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ROTATIONAL MOTION OF RHODOPSIN IN THE VISUAL RECEPTOR MEMBRANE AS STUDIED BY SATURATION TRANSFER SPECTROSCOPY

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Summary

The saturation transfer spectrum of a nitroxide spin probe rigidly bound to rhodopsin in frog rod outer segment membrane has been measured. The rotational correlation time at 20° C was obtained as 20, 3.6, and 13 μs from the peak height ratio in the low field, central, and high field part of the spectrum, respectively. The difference in the obtained values suggests an anisotropic rotation of rhodopsin in the membrane. Glutaraldehyde treatment restricted the rotational motion. The correlation time became 45, 9.6, and 45 μs as estimated from the low field, central, and high field part of the spectrum, respectively. Comparison with the data by Cone ((1972) Nat. New Biol. 236, 39—43) suggests oscillatory rotation within a limited angle. Photobleaching affected the spectrum slightly but definitely.

The lipid bilayer portion of biological membranes usually has a viscosity of the order of several poises and the proteins in the membranes should have considerable lateral and rotational mobilities [1]. These motions will be restricted when the proteins are aggregated or anchored through interaction with intracellular fibrous network. The protein mobility and its control may be of crucial importance in regulation of the membrane physiological phenomena.

As a measure of the protein mobility, we have studied rotational motion of a membrane protein by means of saturation transfer spectroscopy. Classical ESR measurements of nitroxide spin labels are sensitive to rotational motions with the correlation time ranging from 10^{-10} to 10^{-7} s. The saturation transfer spectroscopy has been recently developed by Hyde, Dalton. Thomas, and McConnell [2—5]. The new method enables us to detect slower rotational motions with correlation times from 10^{-7} to 10^{-3} s and is, therefore,

particularly suitable for the study of rotational motions of proteins in membranes. Rhodopsin was chosen as a membrane protein because its rotational motion has been studied by transient photodichroism [6] and the results can be directly compared with each other. According to a recent personal communication, Devaux et al. have also studied motion of rhodopsin by the same technique [7].

Rod outer segment membranes were isolated from retinae of dark-adapted frog (*Rana catesbeiana*) [8]. The membranes were used fresh or stored under argon at −80° C to protect from oxidation of lipid alkyl chains. The buffer used for the following experiments was 80 mM KCl/40 mM NaCl/15 mM $CaCl_2$/10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, pH 7.4, and bubbled thoroughly with nitrogen for the protection of oxidation. The rod outer segment membranes were pretreated with 20 mM iodoacetamide at 0° C for 2 h and washed twice. The membranes were then reacted with 3-maleimide-2,2,6,6-tetramethyl-1-piperidinyloxyl at a molar ratio of 100:1 at 0° C for 8 h and washed 7 times. The spin label reagent was synthesized in this laboratory and purified by recrystallization (m.p. 104—105° C). When necessary, the spin-labeled membranes were treated with 5% glutaraldehyde (Ladd, 70%) at 0° C for 2 h and washed once. The membrane suspension was taken into a quartz capillary tube (inside diameter 0.7 mm) and the ESR spectrum was measured with a Varian E-12 X-band spectrometer. The temperature was controlled within ± 1° C. A "÷ 2" circuitry, 50 kHz active filter, and a capacitor to resonate cavity coils at 50 kHz were attached to the spectrometer in order to detect the second harmonic out-of-phase absorption for the saturation transfer spectroscopy. The incident microwave power was 63 mW on the dial and the field modulation amplitude at 50 kHz was 4.2 G. As a reference, a complex of 5-nitroxide stearic acid with bovine serum albumin (Sigma, fatty acid-free) was prepared at a molar ratio of 2:1. The complex was dissolved in glycerol/water (82:18, v/v) and the saturation transfer spectrum was measured at various temperatures from 30° C to −20° C. Since the cavity *Q* factor was considerably different between water and water/glycerol, the incident microwave power was adjusted so that the effective microwave field strength may become identical. The rotational correlation time was calculated using 3.6 nm as the radius of albumin [9] and viscosity of the aqueous glycerol [10]. A graph relating the peak height ratio to the correlation time was made as shown in Fig. 1.

A conventional ESR spectrum of the spin-labeled rod outer segment membrane is shown in Fig. 2a. The overall splitting value was 65.7 ± 0.20 G at 20° C. A trace amount of narrow component (see arrow) can be seen in addition to the rigid component. The narrow component increased when the molar ratio of spin label reagent to rhodopsin was decreased. Since it perturbs estimation of the correlation time from the saturation transfer spectrum, a higher ratio was employed for the spin labeling. The pretreatment with iodoacetamide also decreased the narrow component. The trace narrow component did not practically affect the saturation transfer spectrum. The number of spin labels attached to one mole of rhodopsin was 2.2, in agreement with the previously published data [11,12]. The spin label signal gradually decreased owing to reduction of the nitroxide moiety (see Fig. 3a).

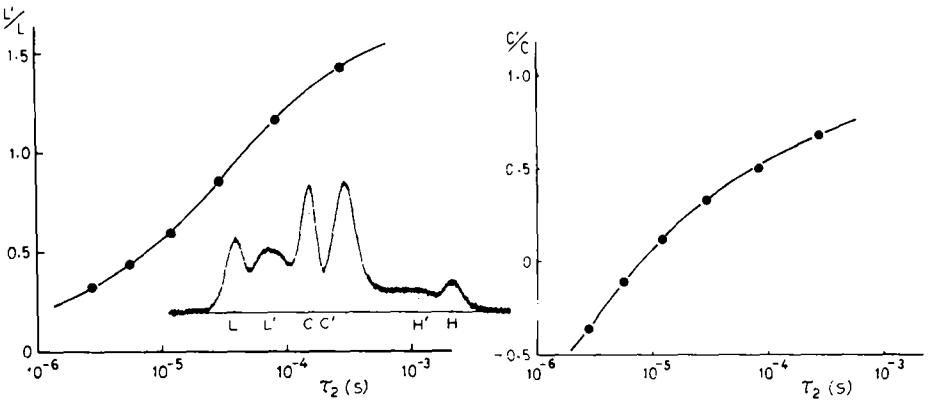

Fig. 1. Dependence on the rotational correlation time τ_2 of the peak height ratios of the saturation transfer spectrum. The second harmonic out-of-phase absorption of a complex of 5-nitroxide stearic acid with bovine serum albumin in glycerol/water was measured at various temperatures.

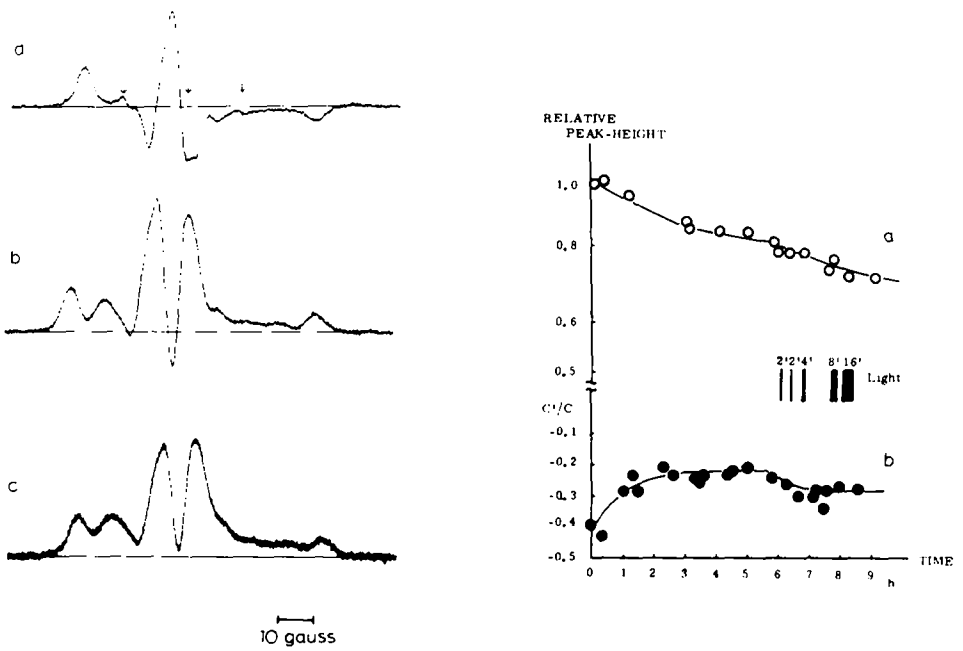

Fig. 2. ESR spectrum of spin-labeled rod outer segment membranes at 20° C. (a) Conventional measurement: first harmonic in-phase absorption spectrum at an incident microwave power of 1 mW and with field modulation (10 kHz) amplitude of 2 G. (b) Saturation transfer spectrum: second harmonic out-of-phase absorption with an incident microwave power of 63 mM and field modulation (50 kHz) amplitude of 4.2 G. (c) Saturation transfer spectrum of glutaraldehyde-fixed membranes.

Fig. 3. (a) Decay of the ESR signal of spin-labeled rod outer segment membranes. The low field peak height of the conventional ESR spectrum was plotted against time at 20° C. (b) Dependence of the peak height ratio C'/C of the saturation transfer spectrum on the storage time in the dark (before 5.8 h) and on intermittent illumination using a cut-off filter at 620 nm at 20° C. The inset shows the time of illumination in minutes.

The saturation transfer spectrum of the spin-labeled membrane is shown in Fig. 2b. The peak height ratios L'/L, C'/C, and H'/H were measured for more than 7 spectra, yielding 0.75 ± 0.015, −0.27 ± 0.029, and 0.49 ± 0.026, respectively. The C'/C ratio was smaller immediately after the spin labeling, increased gradually with time, and reached a stable value of −0.27 after a few hours (see Fig. 3b). The rotational correlation time was obtained by referring to Fig. 1 as 20, 3.6, and 13 μs from the L'/L, C'/C (stable value), and H'/H, respectively. The difference in the values is probably due to use of model spectra obtained with bovine serum albumin. This protein has a nearly spherical shape and the rotational motion must be isotropic. The result, therefore, suggests anisotropic motion of rhodopsin in the membrane. Generally, the saturation transfer spectrum should be greatly dependent on the orientation of the spin label with respect to the protein rotational axis. When the spin label y axis coincides with the rotational axis, for example, the correlation time would be underestimated. This tendency is more pronounced in the central part of the spectrum. For correct estimation of the correlation time we need to have suitable model spectra or computer-simulated spectra for the anisotropic rotation which is in progress in this laboratory.

The glutaraldehyde-fixed membranes gave the saturation transfer spectrum as shown in Fig. 2c. All the peak height ratios increased markedly, indicating reduction of the rotational motion. The L'/L, C'/C, and H'/H were 1.00 ± 0.011, 0.051 ± 0.020, and 0.80 ± 0.011, which led to the correlation time of 45, 9.6, and 45 μs, respectively. The conventional ESR spectrum (not shown) was affected only slightly. The overall splitting value was 66.4 ± 0.25 G at 20° C. A sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated extensive crosslinking of rhodopsin molecules.

Cone has obtained 20 μs at 20° C for the rotational relaxation time of rhodopsin in frog retina [6]. The value is similar to that obtained in the present study. After glutaraldehyde treatment, Cone has observed no decay of the dichroic ratio even after 10 s at 6° C. The present result also indicated a restriction of rotational motion on glutaraldehyde treatment. However, the correlation time was much less than 10 s. The difference may be reasonably explained by considering the difference in the principle of the measurement. The saturation transfer spectrum would be affected by rotations with the angle large enough to jump over a spin packet. A few degrees of oscillatory rotations would be enough to modify the spectrum.

Finally, the spin-labeled membrane was bleached stepwise by illumination with light at the wavelength longer than 620 nm. The effect on the saturation transfer spectrum was slight but definite. The C'/C value decreased gradually and became −0.31 ± 0.027 on complete photobleaching (Fig. 3b). The L'/L value was almost unchanged, 0.77 ± 0.016. Whether this change is due to change in the rotational motion or in conformational change is to be studied. The photobleaching did not affect the conventional ESR spectrum.

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