Carbocyanine dyes used as fluorescent triplet probes for measuring slow rotational diffusion of lipids in membranes

Pauline Johnson and Peter B. Garland

Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland

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A method for measuring the slow rotational diffusion of lipids or lipid domains in membranes has been developed. It covers the time range from 20 μs–5 ms, and has a sensitivity $>5 \times 10^4$ molecules of probe. The method uses acyl-substituted carbocyanine dyes as fluorescent triplet probes and a laser-microscope combination for excitation and measurement of the triplet state. Rotation rates in dimyristoylphosphatidylcholine vesicles were sensitive to the liquid-to-gel transition. Slow rotations with relaxation times of about 100 μs were detected at the transition temperature region.

Carbocyanine dyes *Fluorescent probe* *Triplet state* *Liposome* *Rotational diffusion* *Membrane*

1. INTRODUCTION

The rotational diffusion of phospholipid or phospholipid-like molecules in membranes in the liquid crystalline state is fast, with relaxation times in the nanosecond region [1]. Three different types of circumstance could bring about much slower rotation: transition to the gel-phase, binding to protein, and lateral phase separation into domains of gel-phase diffusing in surrounding liquid phase. In the last of these cases the phospholipid molecules in the gel-phase domains could be regarded as rotationally immobile except by rotation of the whole domain, like an iceberg.

Fast rotational relaxations of lipid probe molecules can be measured by fluorescence depolarization methods [2]. The time scale is limited by the life time of the probe molecule, typically a few nanoseconds. Longer relaxation times in the microsecond to millisecond range can be measured by electron paramagnetic resonance spectroscopy methods, but the sensitivity is exceedingly low and measuring times are long [3]. Smith et al. [4] introduced a fluorescence photobleaching method for measuring very long rotational relaxation times of lipid probes (carbocyanine dyes) in the time range from many milliseconds upwards. This technique involved irreversible bleaching of the dye by an intense laser flash as the initial photoselection step. The quantum yield for irreversible photobleaching is low ($\sim 10^{-6}$–10^{-5} [5]) and consequently very fast photobleaching can be achieved only at excessive laser intensities and unacceptable temperature increases at the sample. For example, irreversible photobleaching of carbocyanine dyes in multibilayer membranes in 1 μs required a laser intensity of 6 $W.\mu m^{-2}$ [4]. The corresponding temperature rise at the sample would have been in excess of 100°C [6,7]. Nevertheless, at lower bleaching intensities the photobleaching method [4] gives access to long rotational times that cannot otherwise be measured. The photobleaching method is similar in principle to that which we have developed for use with triplet probes and much lower laser intensities (the fluorescence depletion method [6,8]). Triplet probes are well suited for the microsecond to millisecond time range [9], corresponding to the gap in the range of fluorescent methods available for measuring lipid rotation. Accordingly we have explored the use of carbocyanine dyes as fluorescent triplet probes. We find that they can be used to report on their

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rotational diffusion on a time scale from a few microseconds to several milliseconds.

2. MATERIALS AND METHODS

N,N'-di(octadecyl)indocarbocyanine and *N,N'*-di(tetradecyl)indocarbocyanine, abbreviated as C_{18}-dil and C_{14}-dil, respectively, were from Molecular Probes Inc. (Junction City, OR 97448). Dimyristoylphosphatidylcholine was from Sigma (St Louis MO). Large diameter liposomes were made by mixing stock solutions of lipid and probe in methanol in the appropriate ratios (1000–50000:1) such that the final solution contained ~1 μmol lipid. The solution was then rota-evaporated to dryness and the residue re-dissolved in 0.5 ml chloroform. This, or the solution after aqueous extraction, was then evaporated to dryness on a microscope coverslip under nitrogen. A drop of water was added to the lipid mass which was then covered with a circular cover slip and left overnight in water-saturated air above the main transition temperature (>21°C for DMPC). The slides were then incorporated into the anaerobic chamber and viewed under the microscope. Vesicles of large diameter (>20 μm) and with brighter fluorescence at the perimeter than the centre (i.e. paucilamellar) were selected for study by positioning them under an argon ion laser beam focussed to ~10 μm diam. with the 20× objective of a fluorescence microscope. Fluorescence depletion measurements were made with the laser-microscope apparatus in [6,8]. Briefly, it uses an acousto-optically modulated argon ion laser and a fluorescence microscope to obtain an intense, focussed laser spot that can cause 20–30% population of the triplet state of a fluorescent molecule in a few microseconds. The resultant ground state depletion is monitored as a fall in fluorescence (550 nm) excited with a greatly attenuated laser beam (514.5 nm). With suitable polarization optics the decay of anisotropy of the ground state depletion is measured throughout the life time of the triplet state, typically a few milliseconds.

The triplet state is usually quenched by oxygen, and anaerobic conditions were used. The preparation of anaerobic microscope slides in a glove box [6,8] is time-consuming and inconvenient. Here, we devised a new method of preparing anaerobic samples on microscope slides. We used a microscope chamber (TCSC-1, Bachofer GmbH, Laboratoriumsgeräte, D-7410 Reutlingen) that was divided into upper and lower compartments by a 4 μm thick Teflon membrane. The upper compartment contained the sample, the lower compartment sodium dithionite solution (10 mM in 10 mM NaOH). Oxygen diffuses from the upper to lower compartment where it is reduced. About 10–15 min are sufficient to achieve satisfactory oxygen removal from the upper compartment, even when the sample is under a coverslip (see fig. 1). The Teflon membrane does not interfere with phase-contrast illumination of the sample from below.

3. RESULTS

Both C_{18}-dil and C_{14}-dil in multilamellar vesicles were readily converted to the triplet state as shown by reversible fluorescence depletion (fig. 2). The light energy delivered in each high intensity laser pulse was 10 nJ.μm^{-2}. This is 5–10× greater than the intensity required for comparable triplet formation from fluorescein, 50–100× that for eosin, and 0.25–0.5× that for rhodamine [6]. The life time is long (several milliseconds), relatively independent of temperature, and not greatly shortened by the presence of oxygen. The reversible bleachings (fig. 2) are not due to irreversible bleaching followed by lateral diffusional recovery. We calculated the recovery times for the laser spot size and known lateral diffusion coefficients, and also used the same apparatus to measure the diffusional recovery times [10,11].

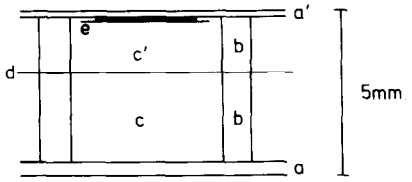

Fig. 1. Anaerobic microscope slide chamber. A diagrammatic cross section is shown. A sample is placed under a circular coverslip (e) on a larger rectangular coverslip (a'). A sandwich is assembled using 2 silicone rubber rings (b) and a Teflon membrane (d) to create the upper (c') and lower (c) compartments. The whole assembly sits on a microscope slide in a metal chamber with viewing ports. The lower compartment was filled with sodium dithionite in dilute NaOH by injecting in through the silicone rubber ring (b).

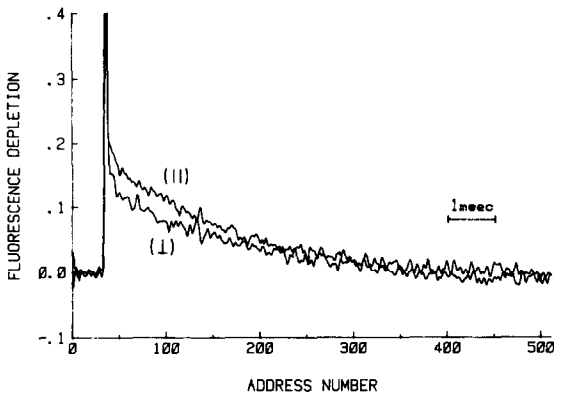

Fig. 2. Fluorescence depletion signals from diI-C_{18} in dimyristoylphosphatidylcholine vesicles at 13°C. Molar ratio 50 000 : 1. The liquid-to-gel transition temperature is 21–22°C [15] and was confirmed by measuring the lateral diffusion coefficient of diI-C_{18}. The fluorescent photon count recordings (20 μs/address, undepleted signal 7200 counts/address after 512 sweeps) were manipulated with a Hewlett-Packard HP-85 microcomputer to be plotted as the fractional fluorescence depletion, which is zero before the bleach pulse (gating artefact), about 0.15–0.2 immediately after the pulse, and declines back to zero over several ms. The upper trace is the (||) or parallel signal, the lower trace the (⊥) or perpendicular signal [6]. The anisotropy parameter (defined as $(\| - \perp)/(\| + 2\perp)$ see [9]) did not decay with time.

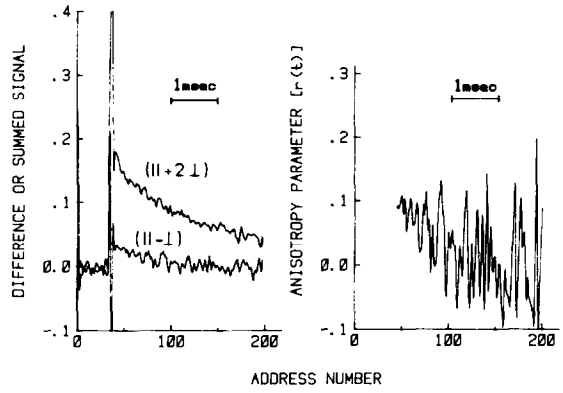

Fig. 3. Slow rotation of the lipid probe. Experimental conditions as for fig. 2 but at 18°C. The number of sweeps was 1024. The left-hand pair of recordings is for the difference $(\| - \perp)$ and sum $(\| + 2\perp)$ of the depletion signals, the latter being on a ×2 reduced sensitivity. The right-hand trace is the anisotropy parameter.

The triplet life times (fig. 2) were 1–2 orders of magnitude too fast for diffusional recovery in multilamellar vesicles above their gel–liquid crystalline phase transition temperature, and $\geqslant$3 orders of magnitude too fast below the transition temperature. The decay of the triplet state followed a single exponential. The ratio of the parallel (||) to perpendicular (⊥) depletion signals was 1.0 above the transition temperature and 1.3 below the transition temperature (fig. 2). In some, but not all, experiments we detected a decay of anisotropy as the temperature was lowered through the transition temperature (fig. 3). Otherwise anisotropy was undetectable above the transition temperature and time-independent below it.

4. DISCUSSION

Long chain acyl-substituted carbocyanine dyes were selected as probes for rotational diffusion because the fluorescent part of the dye is not free to rotate around the membrane normal except by rotation of the whole molecule. Probes such as acyl-substituted aminofluoresceins are not suitable because the chromophore can rotate rapidly about its linkage to the acyl chain. Acyl carbocyanines are considered to lie with their long axis parallel to the membrane surface [12] with an angle of up to 10° [13] or 28° [14] between their absorption and emission dipole moments. With the optical geometry used in our experiments the only motion capable of causing anisotropy is rotation around the membrane normal. Wobbling of the molecule about its long axis (along the chromophore, parallel to the membrane surface) would not depolarize. Accordingly, we can be confident that slow rotational diffusion on the micro-to-millisecond time scale does not occur beneath the transition temperature, and was too fast to resolve above. At the transition temperature we have observed slow rotation (100 μs) consistent with either the (temporary) existence of gel-phase domains of lipid (icebergs) or slow independent rotation of the probe itself.

The sensitivity of the method is high. Satisfactory measurements with a time resolution of 20 μs could be made in 1–2 min of signal accumulation with a molar ratio of lipid to probe of 50 000 : 1, corresponding to $\sim 3 \times 10^4$ molecules of probe in each bilayer (and there was probably more than

one) at the focussed laser spot, 10 μm diam. Triplet formation was also observed with other lipid vesicles and alternative carbocyanine probes. Thus the method is likely to be generally useful and also applicable to living cells. The availability of a brominated carbocyanine dye with a higher quantum yield for triplet formation could enable the time resolution to be taken down to a fraction of a microsecond.

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