Mobility of bacteriorhodopsin in lipid vesicles

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BR† has been the subject of a wide variety of biophysical studies. Particular attractions are its ease of preparation in pure form, the availability of much structural information and its intrinsic chromophore, which facilitates a range of spectroscopic investigations (for review, see Stoeckenius et al., 1979). We have used BR to investigate various aspects of protein diffusion in membranes. In the native purple membrane, BR does not of course diffuse because it exists as a crystalline lattice. To observe diffusion, we have reconstituted BR into dimyristoylglycerophosphocholine vesicles (Cherry et al., 1978). This has provided a well characterized model system in which it has proved possible to make reasonably accurate measurements of rotational and lateral diffusion coefficients.

Rotational diffusion of BR may be measured by exploiting the properties of its intrinsic chromophore, retinal. Excitation of BR by a brief laser pulse produces a transient decrease in absorption (depletion) at 570 nm of milliseconds duration owing to molecules entering the photochemical cycle. When the exciting pulse is linearly polarized, the absorption transients are dichroic due to photoselection. From the decay of dichroism, or specifically the anisotropy $r$, rotational motion may be determined. The anisotropy at time $t$ after excitation is defined by the following expression:

$$r(t) = \frac{A(t) - A_1(t)}{A(t) + 2A_1(t)}$$

where $A(t) = A(t)$ and $A_1(t)$ are the absorbance changes for light polarized parallel and perpendicular with respect to the polarization of the exciting flash. For a protein that rotates strictly only around the membrane normal

$$r(t) = \frac{r_0}{A_1 + A_2 + A_3}$$

where $r_0$ is the anisotropy at $t = 0$, $\phi_1$ is the relaxation time ($\phi_1 = 1/D_{\perp}$, where $D_{\perp}$ is the rotational diffusion coefficient) and $A_1$, $A_2$, and $A_3$ are functions only of the orientation of the transition dipole moment (Cherry, 1978).

In dimyristoylglycerophosphocholine vesicles at temperatures well below $T_c$ (temperature of gel-to-liquid-crystalline-phase transition), BR crystalizes into the same hexagonal lattice as occurs in the native purple membrane (Cherry et al., 1978). However, above the $T_c$, the lattice disaggregates. At phospholipid/protein mole ratios greater than about 100, BR is completely dissociated into monomers (Cherry et al., 1978; Cherry & Godfrey, 1981). Thus under appropriate conditions of temperature and phospholipid/protein mole ratio, BR is able to undergo free diffusion in the lipid bilayer. Rotation is monitored by the decay of flash-induced anisotropy, as indicated above. An important question is whether or not the observed $r(t)$ is adequately described by eqn. (2). Curve-fitting procedures show that this is the case provided BR is fully dissociated (Cherry & Godfrey, 1981). As might be expected, low phospholipid/protein mole ratios or temperatures below $T_c$ produce deviations from eqn. (2) due to the onset of BR self-aggregation. Apart from $r_0$, the only adjustable parameters in the curve fitting are $\phi_1$ and the angle $\theta$ between the transition dipole moment (which lies along the long axis of all-trans retinal) and the membrane normal. The best-fit value of $\theta$ is $79 \pm 2^\circ$. This is in reasonable agreement with determinations using other methods and hence gives further confidence in the validity of eqn. (2).

Meaningful values of $\phi_1$ are only obtained when the phospholipid/protein mole ratio is sufficiently high for BR to be fully dissociated (above the $T_c$). $\phi_1$ depends somewhat upon the phospholipid/protein mole ratio and temperature, but is typically of the order of 15 ns between 25 and 37°C and for a phospholipid/protein mole ratio of 140–210. It should be emphasized that the relaxation times for BR are probably the most accurate currently available for a membrane protein. This suggested to us that if lateral diffusion coefficients could also be obtained, it might be possible to test the equations of Saffman & Delbrück (1975). These equations are:

$$D_{\parallel} = \frac{kT}{4\pi a^2 \eta}$$

$$D_{\perp} = \frac{kT}{4\pi \eta} \ln (\eta h/\eta_\perp a) - \gamma$$

where the protein is modelled as a cylinder of radius $a$ spanning a membrane of width $h$. The membrane is treated as a fluid continuum of viscosity $\eta$ surrounded by an aequous phase of viscosity $\eta_\perp$. $D_{\parallel}$ is the (two-dimensional) lateral diffusion coefficient and $\gamma$ is Euler's constant ($\approx 0.5772$). Eqsns. (3) and (4) are approximations that are valid when $\eta_\perp \ll \eta$ (Hughes et al., 1982). Although they are frequently used to interpret diffusion measurements, their validity has never been tested.

Lateral diffusion may be measured by fluorescence microphotolysis (FM), also known as 'FPR' and 'FRAP' (Peters et al., 1974; Axelrod et al., 1976; Edidin, 1981). In the case of BR, the intrinsic chromophore is not suitable for such measurements. We therefore labelled BR in the reconstituted vesicles with the probe eosin-5-isothiocyanate. The vesicles were then dehydrated and subsequently rehydrated on a microscope cover slip to produce large multimamellar structures suitable for FM measurements. The multimamellar vesicles were viewed in a fluorescence microscope and eosin in a small spot of a few microns diameter was bleached by brief illumination at high intensity from an argon ion laser.

Lateral diffusion coefficients were obtained from the rate of recovery of fluorescence in the bleached area using the analysis of Axelrod et al. (1976). The measurements were performed at a variety of temperatures and phospholipid/protein mole ratios. The lateral diffusion of the lipid analogue 'diO-C18(3)' (3,3'-dioctadecylxylctricarbocyanine) was also measured in the same vesicles.

One way of testing the validity of eqns. (3) and (4) is to use them to calculate the protein's size from the measured diffusion coefficients. By eliminating the membrane viscosity we obtain:

$$D_{\perp}/D_{\parallel} = \alpha^2 \ln [kT/(4\pi a^2 \eta_\perp D_{\parallel}) - \gamma]$$

Table 1, we list values of $D_{\parallel}$ and $D_{\perp}$ (obtained under conditions where BR is fully dissociated) together with values of $\alpha$ calculated from eqn. (5). Upon averaging these values, we obtain the diameter of BR (=2a) as $4.3 \pm 0.5$ nm. This may be compared with the structural model of Henderson & Unwin

\begin{table}[h]
\centering
\caption{Calculation of BR radius from $D_{\parallel}$ and $D_{\perp}$}
\begin{tabular}{cccc}
Phospholipid/protein & $^\circ$C & $D_{\perp}$ (um$^{-2}$s$^{-1}$) & $D_{\parallel}$ (s$^{-1}$) & $\alpha$ (nm) \\
\hline
140 & 28.5 & 1.8 & 7.8x10$^4$ & 1.97 \\
140 & 32 & 2.3 & 7.6x10$^4$ & 2.35 \\
210 & 24.5 & 1.4 & 6.4x10$^4$ & 1.89 \\
210 & 28.5 & 2.4 & 7.9x10$^4$ & 2.39 \\
\end{tabular}
\end{table}

† Abbreviation: BR, bacteriorhodopsin.
Table 2. Effect of phospholipid/protein mole ratio (L/P) on $D_k$ and membrane viscosity at 32°C

<table>
<thead>
<tr>
<th>L/P</th>
<th>$D_k$ for BR ($\mu$m$^2$ s$^{-1}$)</th>
<th>$D_k$ for 'DiO-C18(3)' ($\mu$m$^2$ s$^{-1}$)</th>
<th>$\eta$* (Pa s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>210</td>
<td>3.4</td>
<td>6.9</td>
<td>0.1</td>
</tr>
<tr>
<td>140</td>
<td>2.3</td>
<td>4.3</td>
<td>0.18</td>
</tr>
<tr>
<td>90</td>
<td>1.3</td>
<td>2.5</td>
<td>0.35</td>
</tr>
<tr>
<td>30</td>
<td>0.15</td>
<td>0.73</td>
<td>(4.3)†</td>
</tr>
</tbody>
</table>

* Calculated from $D_k$ of BR using eqn. (4) and taking $a=2$ nm, $h=4.5$ nm.
† Value for pure dimyristoylglycerophosphocholine.
‡ Probably not meaningful because of steric restrictions to diffusion at such low L/P.

(1975) in which the widest cross-sectional diameter in the plane of the membrane is 3.5 nm. The reasonable agreement between these two determinations of diameter provides a good argument for the validity of eqns. (3) and (4). The results also confirm the earlier conclusion that BR is monomeric above the Tc at sufficiently high phospholipid/protein mole ratios (Cherry et al., 1978). The membrane viscosity can be calculated by substituting the value of $a$ in eqn. (4). In fact, because $D_k$ is rather insensitive to changes in $a$, reasonable values of $\eta$ should be obtained even when a degree of self-aggregation is present. Thus $\eta$ can be calculated over a wider range of phospholipid/protein mole ratios than is possible for the determination of $a$. The procedure we have adopted is to take a fixed value of $a=2$ nm and calculate $\eta$ from $D_k$ using eqn. (4) with $h=4.5$ nm). The way in which $\eta$ varies with phospholipid/protein mole ratio is illustrated by the results at 32°C listed in Table 2, in which the lipid and protein diffusion coefficients are also given. It is clear that the calculated viscosity increases with decreasing phospholipid/protein mole ratio, corresponding to a decrease in both the protein and lipid lateral diffusion coefficients.

A proper interpretation of the dependence of $D_k$ on phospholipid/protein mole ratio is likely to be complex. The important fact is that $D_k$ decreases with decreasing $D_k$ and, moreover, that the protein concentration in many cell membranes is in the range where the changes in $D_k$ become large. Thus even when restrictions to mobility by peripheral proteins and cytoskeletal structures are absent, $D_k$ of proteins in biological membranes is likely to be markedly less than in dilute model systems. When comparing biological membranes with each other and with model systems, it is essential to take the phospholipid/protein mole ratio into account.

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