TEMPERATURE SENSITIVITY OF FLUORESCENCE PROBES IN THE PRESENCE OF MODEL MEMBRANES AND MITOCHONDRIA

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1. Introduction

Biological membranes have been hypothesized to undergo phase transitions as a requirement of their physiological functions [1,2]. Various techniques have been applied to study the proposed phase transitions in both model and natural membranes, including X-ray analysis [3], electron microscopy [4,5], thermal capacitance measurements [6] and recently use of spin labeled probes [7] and fluorescent probes [8,9]. Use of fluorescent probes appears especially promising in the detection of changes in membrane structure in view of the well established response of fluorescence probes to the energy state of biological membranes [10] and their extreme sensitivity to environment [11]. In this report phospholipid dispersions obtained by ultrasonication were used to calibrate fluorescence polarization responses observed in natural membranes. Fluorescent probes demonstrated by X-ray analysis to be located in different regions of model membranes [12] were chosen for this work. These include 8-anilino-1-naphthalene sulfonate (ANS), which on the basis of model systems is demonstrated to be a probe of the aqueous interface; dansylphosphatidylethanolamine (DPE) and octadecyl naphthalene sulfonate (ONS), shown to be probes of the polar head group region, and 12-(9-anthroyl)-stearic acid (AS) which is a probe for the hydrocarbon region of the membrane. Each of these dyes was found to be sensitive to the melting of the hydrocarbon chains of dipalmitoyl lecithin, evidenced by a discontinuity in the Perrin plots at 40°. Incorporation of cholesterol in a 1:1 molar ratio with dipalmitoyl lecithin completely abolished the discontinuity in the Perrin plot of AS polarization. Perrin plots of AS or ANS fluorescence polarization in the presence of pigeon heart or rat liver mitochondria, or in the presence of egg lecithin were linear under the conditions used, in contrast to the biphasic curve obtained with pure dipalmitoyl lecithin.

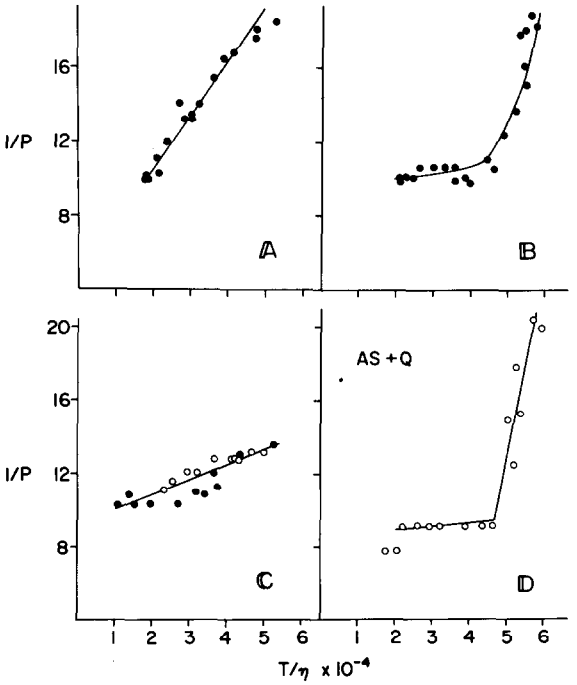

Fig.1. Perrin plots of AS in the presence of artificial membranes. All samples were sonicated in the presence of 10 μM AS and 1 mM phosphate buffer, pH 7.2. Excitation was at 380 nm; emission measured at 450 nm. A) 1 mg egg lecithin/ml; B) 1 mg dipalmitoyl lecithin/ml; C) 1 mg dipalmitoyl lecithin/ml and 0.5 mg cholesterol/ml; D) 1 mM dipalmitoyl lecithin and 0.1 mM coenzyme Q_7.

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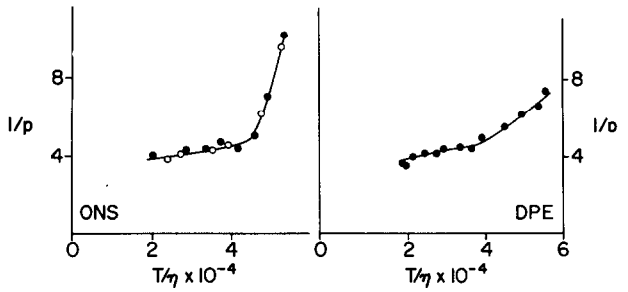

Fig.2. Polarization of ONS and DPE incorporated into dipalmitoyl lecithin micelles. Dipalmitoyl lecithin (1 mg/ml) was sonicated with 10 μM ONS (left) or 10 μM DPE (right) in the presence of 0.5 mM phosphate buffer, pH 7.2. Excitation for ONS, 350 nm; emission, 430 nm. Excitation for DPE, 340 nm; emission, 490 nm. T/η was varied by increasing (•) or decreasing (○) the temperature.

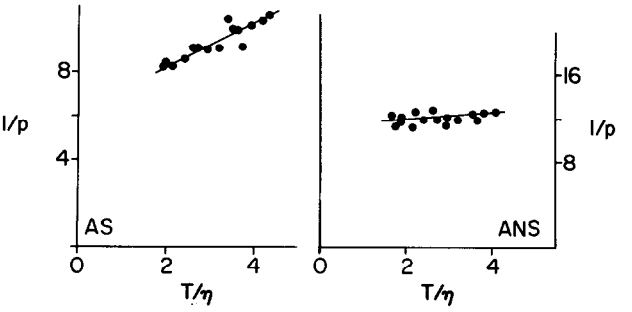

Fig. 3. Polarization of AS and ANS in the presence of pigeon heart mitochondria.

Left: Mitochondria (1 mg/ml) were incubated in the presence of 10 μM AS, 0.225 M mannitol, 0.075 M sucrose and 0.05 M morpholinopropane sulfonate, pH 7.2 for 1 hr. Excitation, 380 nm; emission, 450 nm.

Right: Mixture contained 1 mg mitochondrial protein/ml, 30 μM ANS, 225 M mannitol, 0.075 M sucrose, 0.05 M morpholinopropane sulfonate and 0.2 mM KCN. Excitation, 360 nm; emission, 470 nm. Due to low fluorescence intensity, unpolarized light was used for excitation.

2. Materials and methods

AS, ONS and DPE were the gracious gifts of Drs. A.S. Waggoner and L. Stryer [13]. ANS was obtained from Eastman Organic Chemicals and was recrystallized twice from hot water as the ammonium salt. Coenzyme Q and egg lecithin were the gifts of Drs. K. Folkers and K. Gulik, respectively. L-alpha-lecithin dipalmitoyl (synth) was obtained from Schwarz/Mann. Aqueous suspensions of phospholipids were dispersed ultrasonically with a Branson sonifier prior to each experiment for 1 min.

Fluorescence was measured at 90° from the exciting beam using a Hitachi MPF-2A fluorescence spectrometer. Fluorescence polarization was measured with the aid of a 105PB Polacoat lens inserted between the exciting beam and the cuvette and between the cuvette and the photodetector.

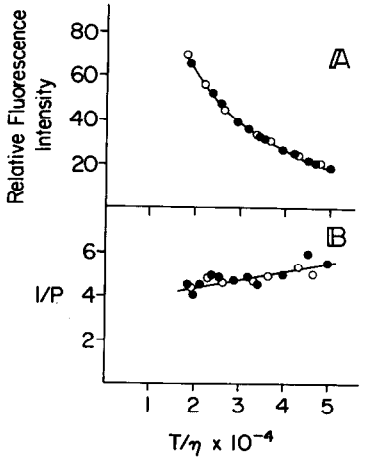

Fig. 4. Interaction of ANS with mitochondrial lipid. Mixture contained about 4 mg mitochondrial lipid/ml, 5 mM PO_4 buffer, pH 7.4 and 30 μM ANS. Excitation, 360 nm; emission, 470 nm.

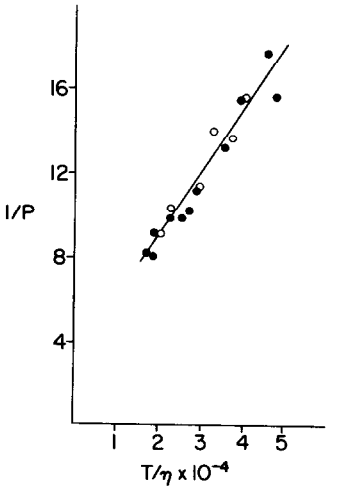

Fig. 5. Polarization of AS in the presence of mitochondrial lipid. Mitochondrial lipid (about 4 mg/ml) was sonicated in the presence of 10 μM AS and 5 mM PO_4 buffer, pH 7.4. Excitation, 380 nm; emission, 450 nm.

Polarization P was defined as

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are corrected light intensity parallel and perpendicular to the exciting beam. Results were plotted according to the Perrin equation [14]. Except where noted, polarized light was used for excitation.

Pigeon heart mitochondria were prepared according to Chance and Hagihara [15] and rat liver mitochondria essentially according to Schneider [16]. Mitochondrial lipid was extracted with 90% acetone [17].

3. Results and discussion

3.1. *Fluorescence polarization of dyes incorporated into artificial membranes*

A plot of reciprocal polarization versus temperature over viscosity is linear for AS incorporated into egg lecithin (fig. 1A). In contrast, the Perrin plot for AS polarization in the presence of dipalmitoyl lecithin shows a sharp discontinuity at around 40°, at which temperature other studies have indicated melting to occur [18]. Incorporation of cholesterol in a 1:1 molar ratio to dipalmitoyl lecithin abolishes the discontinuity in the curve and results in lower polarization values for AS below the melting point of dipalmitoyl lecithin and higher values above the melting point (fig. 1C). These results suggest that cholesterol disrupts the crystalline array of lecithin fatty acid chains, but under conditions when the chains are melted, reduced mobility occurs in the presence of cholesterol. A similar conclusion was obtained using spin-labeled fatty acid in the presence of saturated lecithins [19]. Incorporation of oxidized coenzyme Q, which is known to quench AS fluorescence both in model systems and in mitochondrial membranes [2], into dipalmitoyl lecithin micelles, results in an increased polarization of AS below 40°, but at the concentrations of Q used (1:10 molar ratio with dipalmitoyl lecithin) the discontinuity in the Perrin plot persists (fig. 1D).

It is of interest to note that the change in hydrocarbon fluidity at the melting point of dipalmitoyl lecithin, inferred from AS depolarization, is also observed using ONS (fig. 2A) or DPE (fig. 2B) as fluorescent probes. A similar result was obtained with ANS (not shown) in support of previous findings [21,22]. Our results concerning DPE polarization are essentially in agreement with those of Lusson and Faucon [23]; however, their failure to observe changes in ANS polarization at the melting point of dipalmitoyl lecithin is unresolved.

3.2. *Fluorescence polarization of ANS and AS in the presence of mitochondria*

The Perrin plots of AS and ANS in the presence of pigeon heart mitochondria (fig. 3) or in the presence of lipid extract of mitochondria (figs. 4 and 5) are linear. Similar results were obtained when rat liver mitochondria were substituted for pigeon heart mitochondria. While polarization of AS and ANS decreases with increasing temperature, the absence of a discontinuity in the curve from 0 to 40° is indicative that a structural change such as a phase transition does not occur under these conditions.

In contrast to the lack of any real evidence for a phase transition occurring in mitochondria as detected by ANS or AS, the spin-labeled compound 12-nitroxide stearate has been reported to be sensitive to phase transitions in a variety of mitochondria. Reasons for the discrepancy between the results observed with AS or ANS and the spin-labeled probe include the possible different binding properties of the various molecules of the membrane, the possible perturbation of the system by any of the probes resulting in alteration of the membrane which masks structural changes, or in the the case of the fluorescent probes, scatter of the experimental points may have obscurred a small but significant deviation from linearity in the Perrin plots.

While discontinuities in the Perrin plots are generally thought to reflect changes in the microviscosity of the dye environment, and polarization values are roughly correlated to "mobility" of the fluorescent dye, changes in fluorescence lifetime of the excited state as a function of temperature will also affect polarization values. A systematic study of fluorescent lifetime of the dyes used in this study has been undertaken, and a correlation between polarization values and the microviscosity of the probe environment should be possible to make.

Acknowledgements

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