F_o} = \frac{1}{n}\left(\frac{\Delta F}{F_o}\right)_{max} \frac{nKc_D^*}{1 + nKc_P^*} \tag{A9}$$

A nonlinear least squares fit of the relative fluorescence change, $\Delta F / F_o$, due to the dye (quencher) at constant protein concentration to Eq. A8 as a function of the dye concentration allows the determination of the parameters $(\Delta F / F_o)_{max}$ and K. The advantage of the tryptophan quenching method over the dye fluorescence method is that here there are only two fitting parameters rather than

three, because non-associated dye has no effect on the protein fluorescence.

In the case of membranes containing both lipid and protein components a difficulty arises because of the possibility that the dye could associate with the lipid and the protein components with different association constants. An exact mathematical treatment of the dye fluorescence would, therefore, require an extension of Eq. A2 so as to include the fluorescing states of both lipid-associated and protein-associated dye. If the molar fluorescences of protein-associated and lipid-associated dye are not significantly different, however, such a treatment becomes unfeasible. An approximate method for the treatment of biological membranes exists in determining apparent association constants via the dye fluorescence (Eq. A5) and the tryptophan fluorescence (Eq. A8) methods in terms of either the protein or the lipid content of the membrane. The dye fluorescence method yields an apparent association constant to protein plus lipid, whereas the tryptophan fluorescence yields an apparent association constant of dye in the direct vicinity of the protein. If the two values are significantly different, a preferential interaction of dye with either the protein or the lipid must be occurring.

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