ROTATIONAL DIFFUSION OF BACTERIORHODOPSIN IN LIPID MEMBRANES

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1. Introduction

New techniques for measuring the diffusion of proteins in cell membranes have recently been reported [1–7]. Such measurements should in principle test to what extent proteins are freely floating in the fluid lipid bilayer, as envisaged in currently popular concepts of membrane structure [8]. They may also provide a method of investigating structural features of membranes which restrict or prevent diffusion.

Before these aims can be fully achieved, it is necessary to have a sound basis for interpreting diffusion measurements in membranes. The familiar Stokes-Einstein equations are not applicable in two dimensional systems and indeed it is not certain that diffusion in lipid bilayers can in any case be treated by classical hydrodynamics. Model calculations of diffusion in membranes have recently been presented [9] but the results have yet to be critically tested.

Experimental data which are sufficiently unambiguous to test theoretical predictions are most likely to be obtained with simple model systems rather than with cell membranes. Here we report the incorporation of bacteriorhodopsin, one of the best characterised membrane proteins, into phospholipid bilayers. We propose that this system should prove valuable for a detailed investigation of diffusion in membranes and give results of preliminary measurements of protein rotation.

2. Materials and methods

Purple membranes were isolated from *Halobacterium halobium* (strain R_1M_1) as described by Oesterhelt and Stoeckenius [10]. The membranes were solubilised by suspending 1 mg in 4 ml 0.1 M acetate buffer, pH 5.0, containing 0.1% Triton X-100 for 24–30 h in the dark at room temperature. Membrane reconstitution followed a method proposed by Henderson [11]. After adding the requisite quantity (1–5 mg) of phosphatidylcholine (either dimyristoylphosphatidylcholine (DMPC) or dipalmitoylphosphatidylcholine (DPPC)), Triton was removed by prolonged dialysis against 0.1 M acetate buffer, pH 5.0, containing 0.02% NaN_3. Following dialysis, the sample was purified by centrifugation through a sucrose density gradient (4.5–40%) to remove any non-recombined lipid and protein. Further details of the procedure will be presented elsewhere [12]. Protein was determined by the method of Lowry et al. [13] and lipid by phosphorus analysis [14].

Rotational diffusion of bacteriorhodopsin was measured by observing the decay of dichroism of flash-induced transient absorbance changes; the flash photolysis apparatus and general principles of the method were described previously [1,2]. Spectroscopic and other properties of bacteriorhodopsin are summarised in recent reviews [11,15]. In the present experiments, we detected ground-state depletion of the 568 nm absorption band of bacteriorhodopsin following excitation by a plane-polarised light flash of duration 1–2 μs and wavelength 540 nm from a dye laser. Bacteriorhodopsin was light adapted by the 100 W tungsten–halide lamp used as the source of the measuring beam. The results are analysed by calculating the anisotropy parameter $r(t)$ given by

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \quad (1)$$

where $A_{\parallel}(t)$ and $A_{\perp}(t)$ are the absorbance changes at time t after the flash for light polarised parallel and perpendicular with respect to the polarisation of the exciting light. Rotational measurements were normally made at 25°C in the case of DMPC and at 45°C in the case of DPPC, i.e., above the transition temperature of the lipids.

3. Results and discussion

Figure 1 shows a freeze-fracture electron micrograph of reconstituted bacteriorhodopsin–DMPC vesicles. The vesicles appear to be predominantly unilamellar with a diameter in the order of 0.3–0.5 μm. The large size of the vesicles is important, since tumbling of the vesicles is sufficiently slow not to complicate the measurements of protein rotational diffusion. This is a distinct advantage over reconstituted systems previously reported in which the vesicle size is much smaller [16]. The incorporation of bacteriorhodopsin is demonstrated by the presence of membrane particles. The random distribution of particles is, however, in marked contrast to the regular hexagonal array seen in native purple membranes [17].

Figure 2 shows a typical plot of the time dependence of the anisotropy parameter calculated from flash-induced transient absorbance changes. It can be seen that r initially decays but subsequently reaches a constant value. Both DMPC and DPPC measured above their respective transition temper-

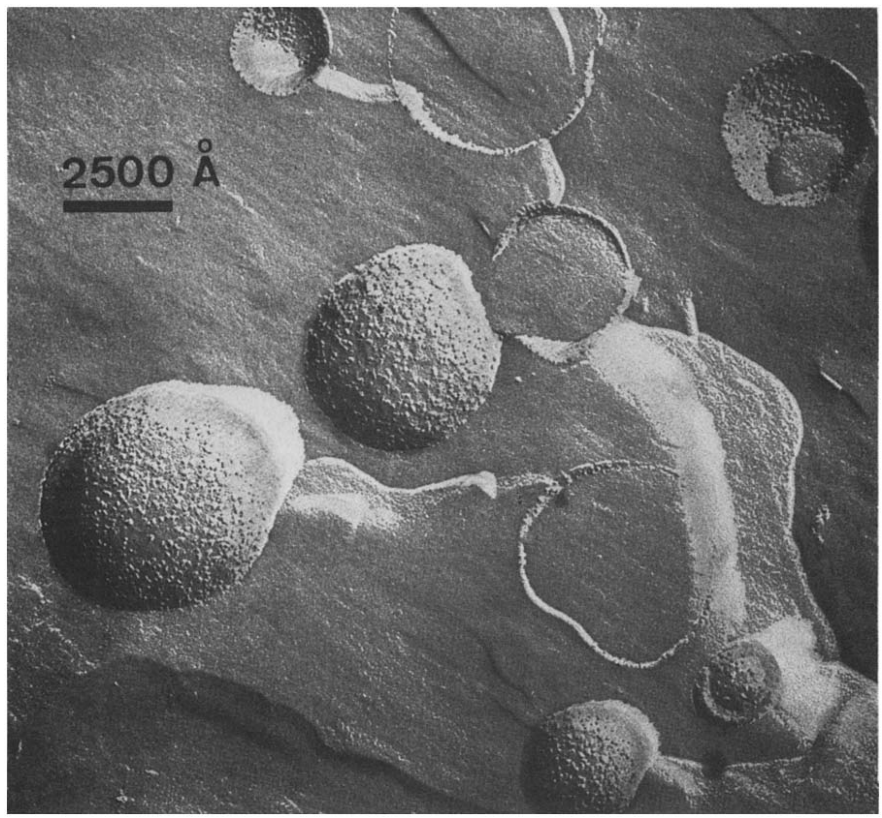

Fig.1. Freeze-fracture electron micrograph of reconstituted bacteriorhodopsin–DMPC vesicles frozen from above the lipid phase transition. Protein:phospholipid ratio 2.35. Samples were mounted on gold discs and frozen in liquid propane at a temperature of −180°C to −190°C using a method developed by Müller, M. (unpublished). Freeze-fracturing was carried out in a Balzer's 300 apparatus. Specimens were replicated with Pt-C, backed with SiO, and examined in a Philips 200 transmission electron microscope.

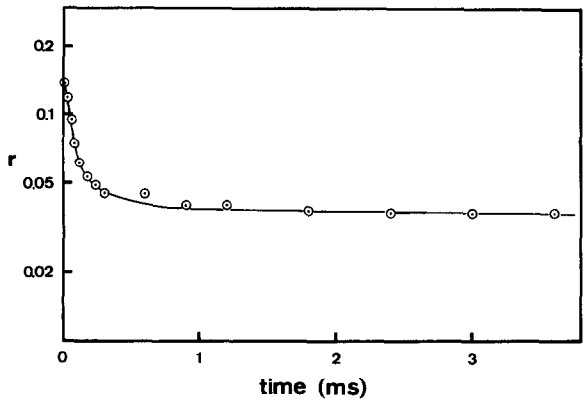

Fig.2. Time dependence of anisotropy parameter (r) calculated from transient absorbance changes measured at 570 nm with bacteriorhodopsin–DMPC vesicles. Protein : phospholipid ratio 1.23, T = 25°C.

atures give plots of similar overall shape. However, the rate of initial decay varies with the amount of excess lipid added to the reconstitution mixture, becoming increasingly rapid as the protein : lipid ratio is decreased.

The initial decay of r implies rotational motion. Since the sample does not contain vesicles small enough to tumble at the rate implied by this initial decay, we conclude that we are detecting rotation of bacteriorhodopsin in the membrane. This conclusion was substantiated by cooling the samples below the phase transition of the lipids; the initial decay of r is reversibly abolished under these conditions [12].

Quantitative evaluation of the transient dichroism measurements is described elsewhere [3,18]. Briefly, the shape of the curve in fig.2 is consistent with rotation of bacteriorhodopsin being confined to a single axis normal to the plane of the membrane. The time-independent part of the curve arises because the inability of the protein to tumble across the membrane prevents the system from becoming fully randomised. If the angle θ between the transition moment of the 568 nm absorption band and the normal axis is known, the diffusion coefficient $D_{\parallel}$ for rotational motion about the normal axis may be determined. Taking $\theta = 75°$ ([17,18], Heyn, M. P., Cherry, R. J. and Müller, U. unpublished) we determine the values of $D_{\parallel}$ shown in table 1. It is clear that there is a very marked dependence of $D_{\parallel}$ on the protein : lipid ratio.

Table 1
Rotational diffusion coefficient ($D_{\parallel}$) of bacteriorhodopsin in reconstituted DMPC vesicles at 25°C as a function of protein : lipid ratio

Protein : phospholipid (w/w) in purified vesicles[a]	$D_{\parallel}$ (s^{-1})[b]
0.25	$(11 \pm 4) \times 10^4$
0.37	$(6 \pm 2) \times 10^4$
0.60	$(3 \pm 1) \times 10^4$
0.91	$(5.7 \pm 1.3) \times 10^3$
1.69	$(2.3 \pm 0.5) \times 10^3$

[a] The ratio protein:total lipids is slightly lower (maximum correction 14%) since the purple membrane contains sulfolipids and glycolipids in addition to phospholipids

[b] The higher values of $D_{\parallel}$ may contain a small additional inaccuracy due to distortion of the 568 nm depletion signal at short times by overlapping positive transients

A similar dependence of $D_{\parallel}$ on the protein:phospholipid ratio was also observed with DPPC vesicles measured at 45°C.

Since the lipids of the purple membrane are solubilised together with bacteriorhodopsin, it is conceivable that the variation of $D_{\parallel}$ is due to an effect of the bacterial lipids on the membrane viscosity. To check this possibility, we extracted lipids from purple membrane and added them to the Triton solution together with DMPC. In this way we could prepare samples in which the ratio of bacterial lipids to DMPC was varied while maintaining the total lipid : protein ratio constant. We found little variation in $D_{\parallel}$ when the ratio bacterial lipids : DMPC was varied from 1 : 3 to 1 : 15. Hence we conclude that the large variations in $D_{\parallel}$ in table 1 are not due to variations in the lipid composition.

The dependence of $D_{\parallel}$ on protein concentration could, at least in part, be due to protein aggregation. The aggregation state of bacteriorhodopsin in these samples has been investigated using electron microscopy, X-ray diffraction and CD measurements; the results of these investigations will be presented elsewhere [12]. In summary, bacteriorhodopsin appears to be monomeric above the lipid phase transition in the lower protein : lipid ratio samples. In the highest protein : lipid ratio samples there is no evidence of a crystalline lattice but some exciton coupling is detected in the CD spectrum. This indicates that protein aggregation does occur [19] and may in part account for the slower protein

rotation observed in the samples containing most protein. It is unlikely, however, to account for the variation in $D_{\parallel}$ observed in lower protein : lipid ratio samples, where no exciton coupling is detectable. Further, since $D_{\parallel}$ changes by a factor of 50 over the range of composition examined, it would be necessary to assume that the aggregation state also changed by this factor in order to explain the data entirely on the basis of protein aggregation. There is no indication from freeze-fracture electron micrographs that such large aggregates actually occur.

We therefore consider it highly probably that a second factor is also important in explaining our data, namely that the membrane viscosity itself is dependent on protein concentration. Such an effect would not be particularly surprising since proteins dissolved in aqueous solution produce large viscosity changes in the range of concentration applicable to the present experiment [20]. For example, the viscosity of aqueous solutions of horse serum albumin increases by a factor of 10 over the concentration range 0–235 g/litre [21]. Furthermore, semiempirical equations for the viscosity of colloidal dispersions predict order of magnitude changes in viscosity when the volume fraction occupied by particles reaches 0.4 [22]. The details of the molecular interactions which give rise to those effects are so far not well characterised, even in simple systems. Apart from a brief discussion by Edidin [23], the possible effect of protein concentration on membrane viscosity appears to have been largely overlooked. The preceding considerations suggest that the viscosities of cell membranes, which often have protein concentrations of 500 g/litre and higher, may be markedly greater than those of protein-free lipid bilayers.

The rotational diffusion of rhodopsin in the disc membrane of frog-rod outer segment has previously been measured by Cone [24] who obtained a value of $D_{\parallel} = 2 \times 10^4\ s^{-1}$ at 20°C. According to Saibil et al. [25], protein occupies one-third of the volume of the hydrophobic region of the disc membrane so that the protein : lipid ratio is about 0.5. Comparison with table 1 shows that the rhodopsin data and the present measurements correlate rather well, although the different lipid compositions of the two systems should not be overlooked. Bacteriorhodopsin itself is immobilised in the native purple membrane [26]. However, bacteriorhodopsin rotation has been measured in the reconstituted apo-brown membrane of *Halobacterium halobium*, yielding a value of $D_{\parallel} = 2.3 \times 10^3\ s^{-1}$ at 22°C [18]. Comparison of this result with the present data, however, awaits the determination of the protein : lipid ratio in this membrane.

In conclusion, the experiments reported here constitute a first step towards obtaining a comprehensive set of diffusion data all obtained with the same simple system. Measurement of both rotational and lateral diffusion of bacteriorhodopsin, lipid diffusion and membrane 'viscosity' experienced by small probe molecules may be envisaged. Such experiments should provide the necessary experimental basis for critically testing theoretical predictions.

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