Calibration of Membrane Viscosity of the Reconstituted Vesicles by Measurement of the Rotational Diffusion of Bacteriorhodopsin *

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Membrane viscosity of the reconstituted vesicles was calibrated by rotational diffusion of bacteriorhodopsin (BR) in dimyristoylphosphatidylcholine (DMPC) and egg phosphatidylcholine (PC) vesicles. Rotational diffusion of BR in the vesicles was measured by flash-induced absorption anisotropy decay. BR was, for the first time, reconstituted successfully into DMPC and egg PC vesicles. From the measurement of flash-induced absorption anisotropy decay of BR, the value of rotational diffusion coefficient D was obtained from each curve fitting by a global fitting procedure and, in turn, membrane viscosity η was estimated from D. The results have shown that membrane viscosity is temperature-dependent. It was decreased as temperature increased, but a transition occurred in the region of the respective phase transition of DMPC and egg PC, respectively. The decrease of η was fast near the phase transition for DMPC and egg PC. Few effects of lipid/BR ratio and glycerol or sucrose in suspension medium on membrane viscosity were found.

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Membrane viscosity is an important physical property and one of key parameters in the determination of oligomeric states of proteins in membranes based on their rotational diffusion mobility. The rotational mobility of protein in membrane is affected by membrane viscosity and by the area and the shape of the cross section of the rotating particles in the membrane.[1] Membrane proteins have been modelled as cylinders with circular or elliptical cross sections in the membrane.[2] The expression for the rotational diffusion coefficient D of protein in membrane has been derived by Saffman and Delbrück[1] for rotating particles with circular cross sections, and extended to particles with elliptical cross sections.[2] Thus, we have

$$D = kTF/4SH\eta. \tag{1}$$

In other words, membrane viscosity is

$$\eta = kTF/4DSH, \tag{2}$$

where k is Boltzmann constant ($k = 1.38\times10^{-23}$J/K), T is the absolute temperature, F is the shape factor of the protein particle, S is the cross-sectional area in the membrane, and H is the thickness of the membrane. If the cross section is a circle with its radius a, then $S = \pi a^2$ and $F = 1$. If it is an ellipse with its major and minor axes of $2a$ and $2b$ ($a > b$), then $S = \pi ab$ and $F = 2/(1 + a^2/b^2)$.

Measurement of D can provide a determination of the cross-sectional area of membrane proteins. If the membrane viscosity is known, we can know the oligomeric state of protein from the cross-sectional area of the membrane. In other words, if F/S is known, the value of η can be obtained.

To calibrate the membrane viscosity, it is better to use a well-characterized protein-vesicle system as a model. BR molecules are arranged in trimer clusters and the trimers form a two-dimensional hexagonal crystal lattice.[3,4] The orientation of retinal chromophore with respect to the normal to the bilayer of purple membrane was at 78°C.[5] In the reconstituted lipid vesicles, BR molecules can rotate with respect to the normal. BR vesicles are ideal for calibration of the membrane viscosity as the BR structure at different resolutions is available[6,7] and BR molecules have been reconstituted into lipid vesicles.[8,9] Therefore, it is easy to measure the rotational diffusion coefficient accurately by flash-induced anisotropy decay of its intrinsic retinal chromophore. The membrane viscosity of DMPC and egg-PC vesicles has been estimated from the rotational diffusion measurement of BR in vesicles by a simplified fitting procedure.[8] The BR-vesicle system can be used as a model for other protein-vesicle systems.

In this work, BR was reconstituted into DMPC and egg PC vesicles successfully. The absorption anisotropy decay of BR in the vesicles was measured. Then the values of D were obtained from a global procedure for a heterogeneous rotational diffusion system at different temperatures. Finally, η values were calculated from D. The result shows that η depends on temperature. The membrane viscosity η decreases with increasing temperature and a transition occurs in the region of respective phase transition of DMPC

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and egg PC. Few effects of the lipid to protein ratio and glycerol or sucrose in suspension medium on membrane viscosity are found.

For preparation of vesicles and analyses of molar ratios of lipid to protein of vesicles, purple membrane was purified from halobacterium halobium (strain R_1M_1), bacteriorhodopsin was solubilized by Triton X-100 (molar ratio of triton X-100: BR=160:1), and reconstituted into lipid vesicles with DMPC or egg PC by the solubilization-dialysis procedure have been described previously with minor modifications.[10] The dialysis lasted for nine days rather than five or seven days, with the first seven days at 4°C then at 18°C for two days. The whole dialysis procedure was kept in the dark. The reconstituted vesicles were sorted into several parts by centrifugation. The sediments of the samples were harvested at different speeds from 10 000 rpm to 30 000 rpm with a step of 2000 rpm. The protein concentrations of bacteriorhodopsin in vesicles with different rpm were determined by the Lowry–Hartree method with some modifications. Only those vesicles collected at 20 000 rpm were tested. All the samples were flushed with argon prior to measurement to obviate possible bleaching of chromophore.[11]

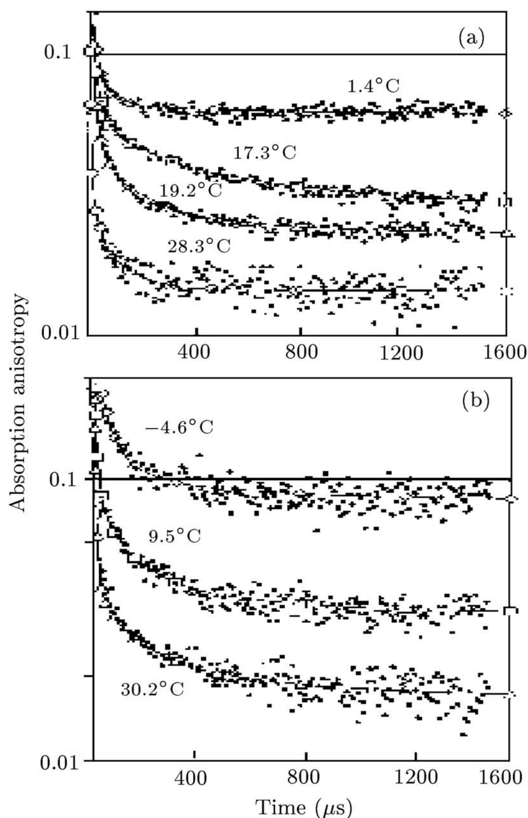

Fig. 1. Flash-induced absorption anisotropy decay of bacteriorhodopsin in (a) DMPC and (b) egg PC vesicles measured at different temperatures. The dots are the experimental data and the solid lines are the fits of Eq. (3).

The flash-induced absorption anisotropy decay was measured with a transient dichroism apparatus similar to that described in detail elsewhere.[12] Excitation was carried out by an Nd:YAG laser (JK Laser Ltd) using frequency-double emission at 532 nm. The pulse width was about 15 ns and the repetition rate was 10 Hz. Transient absorption changes at time t after the flash arising from ground-state depletion were simultaneously recorded at 570 nm for light polarized parallel $[A_{\|}(t)]$ and perpendicular $[A_{\perp}(t)]$ with respect to the polarization of the exciting flash. The signals were averaged in a Datalab DL 102 A signal averager to modify the signal-to-noise ratio. Data were analysed and plotted by calculating the absorption anisotropy $R(t)$ defined by

$$R(t) = [A_{\|}(t) - A_{\perp}(t)]/[A_{\|}(t) + 2A_{\perp}(t)]. \quad (3)$$

For a homogeneous population of membrane proteins rotating only with respect to an axis normal to the plane of the membrane, $R(t)$ is given by[12]

$$R(t) = [R_0/(A_1 + A_2 + A_3)] \times [A_1 \exp(-t/\Phi) + A_2 \exp(-4t/\Phi) + A_3], \quad (4)$$

where $D = 1/\Phi$, R_0 is the experimental value of the anisotropy at $t = 0$, and A_1, A_2 and A_3 are constants depending on the orientation of the transition dipole moment of the absorption band.

Figure 1(a) shows the anisotropy decay of BR-DMPC vesicles at the ratio 118 of the molar lipid to protein, suspended in 0.1 mol/L NaAc buffer with 0.02 wt% NaN_3, pH 5.0. Figure 1(b) exhibits that of BR-egg PC vesicles, with the ratio 167:1 of the molar lipid to protein, suspended in the same buffer with 50 wt% glycerol. The anisotropy decay is temperature-dependent. The higher the temperature, the more rapid the anisotropy decay. However, the duration within which the anisotropy decays to a constant is different for each.

A single BR molecule can be modelled as an ellipse with its long and short axes of 3.5 nm and 2.5 nm from the values of the rotational diffusion coefficient at different temperatures.[13] The viscosity of the membrane at different temperatures can be obtained from Eq. (2). As shown in Fig. 2, the membrane viscosity is temperature-dependent. It seems that η decreases when the temperature increases, but a transition occurs in the region of phase transition of lipid. In the case of DMPC, this transition is at about 22°C, but it is lower than 0°C for egg PC. This may be due to the fact that there are different structures of lipid between gel phase and liquid crystal phase of lipid.

The results in Fig. 2(a) show that there is little effect of solution viscosity (glycerol and sucrose) on the viscosity of membrane. We can also see from Fig. 2(b) that there is little effect of the lipid-to-protein ratio on the membrane viscosity.

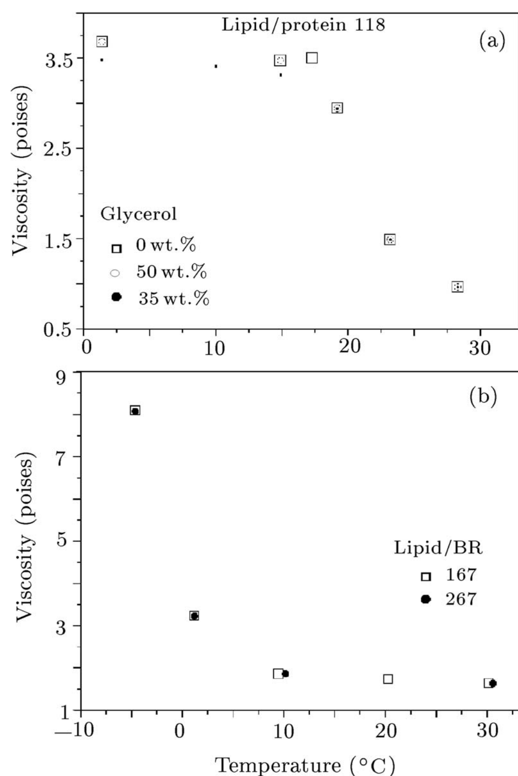

Fig. 2. Dependences of (a) DMPC and (b) egg PC vesicles on temperature with different glycerol contents. The vesicles were suspended in 0.1 mol/L sodium acetate, 0.02 wt% sodium azide buffer (pH 5.0).

In conclusion, BR can be used as a calibration protein in lipid vesicles for the oligomeric state of the other membrane protein based on the measurement of rotational diffusion. The membrane viscosity of the reconstituted vesicles and the rotational diffusion coefficient of BR in the vesicles are temperature-dependent. In the case of DMPC vesicles, the phase transition of the lipid is about 22°C, while in the case of egg PC vesicles, the phase transition of the lipid is lower than 0°C. There is little effect of the lipid-to-protein ratio and solution viscosity on the membrane viscosity.

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