Biochimica et Biophysica Acta, 732 (1983) 347–351
Elsevier

BBA 71749

MONITORING MEMBRANE PROTEIN ROTATIONAL DIFFUSION USING TIME-AVERAGED PHOSPHORESCENCE

ELAINE K. MURRAY, COLIN J. RESTALL and DENNIS CHAPMAN

Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, University of London, Rowland Hill Street, London NW3 2PF (U.K.)

(Received March 3rd, 1983)

Key words: Phosphorescence anisotropy; Sarcoplasmic reticulum; Rotational diffusion; Membrane protein; ATPase; (Rabbit muscle)

Rotational motions of membrane proteins have previously been measured using time-dependent phosphorescence techniques. This paper discusses a method of examining membrane protein mobility at temperatures relevant to biological systems, using a technique similar to steady-state fluorescence. The method is demonstrated using sarcoplasmic reticulum ATPase labelled with erythrosin isothiocyanate, both in its natural condition and crosslinked by incubation with glutaraldehyde. The experimentally-observed dependence of phosphorescence anisotropy on temperature is compared to a calculated anisotropy-temperature curve. Comparison is made between the anisotropy decay curves obtained by time-averaged phosphorescence and steady-state fluorescence.

Introduction

Membrane proteins typically have rotational relaxation times in the range 10 μs–1 ms, their rotational diffusion cannot therefore be detected by fluorescence techniques as fluorescence lifetimes are of the order 1–100 ns. Triplet-state lifetimes however are comparable with membrane protein rotational relaxation times, hence such systems may be studied using phosphorescence depolarisation. Many examples have been reported, such as transient absorption dichroism [1–3] of intrinsic and extrinsic chromophores and, more recently, the flash-induced phosphorescence depolarisation of extrinsic chromophores [4,5]. These methods are highly sensitive, time-resolved, and can provide detailed information about protein rotation. They are, however, expensive, require complex electronics and obtaining data over a range of temperatures may take several days.

It had been suggested that 'steady-state' measurements of phosphorescence depolarisation might be used in an analogous manner to steady-state fluorescence [4]. 'Steady-state' phosphorescence anisotropy measurements of protein tryptophan residues at low temperatures (< -40°C) have been reported [6]. This paper reports a quick and convenient method which uses time-averaged phosphorescence anisotropies to give empirical information about membrane protein mobility over a range of temperatures relevant to biological systems.

Methods

Sarcoplasmic reticulum was isolated from the white muscles of the back and hind legs of rabbits using a method based on that of Nakamura et al. [7]. The white muscle, in a 0.1 M KCl/10 mM Tris-HCl buffer (pH 7.3) was homogenised twice and the mitochondria sedimented by centrifugation at $10\,000 \times g$ for 10 min. Further centrifugation at $30\,000 \times g$ for 1 h caused the sarcoplasmic reticulum to sediment. The pellets were resus-

pended in a KCl buffer (0.6 M KCl/10 mM Tris-HCl, pH 7.3) and incubated for 30 min at 4°C to dissolve the actin, the sarcoplasmic reticulum was then sedimented by centrifugation at 70 000 × *g* for 25 min. The sarcoplasmic reticulum was resuspended in a sucrose buffer (250 mM sucrose/1 M KCl/2 mM dithiothreitol/50 mM Tris-HCl, pH 8) to give a protein concentration of approx. 20 mg/ml and deep frozen.

The sarcoplasmic reticulum was purified by centrifugation through a 20–60° (w/v) continuous sucrose density gradient (1 M KCl/5 mM ATP/2 mM dithiothreitol/50 mM Tris-HCl, pH 8), and deep frozen. The molecular weight of sarcoplasmic reticulum adenosine 5′-triphosphatase (ATPase) was assumed to be 105 000.

Sarcoplasmic reticulum-ATPase was labelled by incubation with erythrosin isothiocyanate (Molecular Probes) in 1 : 1 ratio, 3 mg protein were used per experiment. After incubation for 1 h the unreacted probe was removed by centrifugation at 80 000 × *g* for 30 min and the pellet resuspended in 2.5 ml of 66% glycerol buffer. All these procedures were performed in the dark to prevent aggregation of the protein due to reaction with triplet-state oxygen, the reaction product of light-induced, triplet-state probe and atmospheric oxygen. The sample was poured into a 10 mm glass phosphorescence cuvette, sealed with a 'suba seal' self-sealing cap. Oxygen was removed by bubbling N_2 gas through the sample for 15 min, after which it was possible to expose it to light.

Crosslinking of the proteins was achieved by incubating the sample with 1 ml of 1% glutaraldehyde solution for 20 min prior to labelling.

The fluorescent probe 1,6 diphenyl-1,3,5 hexatriene was introduced into the lipid bilayer by incubation of 0.5 mg sarcoplasmic reticulum in 2.5 ml sucrose buffer with 5 μl of 2 mM diphenylhexatriene in tetrahydrofuran for 30 min.

Phosphorescence and fluorescence anisotropy intensity measurements were obtained using a Perkin-Elmer LS-5 Luminescence spectrometer, equipped with polarisation accessory 5212-3269 10 mm path length glass or quartz (in the case of fluorescence measurements), cuvettes containing 2.5 ml sample suspension were inserted into a four-position thermostatically-controlled cell holder (5212-3124), the temperature of which was controlled by a water bath. The temperature of the sample was determined by monitoring the temperature of 2.5 ml buffer in a similar cuvette in the cell holder, using a mercury thermometer.

Absorption fluorescence and phosphorescence spectra of 10 μM free erythrosin (2′,4′,5′,7′-tetraiodofluorescein) isothiocyanate in 66% glycerol buffer are shown in Fig. 1. The spectra were obtained using the Perkin Elmer LS-5 Luminescence spectrometer. The phosphorescence and fluorescence spectra were obtained using an excitation wavelength of 530 nm under anaerobic conditions. The phosphorescence intensity scale was approx. 100-times more sensitive than the fluorescence. It can be seen from the spectra that a maximum phosphorescence intensity is produced at 690 nm. The experiments were therefore performed using an excitation wavelength of 530 nm and emission wavelength of 690 nm.

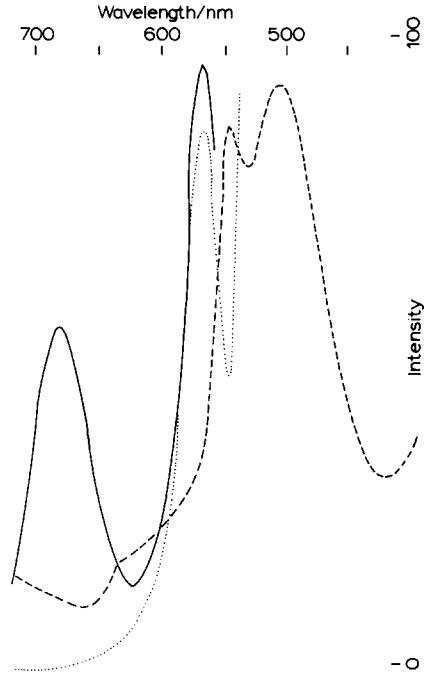

Fig. 1. The absorption (dashed line), fluorescence (dotted line) and delayed emission (solid line) spectra of 10 μM erythrosin isothiocyanate in 66% glycerol buffer. The delayed emission consists of a delayed fluorescence peak at 570 nm and a phosphorescence peak at 690 nm. The emission spectra were taken under anaerobic conditions with an excitation wavelength of 530 nm.

Steady-state fluorescence polarisation measurements have been extensively used to deduce information about lipid regions [8], apparent microviscosity values in biological membranes [9] and oils [10], structural order of lipids and proteins in membranes [11] and the effects of different concentrations of intrinsic molecules in bilayers [12]. A fluorescent probe, for instance diphenylhexatriene, is incorporated into the lipid bilayer, its rotational motion is a measure of the lipid fluidity. Recent evidence from time-resolved fluorescence studies have indicated that this rotational motion is more complex than originally realised, and steady-state fluorescence polarisation values themselves now tend to be used as an empirical measurement of membrane fluidity, without conversion into apparent microviscosity [13].

Phosphorescence anisotropy measurements are an indication of membrane protein mobility as the triplet probe is directly attached to the protein. The measurements differ from the fluorescence analogue in that it is not a steady-state technique. Unlike fluorescence measurements which are taken with constant illumination of the sample, phosphorescence is monitored after exposing the sample to a short burst of illumination. 30 μs is allowed to elapse before measurement takes place in order to eliminate light intensity due to the flash and delayed fluorescence. Phosphorescence emission is detected for a period of 5 ms and the intensity is displayed digitally.

The sample is excited by vertically polarised light and the components of emission parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the excitation beam are measured. Correction is made for the wavelength dependence of the polarisation response of the emission monochromator and photomultiplier by rotating the plane of polarisation of the excitation beam through 90° and measuring the correction ratio, or G factor, $I_{\perp}/I_{\parallel}$.

Results and Discussion

The strength of the interaction between the exciting beam and the phosphorescent probe molecules is proportional to $\mu \cos \theta$ where μ is the excitation dipole moment of the molecule and θ the angle between it and the electric vector of the exciting light. The emission dipole moment of the probe makes a constant angle α with the excitation dipole moment and therefore the relative intensities of the emission components $I_{\perp}$ and $I_{\parallel}$ result from the preferential excitation of the molecules whose excitation dipole is parallel to the excitation beam. This anisotropy is reduced as the protein molecules rotate during the recording interval after the excitation flash.

The phosphorescence anisotropy is defined as the difference in intensity between the parallel and perpendicular components, divided by the total phosphorescence intensity

$$r = \frac{G \times I_{\parallel} - I_{\perp}}{G \times I_{\parallel} + 2 \times I_{\perp}} \tag{1}$$

The triplet lifetime of erythrosin is approx. 1 ms, sufficiently long to detect anisotropy changes due to rotational motion of the order of 10^{-3}–10^{-5} s. Time-dependent, flash-induced anisotropy studies on erythrosin-labelled sarcoplasmic reticulum (Dale, R.E. and Restall, C.J., unpublished data) indicate that there are two separate components of phosphorescence anisotropy decay, one with a relaxation time of around 10 μs and one with a relaxation time of around 100 μs. The time-dependent anisotropy decay would therefore be approximated to

$$r(t) = B_1 \exp(-t/\phi_1) + B_2 \exp(-t/\phi_2) + B_3 \tag{2}$$

where ϕ_1 and ϕ_2 are the two components of rotational relaxation and B_3 the residual anisotropy at infinite time, also known as r_{∞}. The time-averaged anisotropy can be expressed in terms of the time-dependent anisotropy and phosphorescence decays

$$r = \frac{\int_{t_1}^{t_2} r(t) \cdot I(t) \cdot \mathrm{d}t}{\int_{t_1}^{t_2} I(t) \cdot \mathrm{d}t} \tag{3}$$

t_1 and t_2 are the boundaries of the measurement of the time interval. The simplest form of the phosphorescence decay is:

$$I(t) = I_0 \exp(-t/\tau) \tag{4}$$

where τ is the lifetime of the triplet state and I_0 the initial phosphorescence intensity. Although Vadas et al. [5] analyse their phosphorescence anisotropy

data using the Perrin equation, it is not correct to simplify Eqn. 3 to a form of this equation, as can be done for fluorescence anisotropy [5], because the system does not reach a steady-state condition.

On expansion Eqn. 3 becomes:

$$r = \frac{\int_{t_1}^{t_2}(B_1 \exp(-t/\phi_1)\cdot(I_0 \exp(-t/\tau))\cdot dt}{\int_{t_1}^{t_2} I_0 \exp(-t/\tau)\cdot dt} + \frac{\int_{t_1}^{t_2}(B_2 \exp(-t/\phi_2)+B_3)\cdot(I_0 \exp(-t/\tau))\cdot dt}{\int_{t_1}^{t_2} I_0 \exp(-t/\tau)\cdot dt} \quad (5)$$

The time interval of measurement was chosen such that at t_2 the anisotropy will have decayed almost to its value at infinite time. The time-averaged anisotropy for a system having two rotational components can therefore be expressed as:

$$r = B_1\left(\frac{\phi_1}{\tau+\phi_1}\right)\exp(-t_1/\phi_1) + B_2\left(\frac{\phi_2}{\tau+\phi_2}\right)\cdot \exp(-t_1/\phi_2) + B_3 \quad (6)$$

It should also be noted however that τ also exhibits a temperature dependence.

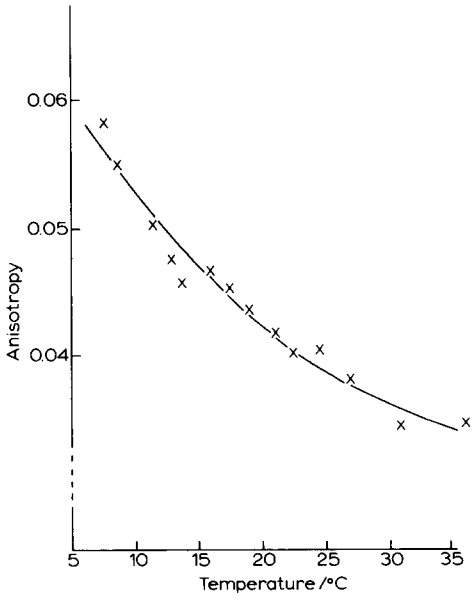

Fig. 2. A comparison between experimentally obtained time-averaged phosphorescence anisotropy of erythrosin-labelled sarcoplasmic reticulum ATPase (×) and that calculated from Eqn. 6 (solid line).

The time-dependent data indicates that ϕ_1 may vary from about 800 μs at 5°C to 80 μs at 37°C, and that ϕ_2 may vary from 12 μs to 8 μs over this temperature range. Fig. 2 illustrates that, by choosing suitable values for B_1, B_2 and B_3, the experimental data agrees well with the values of r calculated by the simple method given above, where B_1 and B_2 take the value 0.02 and B_3 the value 0.035. It is found that the faster rotational component contributes little to r under the experimental conditions. At higher temperatures, r approaches the value of B_3 but at lower temperatures, the term in B_1 also contributes significantly. The time-averaged anisotropy measurements are in agreement with the data obtained by time-dependent methods.

Fig. 3 compares the anisotropy decay curves obtained by steady-state fluorescence of diphenylhexatriene incorporated into the sarcoplasmic reticulum membrane with those obtained using the phosphorescence method. Although the magnitude of the anisotropies obtained by fluorescence is approx. 5-times those obtained by phosphorescence, the temperature dependence of the two curves shows a similar pattern, indicating that both experiments may be affected by the same factors, such as bilayer fluidity and protein concentration.

Comparison between the anisotropy and temperature dependence of mobile and immobile protein was also made, as illustrated in Fig. 4. On crosslinking the sarcoplasmic reticulum-ATPase with glutaraldehyde a higher value for the phosphorescence anisotropy, which is temperature-independent over the range studied, was observed. The technique can therefore be used for studying changes in the mobility of a membrane protein under different conditions. It can be used to provide a rapid, empirical method for investigating the effects of such changes on the various processes involved in the dynamics of membrane proteins. A detailed description of the different motions involved in anisotropy decay requires the analysis of time-resolved spectroscopic information. We propose to carry out further comparisons between time-resolved and time-averaged phosphorescence anisotropy measurements and to develop the latter technique for other investigations into protein mobility. Time-averaged phosphorescence could be

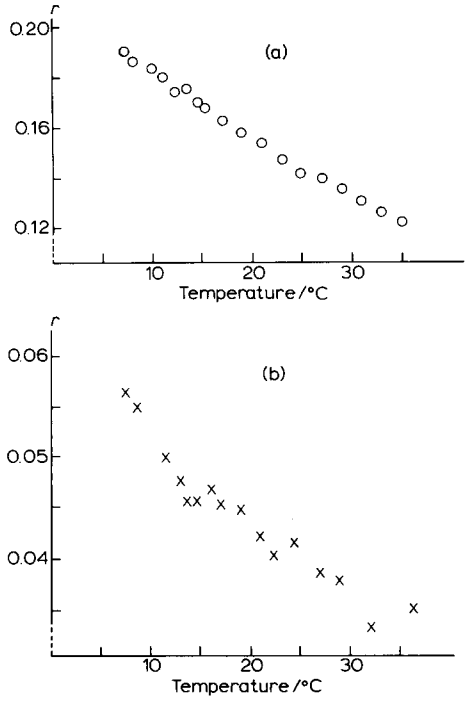

Fig. 3. Fluorescence anisotropy measurements of diphenylhexatriene in the sarcoplasmic reticulum membrane (a) and time-averaged phosphorescence anisotropy measurements of the labelled sarcoplasmic reticulum protein (b) show a similar temperature dependence in their decay curves.

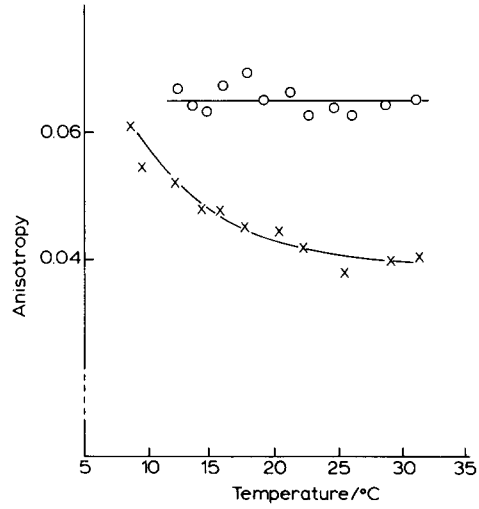

Fig. 4. Time-averaged phosphorescence anisotropy of erythrosin-labelled sarcoplasmic reticulum-ATPase (×) and erythrosin-labelled sarcoplasmic reticulum ATPase crosslinked by incubation with glutaraldehyde (○), showing the higher anisotropy and temperature independence of the latter sample.

useful in laboratories which do not have access to instruments capable of doing time-resolved studies.

Acknowledgements

We wish to thank the SERC and the Muscular Dystrophy Group of Great Britain for financial support for E.K.M. and C.J.R. and the Wellcome Trust for support for D.C.

References

1 Naqvi, R.K., Gonzalez-Rodriguez, J., Cherry, R.J. and Chapman, D. (1973) Nature (London), New Biol. 245, 249–251
2 Cherry, R.J. (1979) Biochim. Biophys. Acta 559, 289–327
3 Chapman, D. and Restall, C.J. (1981) Biochem. Soc. Symp. 46, 139–154
4 Garland, P.B. and Moore, C.H. (1979) Biochem. J. 183, 561–572
5 Austin, R.H., Chan, S.S. and Jovin, T.M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5650–5654
6 Vadas, E.B., Melancon, P., Braun, P.E. and Galley, W.C. (1981) Biochemistry 20, 3110–3116
7 Nakamura, H., Jilka, R.J., Boland, R. and Martonosi, N. (1976) J. Biol. Chem. 251, 5414–5423
8 Shinitsky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394
9 Shinitsky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133–149
10 Hare, F. and Lussan, C. (1977) Biochim. Biophys. Acta 467, 262–272
11 Jahnig, F. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 6361–6365
12 Hoffmann, W., Pink, D.A., Restall, C.J. and Chapman, D. (1981) Eur. J. Biochem. 114, 585–589
13 Van Hoeven, R.P., Van Blitterswijk, W.J. and Emmelot, P. (1979) Biochim. Biophys. Acta 551, 44–54