# PHOSPHORESCENCE DEPOLARIZATION AND THE MEASUREMENT OF ROTATIONAL MOTION OF PROTEINS IN MEMBRANES

Christopher MOORE*, David BOXER and Peter GARLAND

*Department of Biochemistry, Dundee University, Dundee DD1 4HN, Scotland* and **Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North, New Zealand*

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## 1. Introduction

Rotational relaxation times of the order of many microseconds are typical of the presumably uniaxial rotation of membrane proteins [1]. So far measurements of rotational relaxation times have been limited to those proteins which are present at high occupancy either in the orginal membrane, as with the band-3 proteins of erythrocyte ghosts [2], or in highly purified fragments of membrane such as the acetylcholine receptor protein of the electric organ of *Torpedo marmorata* [3], or in reconstituted membranes [4]. This restriction arises from the relative insensitivity of the two measurement methods usually used, either saturation transfer EPR spectroscopy [5] or the decay of linear dichroism following flash photolysis of an attached triplet-forming probe such as eosin [6]. We describe here a method for determining rotational relaxation times ($\lesssim$1 ms) by measurement of the depolarization of laser flash-induced phosphorescence of erythrosin (tetraiodofluorescein) attached to the protein of interest. This new method provides experimental realisation of many earlier suggestions [7,8]: it exceeds the sensitivity of the photodichroism method [6] by a factor of $\sim 10^2$, and the saturation transfer method [5] by $\sim 10^4$. We illustrate the use of our method by describing the slow isotropic rotation of proteins in viscous media, and the anisotropic rotation of $Ca^{2+}$- dependent ATPase in sarcoplasmic reticulum membrane.

## 2. Materials and methods

Erythrosin isothiocyanate was synthesized from 5-aminofluorescein as in [9]. Sarcoplasmic reticulum was purified from rabbit skeletal muscle essentially as in [10]. Residual phosphorylase was removed by centrifugation following amylase treatment, and the membranes were further purified by centrifugation on a discontinuous sucrose density gradient. The $Ca^{2+}$-dependent ATPase of the sarcoplasmic reticulum (SR-ATPase) was assayed by measuring the $Ca^{2+}$-dependent release of ADP from ATP, and had spec. act. 2 units/mg membrane protein at 30°C and pH 7.2. We assumed that ~70% of the membrane protein was SR-ATPase. Bovine serum albumin was obtained commercially. Carbonate dehydratase (EC 4.2.1.1), phosphorylase *b* (EC 2.4.1.1) and rabbit mammary gland fatty acid synthase (EC unlisted) were gifts from colleagues. Erythrosin labelling of proteins or sarcoplasmic reticulum was carried out in the dark at room temperature and pH 8.0 for 1 h at a slight molar excess (~1.2–2.0-fold) of erythrosin isothiocyanate over protein, giving $\lesssim$1 mol erythrosin bound/mol protein. The mixture was adjusted to pH 7.0 and freed of noncovalently-bound erythrosin reaction products by passage through a column of Sephadex G-25. A preliminary treatment of the reaction mixture with activated charcoal was required in the case of albumin. The concentrations of bound erythrosin were calculated from absorption measurements at 540 nm, using $\epsilon_{mM} = 83\ mM^{-1} \cdot cm^{-1}$ and, for sarcoplasmic reticulum, clarification with sodium dodecyl sulphate. Viscosities of glycerol solutions were taken from published tables [11]. Solutions of erythrosin-labelled proteins were stored in the dark, and deoxygenated with argon [6] prior to phosphorescence measurements.

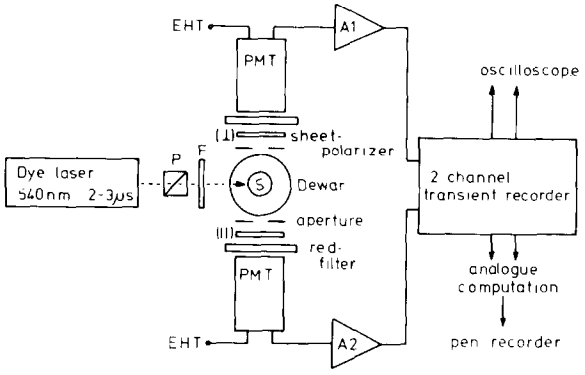


Fig.1. Measurement of polarized phosphorescence. A flash-lamp-pumped dye laser ($1.5 \times 10^{-4}$ M coumarin 6 in methanol) provided the excitation flash, which was polarized with a rotatable Glan-Taylor prism P and passed through a blue–green filter F (Schott glass BG18, 3 mm) to fall on the sample S (1 cm diam. tube) in a Dewar flask. Phosphorescence was viewed at 90° to the laser light path with two photomultiplier tubes PMT (EMI types 9798B with S20 cathode response) which were each shielded from the sample by an aperture, a sheet polarizing filter and a far-red pass filter (Schott RG695, 3 mm). The photomultipliers had independent high voltage supplies (EHT). Anode currents were converted to voltages with operational amplifiers A1 and A2 set to give a response time of 1 μs. Signals were stored in a two channel transient recorder, displayed on an oscilloscope, and plotted out through an analogue computer (see text) on a pen recorder.

Measurements of phosphorescence depolarization were made with the instrument outlined in fig.1. Two photomultipliers in a 'T'-format measured phosphorescence polarized parallel (∥) or perpendicular (⊥) to the plane of polarization of the laser excitation flash. Balancing of the two channels to identical gains was done by varying the photomultiplier high voltage supply until similar signals were given by each channel in response to excitation of the sample when the plane of polarization of the laser had been rotated through 90°. Final and more accurate balancing was then achieved by adjustment of gains in the analogue computing circuit intervening between the 2-channel transient recorder and the pen recorder. This circuit could be switched to display the phosphorescence signals $S_{\parallel}$ and $S_{\perp}$, their difference $S_{\parallel}-S_{\perp}$, the anisotropy parameter $r$ [12] where $r = (S_{\parallel}-S_{\perp})/(S_{\parallel}+2\,S_{\perp})$, and $\log_{10} r$.

## 3. Results

### 3.1. *Photochemistry of erythrosin covalently bound to proteins*

We had shown that erythrosin bound noncovalently to bovine serum albumin in anaerobic aqueous solution at pH 8.0 and 22°C exhibited a delayed emission spectrum with peaks at 555 nm (delayed fluorescence) and 685 nm (phosphorescence) following excitation with a 540 nm laser flash [13]. The quantum yields were $\sim 2 \times 10^{-3}$ for phosphorescence and $\sim 2 \times 10^{-4}$ for delayed fluorescence. The lifetime for phosphorescence was 0.35 ms. We have obtained similar results with covalently bound erythrosin, and fig.2 shows the delayed emission spectrum recorded 0.25 ms after excitation.

### 3.2. *Ability of erythrosin to report on slow rotational movements*

We labelled several proteins with erythrosin isothiocyanate and measured the depolarization of their laser-induced phosphorescence in glycerol solutions covering a wide range of viscosities. For a spherical molecule undergoing isotropic rotation, the anisotropy parameter $r$ of the laser-induced phosphorescence is time-dependent and is given by:

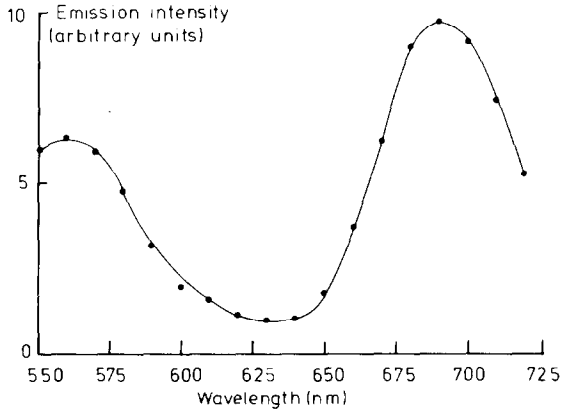


Fig.2. Delayed emission spectrum of erythrosin covalently bound to bovine serum albumin. Intensities were measured 0.25 ms after excitation with a 540 nm dye laser flash, using the apparatus in [13]. Albumin was 8 μM in anaerobic 0.1 M potassium phosphate buffer (pH 8.0), 22°C, with 1.1 mol erythrosin/mol albumin. The spectrum is not corrected for the ~4-fold fall in photocathode sensitivity as the wavelength rises from 550–700 nm.

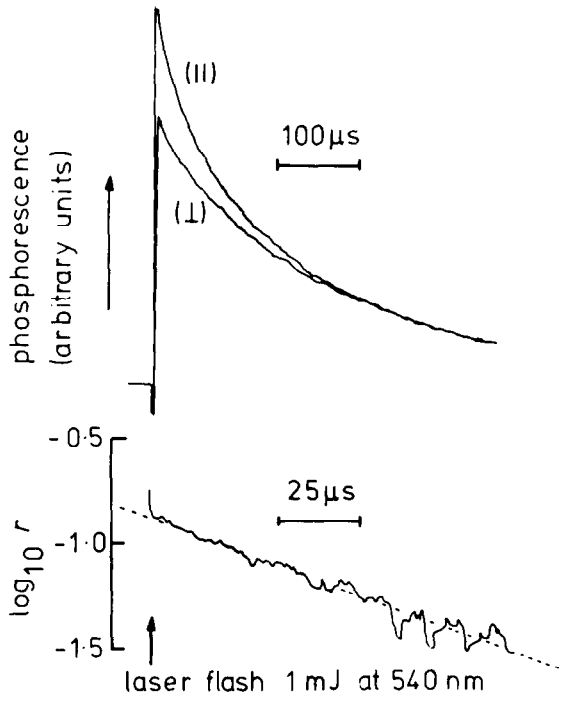


Fig.3. Phosphorescence depolarization of erythrosin-labelled bovine serum albumin. The sample contained $2.4 \times 10^{-7}$ M erythrosin-labelled albumin in 94% (w/v) glycerol at 10°C. The molar ratio of erythrosin to albumin was 1.0. The upper pair of traces shows the phosphorescence intensity polarized parallel (II) or perpendicular (⊥) to the plane of polarization of the laser flash. The lowest trace is the logarithm of the anisotropy parameter $r$, plotted at a 4-fold time scale expansion compared to the upper traces. The rotational correlation time was calculated from the slope (interrupted line) of the log $r$ plot ($\theta^{-1} = -2.303$ slope) and found to be 70 μs.

$$r_t = r_0 e^{-t/\phi} \qquad (1)$$

where $\phi$ is the rotational correlation time. Figure 3 shows the phosphorescence signals for erythrosin-labelled albumin. The plot of log $r$ against time was linear (fig.3) and from its slope we calculated that $\phi$ was 70 μs. Repetition of this experiment at other viscosities and with other proteins gave the results of fig.4 in which $\phi$ is plotted against solution viscosity for carbonate dehydratase (mol. wt 29 000), bovine serum albumin (monomer of 68 000), phosphorylase *b* (dimer of 200 000) and fatty acid synthase (dimer of 515 000). The linear relationship between $\phi$ and viscosity for each protein indicates that the depolarization of erythrosin phosphorescence reports on rotational mobility. If these proteins were spherical and had molecular diameters proportional to their molecular weights, then their rotational correlation times for a given viscosity and temperature would also be proportional to their molecular weights. In fact the ratios of the molecular weights (carbonate dehydratase:albumin:phosphorylase *b*:fatty acid synthase, 1:2.3:6.9:17.8) are in reasonable agreement with the ratios of the rotational correlation times (1:2.5:5.5:13.0) indicated by the relative slopes of the lines in fig.4.

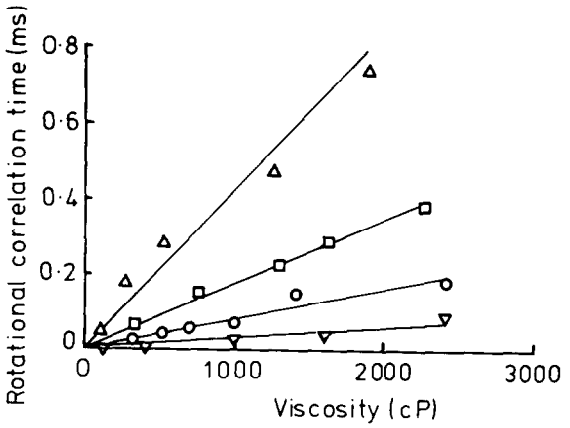


Fig.4. Dependence of rotational correlation time on viscosity. The rotational correlation times of erythrosin-labelled carbonate dehydratase ($3.5 \times 10^{-7}$ M), bovine serum albumin ($1.2 \times 10^{-7}$ M), phosphorylase *b* ($1.9 \times 10^{-7}$ M) and fatty acid synthase ($1.6 \times 10^{-7}$ M) were determined as in fig.3, using a variety of glycerol concentrations over 3–20°C. The concentrations just given are for the proteins; the molar ratios of erythrosin to protein were 1.0 for carbonate dehydratase (–▽–), 1.0 for bovine serum albumin (–○–), 0.5 for phosphorylase *b* (-- –) and 1.1 for fatty acid synthase (–△–).

### 3.3. *Rotation of the $Ca^{2+}$-activated ATPase of sarcoplasmic reticulum*

Our labelled sarcoplasmic reticulum preparation contained 1.23 nmol erythrosin/mg protein. If 70% of the protein were SR-ATPase carrying all of the label, then the molar ratio of erythrosin to SR-ATPase (mol. wt $10^5$) would be 0.18. Polyacrylamide gel electrophoresis of sodium dodecyl sulphate-solubilized membrane followed by inspection of the gels for erythrosin fluorescence prior to Coomassie blue

staining showed that almost all of the fluorescence was associated with the major polypeptide, mol. wt $\sim 10^5$ and assumed to be the SR-ATPase. Figure 5 shows the laser-induced phosphorescence of sarcoplasmic reticulum labelled with erythrosin. The phosphorescence anisotropy parameter decayed from a value of 0.12 immediately after the laser flash to a limiting value of ~0.025. A reasonable interpretation

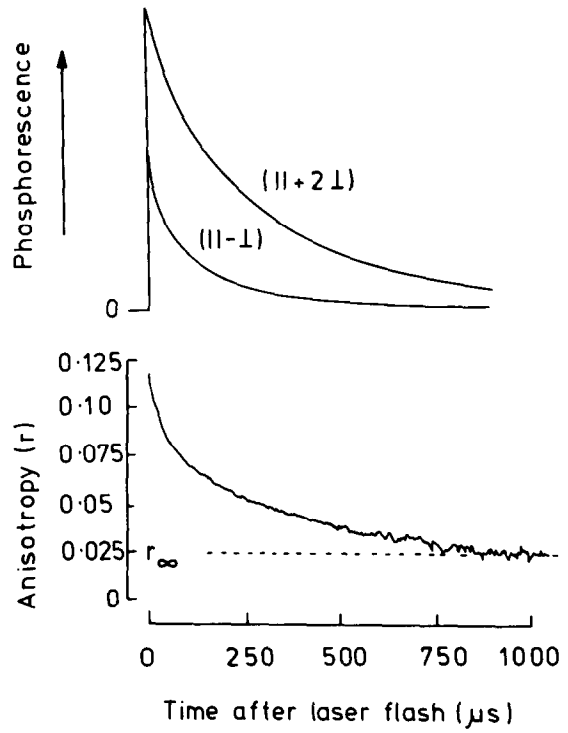


Fig.5. Depolarization of phosphorescence of erythrosin-labelled sarcoplasmic reticulum. The erythrosin-labelled membrane suspension was suspended in 250 mM sucrose, 100 mM KCl and 20 mM 3-(*N*-morpholino)propane sulphonate buffer (pH 7.0) at 13°C to a protein concentration of 0.69 mg/ml (erythrosin 0.85 μM). Phosphorescence was excited with repetitive laser flashes at 540 nm, ~1.0 mJ/flash at 1 Hz for 32 flashes, and the signals stored and averaged with a Nicolet 1170 signal averager. The phosphorescence intensities were recorded as ($S_{\parallel}$+2 $S_{\perp}$) and ($S_{\parallel}$–$S_{\perp}$), the latter on a 4-times greater sensitivity. The anisotropy parameter $r$ was computed with the signal averager, and the rotational correlation time calculated from the slope of the plot of log ($r_t$–$r_\infty$) against time, where $r_t$ is the anisotropy at time $t$ and $r_\infty$ is the limiting value or residual anisotropy, shown by an interrupted line in the fig. The overall response time of the measuring amplifiers was 1 μs, and the signals were digitized with a 1.1 μs dwell time and 12-bit resolution.

of this result is that the decay of anisotropy is due to rotation of the erythrosin-labelled SR-ATPase about an axis normal to the plane of the membrane, whereas the residual anisotropy is due to inability of the SR-ATPase to rotate about axes lying in the plane of the membrane. The rotational correlation time calculated from the decay of anisotropy is 210 μs at 13°C, which is in good agreement with estimates made by saturation transfer [14,15] or photodichroism of attached eosin [16].

## 4. Discussion

Our results show that erythrosin isothiocyanate is a sensitive phosphorescence depolarization probe for measuring rotational correlation times $\lesssim$ 1 ms. We have used erythrosin in preference to eosin because the latter has quantum yields for phosphorescence (and delayed fluorescence) several-fold lower. Also, erythrosin isothiocyanate can be prepared as the $^{125}$I derivate thereby providing a sensitive and quantitative means of identifying erythrosin-labelled molecules.

The sensitivity of the phosphorescence depolarization method used with a single excitation flash of 1 mJ at 540 nm and measurement of phosphorescence at 695 nm upwards is limited to samples containing $\geqslant$ 0.05 nmol erythrosin/mg membrane protein. Improvement of the instrumentation to include signal averaging at say a 10 Hz repetition rate with 5–10 mJ/flash, and collection of a greater spectral region of the phosphorescence [13] could improve the sensitivity to ~0.5 pmol erythrosin/mg protein, at which point study of membrane receptors for hormones and neurotransmitters becomes a possibility. The problem of the specific attachment of the probe to a particular protein of low abundance in the membrane may well be solved in individual cases by taking advantage of a naturally occurring ligand. For example, in unpublished work with Professor E. Barnard and Mr M. Lo, we have studied the rotation of acetylcholine receptors of *Torpedo* electric organ membrane by first attaching erythrosin to α-bungarotoxin which in turn binds to the receptor.

The use of delayed fluorescence depolarization of laser excited eosin label for measuring slow rotation of membrane proteins [17] was at least an order

of magnitude less sensitive than our use of erythrosin phosphorescence, and furthermore had an instrumental dead time of 76 $\mu$s due to a mechanical chopper that protected the photomultiplier tube from the laser flash and prompt fluorescence. Thus rotational correlation times $\lesssim 40$ $\mu$s would be difficult to measure. We think that the much higher sensitivity and simpler instrumentation of the phosphorescence depolarization method using erythrosin isothiocyanate offers considerable advantages over methods using saturation transfer [5], photodichroism [6] or delayed fluorescence of eosin [17]. At present the synthesis of only the isothiocyanate derivative of erythrosin has been described [9], but the synthesis of derivatives more specifically reactive, such as with thiol groups, should not be difficult.

In the photodichroism method using eosin the simplifying assumption has been made that the transition moments for excitation and measurement are parallel [2,6]. This is unlikely to be so for phosphorescence. A general equation describing the time dependence of anisotropy for non-parallel excitation and emission moments was given (eq. (4.16) of [18]) for the case of a fluorescent probe undergoing uniaxial rotation on the surface of a much larger sphere that underwent its own slower but independent isotropic rotation. This model, valid for phosphorescence as well as fluorescence, also serves for the uniaxial rotation of a membrane protein (i.e., the probe) if the rotation of the sphere (i.e., the membrane vesicle) is negligible, when the equation becomes:

$$r_t = A \exp(-Dt) + B \exp(-4Dt) + C \qquad (2)$$

where $A = \frac{6}{5} \sin\theta_1 \sin\theta_2 \cos\theta_1 \cos\theta_2 \cos\psi$

$$B = \tfrac{3}{10} \sin^2\theta_1 \sin^2\theta_2 (\cos^2\psi - \sin^2\psi)$$

$$C = \tfrac{1}{10} (3\cos^2\theta_1 - 1)(3\cos^2\theta_2 - 1)$$

and $\theta_1$ and $\theta_2$ are the angles between the axis of rotation and the transition moments for excitation and emission, $\psi$ is the angle between the projections of these moments in the plane normal to the axis of rotation, and $D$ is the rotational diffusion coefficient of the probe about that axis. Otherwise the analysis of the decay of anisotropy (e.g., fig.5) follows that in [4,6] developed for the special case where $\theta_1 = \theta_2$ and $\psi = 0$.

Finally, it can be noted that the phosphorescence lifetime, quantum yield, and sensitivity to quenchers are all environmentally sensitive, and as a by-product may provide information on changes in the conformation or associations of the membrane protein under study.

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