

## Spin-Label Saturation-Transfer Electron Spin Resonance Detection of Transient Association of Rhodopsin in Reconstituted Membranes<sup>†</sup>

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**ABSTRACT:** Rotational diffusion of rhodopsin in reconstituted membranes of phosphatidylcholines of various alkyl chain lengths has been measured by using saturation-transfer electron spin resonance spectroscopy as a function of temperature and lipid/rhodopsin mole ratio. For dipalmitoylphosphatidylcholine, the rotational correlation time is 20  $\mu$ s at physiological concentration, the same as in rod outer seg-

**P**rotein association is a common occurrence in biological membranes. Transient protein-protein interaction can occur on a physiologically significant time scale (Kawato et al., 1981) because the bimolecular collision rate in two-dimensional space is greater than in three-dimensional space (Adam & Delbrück, 1968). Some membrane proteins are highly concentrated in specific domains, including bacteriorhodopsin in purple membranes (Henderson & Unwin, 1975) and acetylcholine receptor in cultured muscle cells (Fischbach & Cohen, 1973; Sytkowski et al., 1973). One of the central problems in membrane biology at the molecular level is to elucidate the nature and the driving force of various types of protein association.

Rotational diffusion of proteins is predicted on theoretical grounds to be particularly sensitive to protein association (Saffman & Delbrück, 1975). Experimentally, rotational diffusion of membrane proteins has been investigated by the techniques of saturation-transfer spectroscopy (Hyde & Thomas, 1980) and triplet dichroic measurement (Cherry, 1979). Information on protein dynamics is accumulating that indicates the existence of various complex modes of association or aggregation<sup>1</sup> of proteins in membranes: (1) association can be transient, leading to somewhat slower rotational correlation times (Kusumi et al., 1980b); (2) there can be coexistence of mobile and immobile populations (Nigg & Cherry, 1979; Kawato et al., 1981); (3) stable oligomers can form (Andersen et al., 1981); (4) there can be massive clustering of proteins under certain conditions of membrane reconstitution even when the lipid phase shows high fluidity (Swanson et al., 1980); (5) phase separation into lipid gel and protein-rich domains can occur below the phase transition temperature of membranes reconstituted with synthetic lipids (Kusumi et al., 1980b); (6) protein-rich domains can occur in plasma membranes (Rousselet & Devaux, 1977); (7) the cytoskeletal system can be involved (Sakaki et al., 1982). Moreover, these studies indicate that association of proteins is often crucial from a biochemical point of view.

The present work has a 2-fold thrust: (1) to contribute to the understanding of the phenomena of association of proteins in membranes using rhodopsin in reconstituted membranes as a model system and (2) to contribute to the understanding of the physiology of vision. Rhodopsin in membranes has been studied in a variety of ways (Applebury et al., 1974; Montal

et al., 1977; O'Brien et al., 1977; Baroin et al., 1979; Davoust et al., 1980; Kusumi et al., 1980a; Fischer & Levy, 1981; Tsuda & Akino, 1981; Deese et al., 1981). Neutron-scattering experiments (Osborne et al., 1978) suggest that the shape of rhodopsin can be crudely approximated as a transmembrane cylinder of about 37-Å diameter, while small-angle X-ray scattering experiments (Sardet et al., 1976) favor a somewhat smaller value, 31.4 Å. The lipid to rhodopsin ratio of rod outer segment (ros)<sup>2</sup> membranes is about 60 to 70 (Daemen, 1973; Stone et al., 1979). Assuming a membrane surface area of 64 Å<sup>2</sup> for a single phospholipid, between 44 and 70% of the ros membrane surface is occupied by rhodopsin. Watts et al. (1979) have shown that between 33 and 43% of the total phospholipid content lies in the first shell of phospholipids surrounding the rhodopsin molecules, a value that is reasonably consistent with phospholipid and rhodopsin dimensions and the lipid to protein ratio. In general, each rhodopsin molecule would be about one rhodopsin diameter away from its nearest-neighbor rhodopsin molecule, assuming a uniform dispersion.

The only systematic study of protein association in well-defined systems as a function of the lipid/protein ratio appears to be that of Kusumi et al. (1980b). They studied reconstituted rhodopsin in membranes of DMPC at ratios of 240/1, 153/1, 43/1, and 22/1, passing through the range of physiological concentration, as a function of temperature across the main phase transition of DMPC. See their Figures 3 and 7. The following are key findings of this study:

(1) Below the phase transition, rotational diffusion is independent of the lipid/protein ratio. Motion is relatively facile (a characteristic correlation time of 10<sup>-4</sup> s). Apparently proteins are segregated out of the gel lipid phase.

(2) For lipid/protein ratios greater than 153/1, protein rotational diffusion "experiences" the phase transition. Above the transition, the protein correlation time decreases by about a factor of 6. Apparently proteins are well dispersed, and the effective correlation time of 12  $\mu$ s is characteristic of rhodopsin monomers in membranes.

<sup>1</sup> In this report, proteins in large-scale stable clusters are said to be "aggregated", while proteins in less stable oligomeric groups are said to be "associated".

<sup>2</sup> Abbreviations: ros, rod outer segment; DLPC, L- $\alpha$ -dilauroylphosphatidylcholine; DMPC, L- $\alpha$ -dimyristoylphosphatidylcholine; DPPC, L- $\alpha$ -dipalmitoylphosphatidylcholine; DSPC, L- $\alpha$ -distearoylphosphatidylcholine; DOPC, L- $\alpha$ -dioleoylphosphatidylcholine; 5-SASL, 5-doyleylstearic acid spin-label, 2'-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinonyloxy.

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(3) For the lipid/protein ratio of 22/1, evidence of the lipid phase transition no longer can be seen, and aggregation of rhodopsin persists at all temperatures. However, rotational diffusion is only 4 or 5 times slower than in well-dispersed samples.

(4) Little correlation was found between changes in lipid fluidity and in rhodopsin rotational correlation time when the rhodopsin concentration was varied, suggesting that association of rhodopsin at lower lipid to rhodopsin ratios is a major reason for the slowing down of rotational diffusion. A convenient display to demonstrate protein association in the fluid phase of the membrane is obtained by plotting the rotational correlation time against a parameterization of the motion of phospholipid alkyl chains.

We pursue this line of research here in greater detail. Systematic saturation-transfer experiments similar to that on rhodopsin in DMPC have been performed with a number of phosphatidylcholines in order to understand the effect of lipid chain length on the tendency for protein association. The results may be related to data about the influence of bilayer thickness on the biochemical properties of enzymes and antibiotics (Boehler et al., 1978; Johannsson et al., 1981; Caffrey & Feigenson, 1981). We continue to refine our saturation-transfer methodology. Careful attention has been paid to control of molecular oxygen and microwave field on the sample. As is appropriate in studies of this type, experiments have been done not only by spin-labeling the proteins but also by using fatty acid probes of the lipid phase.

#### Experimental Procedures

**Sample Preparation.** All procedures involving rhodopsin were carried out under dim red light (19-W bulb, deep red filter no. 3, Asanuma Co., Ltd., or 15-W bulb, red filter no. 1, Eastman Kodak Co.) or in total darkness.

Rhodopsin-reconstituted membranes were prepared as described previously (Kusumi et al., 1980b). Briefly, bovine rod membranes prepared by the method of Papermaster & Dreyer (1974) were solubilized in *n*-octyl  $\beta$ -D-glucopyranoside solution, and rhodopsin was purified on a concanavalin A-Sepharose 4B (Pharmacia) column (van Breugel et al., 1977; Albert & Litman, 1978). Phospholipid was solubilized with cholate and mixed with purified rhodopsin, and the detergents were removed by dialysis. The procedure resulted in a uniform dispersion of protein in lipid as evidenced by appearance of a single well-defined band after sucrose linear gradient (15–60% w/w) ultracentrifugation. Thin-layer chromatography developed with chloroform/methanol/water (65:35:4 v/v) was routinely employed to check for residual detergents and the possible degradation of phospholipids. The amount of residual detergent was not greater than 1 mol % phospholipid after charring. This estimate was confirmed for several samples with tritiated cholic acid (New England Nuclear). No lysophosphatidylcholine was detected by using Dittmer's reagent (Dittmer & Lester, 1964). Rhodopsin is stable during reconstitution, giving an optical purity  $A_{280}/\Delta A_{500}$  ratio of 2.1–2.3 for all phospholipids after dialysis measured in the presence of 2% (w/w) dodecyltrimethylamine oxide and 0.1 M hydroxylamine. Amounts of protein and phospholipid in the sample were determined by the methods of Lowry et al. (1951) and Bartlett (1959), respectively. The reconstituted membranes were suspended in a buffer solution of 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid and 65 mM NaCl, pH 7.8, unless otherwise stated.

Spin-labeling of rhodopsin with maleimide spin-label (4-maleimido-2,2,6,6-tetramethylpiperidinoxy), probably on the carbohydrate-carrying segment (Fung & Hubbell, 1978), was

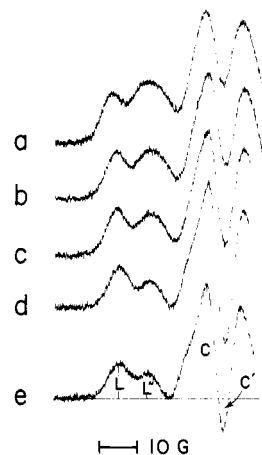


FIGURE 1: Saturation-transfer spectra of spin-labeled rhodopsin in DPPC-reconstituted membranes at (a) 6.6, (b) 27.0, (c) 36.2, (d) 41.8, and (e) 47.2 °C. Conditions are the following: 50-kHz field modulation, 100-kHz 90° out-of-phase detection,  $2H_m = 10$  G, and  $H_1 = 0.27$  G.

carried out as described previously (Kusumi et al., 1980b). The stoichiometry was 1/1 spin-label per protein. 5-SASL was added to reconstituted membranes as described previously (Kusumi et al., 1980b).

**ESR Measurements.** Conventional and saturation-transfer ESR spectra were measured on a Varian E-109 spectrometer. Saturation-transfer spectra were taken according to Thomas et al. (1976).

Oxygen was removed from all samples by using the TPX-capillary technique of Popp & Hyde (1981). The microwave field at the sample was carefully calibrated as described previously (Kusumi et al., 1980b).

The ESR experiments involved measurements at rather high temperatures (up to 56 °C). The thermal stability of DLPC- and DSPC-reconstituted membranes was investigated as follows: incubation at 65 °C for 1 h, cooling, solubilization in a solution of 2% (w/w) dodecyltrimethylamine oxide and 0.1 M hydroxylamine, and examination of the absorption spectrum from 240 to 700 nm. No changes were observed, and the optical purity remained the same. Occasional checks were made in this manner for DMPC-, DSPC-, and DOPC-reconstituted membranes after ESR measurements, and in no case was there evidence of denaturation.

A typical spectrum of spin-labeled rhodopsin is shown in Figure 1. For the most part, anisotropic rotational diffusion of rhodopsin was neglected in our analysis. Approximate rotational correlation times were obtained for the low-field  $L''/L$  parameter by using calibration curves from isotropically diffusing model systems. The correlation time obtained in this manner has been shown (Baroin et al., 1977; Kusumi et al., 1978) to be in agreement with the transient photodichroic data of Cone (1972). This procedure is a reasonable one to estimate changes in protein aggregation or association unless there are concomitant changes in  $\theta$ , the angle between  $p_z$  orbital of the spin-label and the normal to the bilayer.

#### Results and Discussion

**Binding Tightness.** The protein mobility that is observed by saturation-transfer spectroscopy could be a superposition of several types of motions: (1) rotational diffusion of rhodopsin in the membrane; (2) motion of the spin-label with respect to rhodopsin, which might be a consequence of insufficiently tight binding. Alternatively it might reflect internal motions of rhodopsin ("breathing motion"); (3) rotational

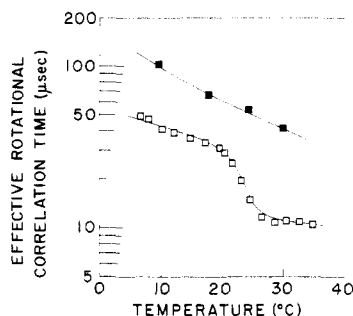


FIGURE 2: Immobilization of rhodopsin after the addition of anti-rhodopsin antibody (sheep). DMPC-reconstituted membrane (DMPC/rhodopsin = 150/1) (□) containing 4 mg of rhodopsin was mixed with approximately 15 mg of antibody (■) at 27 °C.

motion of the membrane vesicles as a whole; (4) lateral diffusion of rhodopsin over the curved surface of the membrane vesicles of a sufficient distance that the orientation of rhodopsin with respect to the polarizing magnetic field is changed.

Because the correlation time obtained by ESR techniques using loosely pelleted samples (Baroin et al., 1977; Kusumi et al., 1978) is in reasonable agreement with transient photodichroic data on intact retinae (Cone, 1972), we believe that mechanism 1 above, rotational diffusion of rhodopsin, dominates. We are confident that mechanisms 3 and 4 are insignificant, but the possibility that insufficient tightness of binding contributes to our results is a serious consideration that merits continued investigation.

If rotational diffusion of rhodopsin is dominant, a large change is predicted when the proteins are cross-linked. This was done in two ways by Kusumi et al. (1978, 1980b): first, treating rhodopsin by dinitrofluorobenzene followed by addition of antibody to the dinitrophenyl group and, second, using glutaraldehyde and bovine serum albumin. We report here use of a third method. Antibody directed to rhodopsin (sheep whole serum, kindly supplied by Dr. D. S. Papermaster, Yale University School of Medicine; Papermaster et al., 1978), partially purified by ammonium sulfate precipitation, was added to rhodopsin-DMPC-reconstituted membranes. Results of saturation-transfer experiments are shown in Figure 2.

In the absence of antibody, rhodopsin at a lipid to protein mole ratio of 150/1 experienced the main phase transition, which occurs at about 23 °C as reported earlier by Kusumi et al. (1980b). Cross-linking increases the apparent correlation time by a factor of 2 below the main phase transition and by a factor of 4–5 above. A substantial dependence of the apparent correlation time on temperature is observed even after cross-linking. All three cross-linking experiments yield essentially the same results. This residual mobility is about 10 times greater than has been reported for cross-linking experiments on other membrane protein systems using saturation-transfer ESR [see Hyde & Thomas (1980) for a review]. It is possible that rhodopsin continues to undergo restricted small-angle rotational diffusion after cross-linking (Kusumi et al., 1978) or alternatively the spin-label binding tightness is less than that in other systems that have been studied. It is also possible that the difference is an experimental artifact associated with our rigorous efforts to maintain the radio-frequency magnetic field at the sample constant for all experiments while other workers have tended to hold the power incident on the cavity constant. Saturation-transfer spectroscopy in the presence of superimposed motions will tend to report the faster motion. It is concluded that the values obtained for rhodopsin largely reflect rotational diffusion of the protein but that there is likely to be a smaller residual

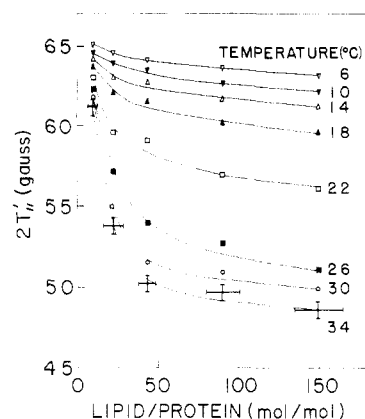


FIGURE 3:  $2T_{||}$  in conventional ESR spectrum of 5-SASL in DMPC-reconstituted membrane is plotted against lipid/protein ratio at various temperatures: 6 (▼), 10 (▽), 14 (△), 18 (▲), 22 (□), 26 (■), 30 (○), and 34 °C (●).

contribution from another type of motion.

**Lipid Motion.** Figure 3 shows plots of the overall separation of the outer extrema of 5-SASL as a function of the lipid/protein ratio and temperature. Rhodopsin was reconstituted in DMPC. The 5-SASL label is sensitive to motions of the lipid alkyl chain in a conical potential well whose axis coincides with the normal to the bilayer. There seems little doubt but that there are at least two populations of lipids, boundary and bulk, characterized by different motions near the terminal methyl groups (Watts et al., 1979, 1981), but 5-SASL does not appear to be a particularly sensitive probe to detect the two populations. We believe this is not a spectroscopic artifact but is because motions close to the polar head group of boundary lipids are in fact less affected by the presence of the protein than are motions in the fluid region of the bilayer. Rotational motions near the membrane surface are affected by lipid packing and seem likely to be correlated with lipid translational diffusion.

The available evidence indicates that there is no significant preferential partitioning of stearic acid spin-labels between boundary and bulk lipids. Watts et al. (1979) found that the integrated intensities of boundary and bulk 14-SASL spectra in *ros* membranes correspond closely to the expected relative amounts of lipids in these two regions. The intensities were the same within experimental error as those obtained from 14-stearoylphosphatidylcholine spin-label. Moreover, using this latter label Knowles et al. (1979) in reconstituted cytochrome oxidase DMPC samples found no preferential partitioning.

It is concluded from this discussion that  $2T_{||}$  is a reasonably good parameterization of the average motion of the lipids. The main phase transition as evidenced by the relative separations of the 18, 22, and 26 °C curves is evident even at a 50/1 lipid/protein ratio, indicating cooperative interaction of the lipids.

The parameter  $2T_{||}$  is extremely sensitive to motional changes when it is close to 50 G (McCalley et al., 1972), and the fact that it hardly changes above the main phase transition between 50/1 lipid/protein ratio and 150/1 lipid/protein ratio is notable. For example,  $2T_{||}$  for 150/1 lipid to protein at 26 °C is very close to  $2T_{||}$  for 50/1 at 30 °C, but the rotational correlation time of rhodopsin, as will be seen, changes from 11 to 17  $\mu$ s. In agreement with the report of Kusumi et al. (1980b), there appears to be little correlation between lipid and rhodopsin motions.

*Effect of Alkyl Chain Length and Rhodopsin Concentration on the Tendency for Protein Association.* Chen & Hubbell

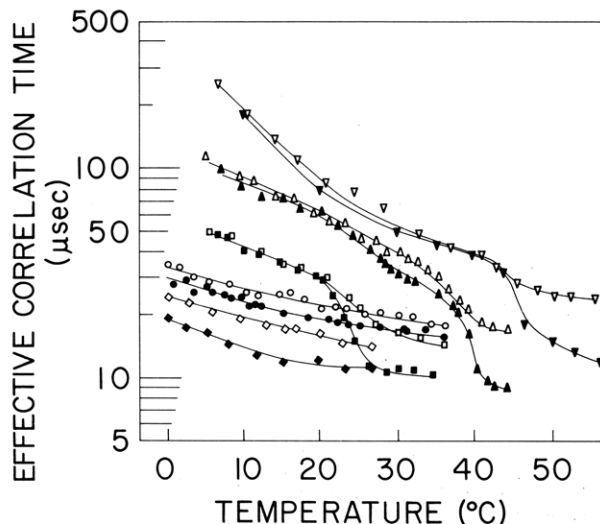


FIGURE 4: Effective rotational correlation time of spin-labeled rhodopsin plotted against temperature. DLPC/rhodopsin = 150/1 (●) and 20/1 (○). DMPC/rhodopsin = 150/1 (■) and 50/1 (□). DPPC/rhodopsin = 150/1 (▲) and 50/1 (△). DSPC/rhodopsin = 150/1 (▼) and 50/1 (▽). DOPC/rhodopsin = 150/1 (◆) and 90/1 (◇). The error in the estimation of the ratio of phospholipid to protein is about  $\pm 10\%$ .

(1973) presented freeze-fracture electron micrographs of rhodopsin reconstituted in membranes composed of various types of lipids including ros membranes. They observed a wide variety of patterns of aggregation or association depending on the lipid type and quenching temperature. The present work is closely related to that of Chen and Hubbell except that saturation-transfer spectroscopy rather than freeze-fracture micrography is used to investigate the tendency of rhodopsin to associate.

Apparent rotational correlation times of spin-labeled rhodopsin in various reconstituted membranes as determined by comparison with the  $L''/L$  ratio of isotropically diffusing model systems have been measured as a function of temperature for 50/1 and 150/1 mole ratio. The results are shown in Figure 4.

The onset of fast rotational diffusion is observed near the main transition temperature for DMPC membranes and at temperatures lower than the main phase transition temperatures for DPPC and DSPC membranes. This result can be explained by "melting" of phospholipid alkyl chains in protein-rich domains as has been observed in other reconstituted membrane systems. Gomez-Fernandez et al. (1980) showed that protein-rich domains melt well below the pure lipid phase transition in  $\text{Ca}^{2+}$ -ATPase-DPPC membranes. Taraschi & Mendelsohn (1980) reached similar conclusions in their Raman studies on glycoporphin-DPPC membranes. Heyn et al. (1981) found that the melting of lipid in protein-rich domains occurs 6–7 °C below the phase transition of bulk lipid in bacteriorhodopsin-DMPC-reconstituted membranes using calorimetric and fluorescence depolarization techniques. Our results are in general agreement with these studies.

DOPC-reconstituted membranes were included in this study because the unsaturated bonds are characteristic of lipids in ros membranes. The behavior of rhodopsin in DOPC membranes is rather similar to that observed in membranes of saturated phosphatidylcholines.

In Figure 5, data from Figure 4 obtained 4 °C above the main phase transition of pure phospholipid membranes are plotted as a function of alkyl chain length for the four fully saturated lipids used in this study.

The values for 150/1 DMPC, DPPC, and DSPC as shown

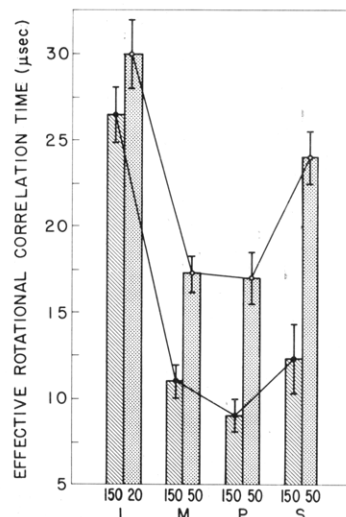


FIGURE 5: Effective rotational correlation time of rhodopsin at  $\sim 4$  °C above the phase transition temperature of each phospholipid membrane, plotted as a function of alkyl chain length and lipid to protein ratio. L, M, P, and S stand for DLPC (12-carbon acyl chains, 5 °C), DMPC (14 carbons, 27 °C), DPPC (16 carbons, 44 °C), and DSPC (18 carbons, 56 °C), respectively. Numbers on the abscissa represent lipid to protein ratio.

in Figure 4 are insensitive to further dilution and are nearly the same. It is concluded that this value, 10  $\mu\text{s}$ , is characteristic of monomeric well-dispersed rhodopsin.

Rotational diffusion of rhodopsin is slower for 50/1 preparations. As previously noted, the effect of 50/1 lipid/protein concentration on rotational diffusion of the lipid fraction as monitored by 5-SASL is slight. It is therefore concluded that the slowing of rotational diffusion at higher concentrations of rhodopsin is the result of protein aggregation or association and is not a consequence of decreased mobility of phospholipid alkyl chains, supporting the conclusion of Kusumi et al. (1980b).

Chen and Hubbell show freeze-fracture micrographs of 100/1 DMPC and DSPC quenched from below the phase transition temperature, demonstrating aggregation of a type suggestive of phase separation—i.e., protein-rich and lipid-rich regions of fairly large dimensions. Our data below the phase transition temperatures show no dependence on concentration. The data blend smoothly into the high concentration data above the phase transition, where evidence for protein association has already been presented. (Of course, no phase transition exists at very high rhodopsin concentration.) We think it likely that rhodopsin aggregation is pervasive below the phase transition.

In the case of DLPC membranes, aggregation seems to persist even above the phase transition temperature, since the observed rotational diffusion of rhodopsin is nearly independent of protein concentration. We suggest that there is a dimensional mismatch between the hydrophobic domains of rhodopsin and the thickness of the membrane. Free energy of the system is then minimized by clustering of rhodopsin.

*Transient Dimers of Rhodopsin.* Saffman & Delbrück (1975) have discussed diffusion in membranes, pointing out that the rotational diffusion constant of a cylinder of radius  $a$  and height  $h$  perfectly embedded in a membrane of thickness  $h$  is

$$D_R = \frac{kT}{4\pi\eta a^2 h}$$

neglecting the viscosity of the medium on either side of the

membrane. For a large cylindrical cluster of  $n$  identical strongly aggregated cylinders,  $D_R$  will be inversely proportional to  $n^2$ , while for a dimer in dumbbell arrangement,  $D_R$  must surely lie between 2 and 4 times less than that for a monomer, with a factor of 3 being a good estimate.

Our data above the phase transition temperature indicate that DPPC is the most effective phospholipid we have examined in solubilizing rhodopsin, and we have assigned the correlation time measured for the most dilute sample in DPPC to the monomeric species.

For DPPC, when the concentration is increased by a factor of 3 to 50/1 lipid to protein molar ratio, the rotational correlation time increases by a factor of slightly more than 2. Our cross-linking studies indicate that rhodopsin antibody increases the apparent correlation time by a factor of 5, a factor that must be much less than the completely cross-linked correlation time. As mentioned earlier, this factor of 5 is somewhat troublesome, apparently indicating some residual mobility. Nevertheless, in a certain region of motion in the range 2–3 times slower than that for monomers, our data should truly reflect dimer and oligomer formation.

A reviewer has raised a question about whether or not change in lipid alkyl chain length might mediate residual spin-label motions. This effect, if it occurs, does not affect our results since we find that rotational diffusion of monomeric rhodopsin is substantially independent of lipid type [see Figure 4, solid data points at highest temperatures for all lipids except DLPC (in which rhodopsin never becomes monomeric in our experience)].

These motional studies provide evidence for the formation of transient dimers. The slightly greater correlation times found in the 150/1 DMPC and DSPC samples compared with those of DPPC are interpreted as a consequence of the presence of a larger number of dimers in equilibrium with monomeric species. As the concentration increases, fewer monomers and more dimers are present. These are transient dimers, forming and dissolving continuously. The distribution of populations is delicately balanced and easily affected by temperature, protein concentration, and lipid chain length. This balance occurs at a rhodopsin concentration that corresponds to the physiological range. The rotational correlation time of spin-labeled rhodopsin in disk membranes was measured by saturation-transfer ESR (Baroin et al., 1977; Kusumi et al., 1978) and found to be in general agreement with photodichroic measurements (Cone, 1972), although some complexity was pointed out because of the anisotropic rotational diffusion of rhodopsin in membranes (Kusumi et al., 1978). The rotational correlation time in disk membranes was estimated at 20  $\mu$ s, similar to the correlation time in DMPC- or DPPC-reconstituted membranes with lipid/rhodopsin ratio of 50/1, and about 2 times longer than that in 150/1 reconstituted membranes. Since saturation-transfer ESR was used for both *ros* and reconstituted membranes, this relative factor of 2 is highly significant even though there is uncertainty in the absolute magnitude of the rotational correlation time because of the anisotropy of the diffusion. Thus it is concluded that transient dimers and oligomers are frequently formed in disk membranes under physiological conditions. The equilibrium between rhodopsin monomer and oligomer and the lifetime of these transient complexes should be critically dependent on the delicate balance of free energy of protein–lipid, protein–protein, and lipid–lipid interactions.

One would like to know the lifetime of the dimeric complex and whether or not specific domains of the rhodopsin surface interact. Poo & Cone (1973, 1974) have measured the

translational diffusion constant of rhodopsin in *ros* membranes, and one can readily calculate from their value that rhodopsin will diffuse about one rhodopsin diameter in  $10^{-5}$  s. We have previously pointed out that dispersed rhodopsin molecules are about one rhodopsin diameter apart. The rotational correlation time of rhodopsin is  $10^{-5}$  s. Moreover, the basic memory time of the spin-label, namely, the spin–lattice relaxation time, has been shown by Kusumi et al. (1982) to be about  $10^{-5}$  s. This coincidence of times limits the amount of detail that can be obtained by our techniques concerning dimer or oligomer formation. The best estimate of the lifetime we can make from our data is that two rhodopsin molecules stay together after collision at least  $10^{-5}$  s.

One might attempt to model the results of this paper by considering the diffusion of rhodopsin in a continuum of viscosity  $\eta$ , as was done by Saffman and Delbrück, where  $\eta$  is determined by the lipid/rhodopsin ratio. We would not favor such a model because we have demonstrated that the diffusive processes depend at least as strongly on the lipid type as on the lipid/rhodopsin ratio, indicating that these processes are regulated by the balance of protein–lipid, protein–protein, and lipid–lipid interactions.

We have established here in a physiologically relevant range of concentration and temperature that pairs of rhodopsin molecules are interacting on a time scale that can affect some of the photochemical intermediate conformational states of rhodopsin following bleaching [e.g.,  $10^{-4}$  s is the order of the lifetime of lumirhodopsin (Cone, 1972; Lewis et al., 1981)].

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