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Lipid-protein interactions in frog rod outer segment disc membranes. Characterization by spin labels

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Freely-diffusing phospholipid spin labels have been employed to study rhodopsin-lipid interactions in frog rod outer segment disc membranes. Examination of the ESR spectra leads us to the conclusion that there are two motionally distinguishable populations of lipid existing in frog rod outer segment membranes over a wide physiological temperature range. Each of the spin probes used shows a two-component electron spin resonance (ESR) spectrum, one component of which is motionally restricted on the ESR timescale, and represents between 33 and 40% of the total integrated spectral intensity. The second spectral component which accounts for the remainder of the spectral intensity possesses a lineshape characteristic of anisotropic motion in a lipid bilayer, very similar in shape to that observed from the same spin labels in dispersions of whole extracted frog rod outer segment lipid. The motionally restricted spectral component is attributed to those spin labels in contact with the surface of rhodopsin, while the major component is believed to originate from spin labels in the fluid lipid bilayer region of the membranes. Calculations indicate that the motionally restricted lipid is sufficient to cover the protein surface. This population of lipids is shown here and elsewhere (Watts, A., Volotovski, I.D. and Marsh, D. (1979) Biochemistry 18, 5006–5013) to be by no means rigidly immobilized, having motion in the 20 ns time regime as opposed to motions in the one nanosecond time regime found in the fluid bilayer. Little selectivity for the motionally restricted population is observed between the different spin-labelled phospholipid classes nor with a spin-labelled fatty acid or sterol.

Introduction

Much available information regarding biological membranes indicates that the lipid-protein interface is important with respect both to function and structure of these assemblies. Vertebrate rod outer segments have for several reasons been shown to be an excellent natural membrane source for the study of lipid-protein interactions in biological membranes. The rod outer segment disc is an easily obtainable membrane system, naturally en-

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Abbreviations: ROS, rod outer segment; ESR, electron spin resonance; 14-PCSL, -PESL, -PSSL, -PGSL, and -PASL: 1-acyl-2-[14-(4′,4′-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, -phosphoserine, -phosphoglycerol, and -phosphoric acid, respectively; *n*-SASL, *n*-(4′,4′-dimethyloxazolidine-*N*-oxyl)stearic acid; ASL, 17-β-hydroxy-4′,4′-dimethylspiro[15-α-androstan-3,2′-oxazolidin]-3′-yloxyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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riched to a very high degree with the integral membrane protein rhodopsin [2]. Therefore, the observations made may be interpreted in terms of rhodopsin-lipid interactions.

Secondly, much research has already been directed to the study of rod outer segment because of the significance for the understanding of the process of visual transduction. Therefore, there exists in the literature reliable structural data regarding the membrane and in particular the protein rhodopsin. The data has emerged from several experimental techniques: X-ray diffraction [3,18], X-ray scattering [4], and neutron scattering [5].

In addition, the rod outer segment disc membrane is a fluid membrane where the lower segments of the lipid chains exhibit fast motions of large amplitude. Rhodopsin has been shown to exhibit fast rotational motion around its long axis [6,7], and fast rates of lateral diffusion in the plane of the membrane [8]. These data indicate that the protein is not clustered or aggregated, and that its entire surface is free to interact with the surrounding lipid. These features of vertebrate rod outer segment membranes make them particularly attractive for study using magnetic resonance techniques [23].

Previous ESR studies in bovine rod outer segment disc membranes have thus established the existence of at least two motionally distinct populations of lipid [1]. One of these is motionally restricted on the ESR timescale (i.e. it has a motional correlation time of greater than approx. 10^{-8} s). The other is a typical ESR spectrum of spin labels undergoing anisotropic motion in a lipid bilayer. However, at the physiological temperature in the bovine rod outer segment system, identification of the motionally restricted component was achieved with difficulty because of the strong temperature dependence of the fluid component. It is thus of considerable importance to investigate rod outer segment membranes from another species with a different physiological temperature range.

In the present work we demonstrate that the motionally restricted lipid is observed in the frog rod outer membrane system, and is revealed as one component of a distinct two-component ESR spectrum over a wide physiological temperature range. Similar experiments performed on dispersions of extracted frog rod outer segment lipid show a one-component spectrum with complete absence of the motionally restricted signal.

The relative proportion of the total ESR signal intensity comprised by the motionally restricted component is determined by computer subtraction, and the resulting motionally restricted spectral lineshapes are examined in detail. These investigations enable a critical study of the dynamics of the probes in each of the environments.

Materials and Methods

Preparation of rod outer segment membranes. All procedures were performed in a darkened laboratory in dim red light. All buffers used were degassed by bubbling with argon for several minutes shortly before use. Batches of 8–10 *Rana pipiens* frogs (supplier West Jersey Biological Supply, Weronah, NJ 08009, U.S.A.) were dark adapted overnight, decapitated and pithed. The retinas were removed and transferred to 4 ml Hepes or phosphate buffer (125 mM NaCl, 3.5 mM KCl, 14 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mM EDTA, 15 mM Hepes or phosphate (pH 7.3)). The retinas were vortexed vigorously for 60 s, and filtered through nylon mesh of pore size 30 μm. The material remaining on the filter was washed with a further 2–3 ml buffer, and the filtrate layered on a sucrose cushion (density 1.14, sucrose dissolved in buffer). The crude material was centrifuged at $40\,000 \times g$ (20 000 rpm) in a Beckman SW41 rotor for 20 min. The rod outer segment membranes were harvested from the interface with a syringe, washed twice in cold buffer, and stored under liquid nitrogen. The A_{280}/A_{500} absorption ratio was routinely 2.7 (2.5 on lysis of the rods), and the yield of rhodopsin was found to be in the range 1–3 nmol per retina. Protein was estimated by the method of Lowry et al. [9], and phosphate by the method of Eibl and Lands [10].

Spin labels. Synthesis of the spin labels employed in this work has been described in detail elsewhere [11]. The androstanol spin label, ASL, was purchased from Syva, CA. The spin labels were kept in stock solutions in chloroform at 1 mg/ml.

Lipid extraction procedures. Lipids were extracted from the frog rod outer segment mem-

branes by the method of Folch et al. [12], as modified by Miljanich et al. [13]. To minimize lipid peroxidation the chloroform used in the extraction contained butylated hydroxytoluene so that the final concentration was 1 mol butylated hydroxytoluene per 200 mol phospholipid. Thin-layer chromatography demonstrated the presence of the three main phospholipid constituents, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine in the resulting lipid extract. Analysis by gas-liquid chromatography of the fatty acid methyl esters prepared from the lipid extract gave a fatty acid composition similar to that reported in Ref. 14. Polyunsaturated fatty acids constituted 65% of the total, indicating negligible lipid peroxidation.

Spin label incorporation procedures. Whenever possible, spin labelling was performed from ethanolic solution of the spin label as previously described [1]. Those spin label species which were insoluble in ethanol were taken up into the rod outer segment suspension from dried films. The rhodopsin content of the sample was determined by absorption spectroscopy, and the phospholipid content was calculated after phosphate analysis [10]. In a typical experiment, 10 nmol of rhodopsin were taken and spin labelled to 1% of the phospholipid content. Samples were washed several times with buffer before transfer to a capillary tube and subsequent ESR measurements.

Electron spin resonance measurements. ESR measurements were made using a Varian E-12, 9 GHz spectrometer, equipped with a nitrogen gas-flow temperature regulating system. Samples sealed in capillary tubes as described above were accommodated within standard 4-mm quartz ESR tubes containing silicone oil for thermal stability. Temperatures were measured with a thermocouple placed inside the quartz tube, slightly above the cavity.

ESR spectra were recorded and stored digitally on disc via a PDP 11/10 dedicated computer. Spectral subtractions were performed using programs written by W. Moeller of this Institute. Apparent order parameters were calculated using the expression

$$S_{app} = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})} \cdot \frac{a'_0}{a_0} \qquad (1)$$

where $2A_{\parallel}$ is equal to the outer, maximum hyperfine splitting ($2A_{max}$) and $A_{\perp}$ is obtained from the inner, minimum hyperfine splitting ($2A_{min}$) according to

$$A_{\perp}(G) = A_{min}(G) + 1.4\left[1 - \frac{A_{\parallel} - A_{min}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})}\right] \qquad (2)$$

The effective isotropic hyperfine splitting constant is given by:

$$a_0 = \tfrac{1}{3}(A_{\parallel} + 2A_{\perp}) \qquad (3)$$

and that corresponding to the single-crystal environment in which the principal values of the hyperfine tensor A_{xx}, A_{yy} and A_{zz} were measured [21] is given by: $a'_0 = \frac{1}{3}(A_{xx} + A_{yy} + A_{zz})$. Recently detailed lineshape simulations have shown that the spectra of lipid spin labels in fluid bilayers contain important contributions from slow molecular motions [30]. Thus the order parameter calculated using Eqn. 1 which assumes fast motion, can only be considered as an apparent value, but is nonetheless useful for making intercomparisons between membranes and lipid dispersions or between different membranes.

Results

Fig. 1 shows typical ESR spectra of 14-PESL, 14-PCSL, and ASL (the phosphatidylethanolamine, phosphatidylcholine and androstanol spin labels, respectively) in frog rod outer segment disc membranes and in dispersions of the extracted lipids at 10°C. All spectra from membranes consist of two components. One component is a relatively narrow, anisotropic spectrum, and is attributed to those spin labels of the total population which are in a fluid bilayer environment. Between the temperatures 0°C and 25°C, this spectral component is seen to comprise between 60 and 70% of the total integrated spectral intensity (see Figs. 2 and 4C, and Table I, below). The second component is clearly visible in the wings of the membrane spectrum, is indicative of labels which are motionally-restricted, and is attributed to those probes in association with the rhodopsin molecules. From the separation, $(H_b - H_f)$, of these two components it can be estimated that the life-

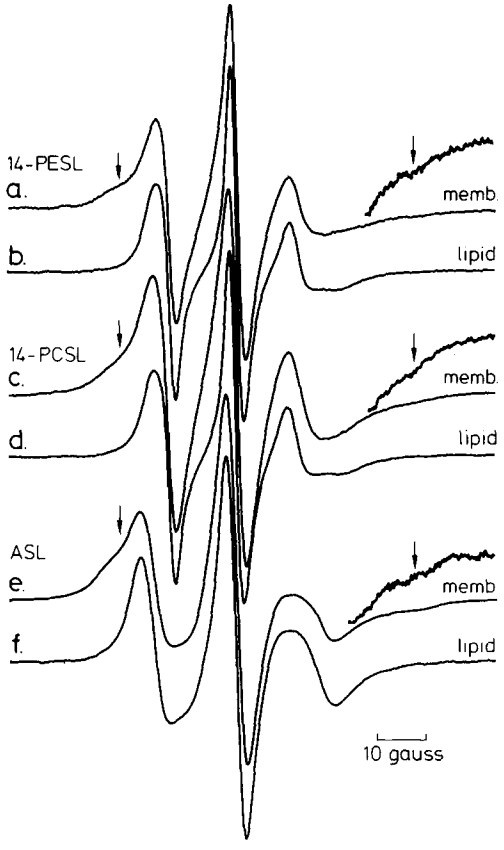

Fig. 1. ESR spectra at 10°C of the phosphatidylethanolamine (14-PESL), phosphatidylcholine (14-PCSL) and androstanol (ASL) spin labels in frog rod outer segment membranes and in dispersions of the extracted membrane lipids. (a) 14-PESL in membranes, (b) 14-PESL in lipids, (c) 14-PCSL in membranes, (d) 14-PCSL in lipids, (e) ASL in membranes, (f) ASL in lipids. The motionally restricted component is indicated by the arrows in the wings of the membrane spectra.

time for exchange between the components must be $\tau_{on,off} > (\hbar/g\beta)(H_b - H_f)^{-1} \sim 10$ ns. In contrast to the two-component membrane spectra, Fig. 1 shows that spectra of labels in dispersions of total extracted frog rod outer segment lipids consist solely of a single, fluid bilayer-like ESR component, the motionally restricted signal being absent.

Analysis of the membrane spectra involves computer subtraction of either one of the two components from the composite membrane spectrum. Fig. 2 shows a typical computer subtraction result. Spectrum (a) is the experimental lineshape of 14-PESL in lysed frog rod outer segment membranes. Spectrum (b) is the fluid lipid difference spectrum which remains on subtraction of a computer-simulated immobilized spectral lineshape from (a), and comprises 61% of the total integrated intensity. The quantitation may also be performed by subtraction of a suitable fluid component of 14-PESL in dispersions of rod outer segment extracted lipids from (a). The endpoint is shown in Fig. 2(c), and accounts for 39% of the total integrated spectral intensity. Thus, both subtraction methods yield consistent quantitative results.

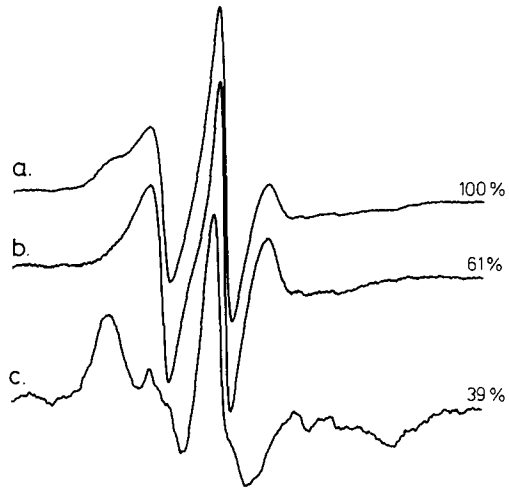

Fig. 2. Spectral subtraction with the composite ESR spectrum of 14-PESL in frog rod outer segment membranes at 0°C. (a) Membrane spectrum, (b) fluid difference spectrum after subtracting a motionally restricted component (of 39% relative intensity) from spectrum (a), (c) motionally restricted difference spectrum after subtracting a lipid dispersion spectrum (of 61% relative intensity from spectrum (a)).

Table I shows results of this quantitation performed for all lipid spin labels employed. The motionally-restricted component is found to account for between 33 and 39% in each case, signifying little selectivity of rhodopsin for a particular lipid species. Differences between PS and PA, the charged spin labels, and PE are considered to be within the experimental error, and thus are not significant. The motionally restricted components obtained from the phospholipid 14-position spin labels are very similar, both with respect to their values of the maximum outer hyperfine splitting, $2A_{max}$ (see Table I), and their linewidths (data not shown).

TABLE I

FRACTION OF MOTIONALLY RESTRICTED SPIN-LABELLED LIPID, f (MEAN VALUES 0–25°C) IN FROG ROD OUTER SEGMENT MEMBRANES, AND OUTER HYPERFINE SPLITTING, $2A_{max}$, OF THIS MOTIONALLY RESTRICTED SPIN LABEL POPULATION AT 0°C

Spin label	f	$2A_{max}$ (gauss)
14-PCSL	0.36 ± 0.04	60.0 ± 0.5
14-PESL	0.33	61.6
14-PSSL	0.39	61.4
14-PGSL	0.37	59.8
14-SASL	0.34	60.7
14-PASL	0.40	61.1
ASL (steroid)	0.36	62.8

Only the steroid spin label, ASL, shows spectral features significantly different from those of the phospholipid labels. It is found that although the proportion of motionally restricted labels is similar, the value of $2A_{max}$ is significantly larger for the steroid label. This result suggests that either the polarity of the environment of this probe, or the motional characteristics of the restricted steroid labels, or possibly both of these, differ from the corresponding features displayed by the motionally restricted phospholipids. In the case of phospholipids, the flexing motions of the lipid chain segments may contribute to the motional averaging of the phospholipid spectral features whereas the steroid is a rigid molecule whose spectra are mainly sensitive to rotation around its long axis. However, since the temperature dependence of the spectra of the latter label shows progressive motional averaging of the restricted component (data not shown) it may be concluded that the steroids in the motionally restricted environment are subject to faster rates of motion as the temperature is raised. Two possibilities explaining this would be either faster rotational rates at the lipid-protein interface, or faster exchange of the molecules between the two environments.

Fig. 3 shows the temperature dependence of the spectra of the 14-PSSL phosphatidylserine spin label in frog rod outer segment disc membranes. At lower temperatures, detection of two spectral components is relatively simple, since the two signals are relatively well resolved. At higher temperatures the resolution is less good, although the

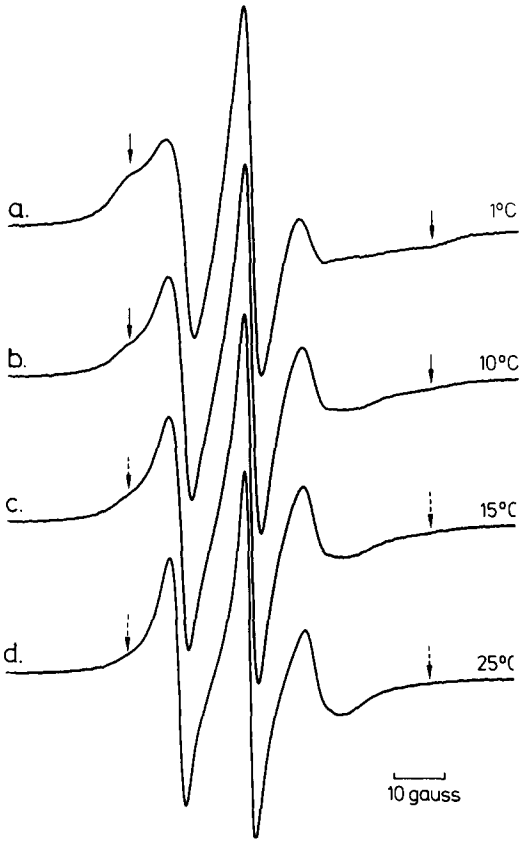

Fig. 3. Temperature dependence of the ESR spectra of the 14-PSSL phosphatidylserine spin label in frog rod outer segment membranes. The motionally restricted component is indicated by the arrows in the wings of the spectra.

motionally restricted component is still identifiable. There are several possible factors contributing to this behaviour. Firstly, higher temperatures cause faster intramolecular motions and disproportionate increase of the lineheight of the narrow component, relative to the broader component. Also, it is reasonable to suspect that higher temperatures cause faster intermolecular motions (i.e., faster exchange between the two membrane environments), which would also give rise to the spectral effects seen in Fig. 3.

Fig. 4 gives a summary of the analysis of the motionally restricted spectra obtained for the frog rod outer segment together with equivalent data from the bovine rod outer segment membrane system. The $2A_{max}$ values (or the maximal line-splitting in gauss, Fig. 4A) give information re-

garding both the motional rate of the probes, and the polarity of the probe environment, while the linewidths (Fig. 4B) are sensitive to motional changes only [15]. It can be seen that the linesplitting decreases, while the linewidths increase, as a function of increasing temperature. These changes are both indicative of increased rate of motion of the spin probes in the motionally restricted environment, which would be consistent with either an increased rate of flexing of the lipid chain relative to the protein surface, or a faster rate of exchange of the probes between the two environments, or both of these. Estimates of the effective rotational correlation times based on simulations for isotropic slow motion [15,17] yield values in the region of 20 ns from both linewidths and linesplitting. Pronounced species differences in the motional characteristics of the restricted lipid component between frog and bovine rod outer segment are not evident in Figs. 4A, B.

The motional characteristics of the fluid lipid component are given in Fig. 5. The temperature dependence of the apparent order parameter (see Eqn. 1) is given for membranes and lipid extracts, for both frog and bovine rod outer segment, and for lipids labelled either on the 5-position or the 14-position of the fatty acid chain. The behaviour is similar for membranes and for dispersions of the extracted lipids, particularly with respect to the difference between the 5- and 14-positions, confirming the existence of substantial areas of fluid lipid bilayer in the membranes. However, in all cases the apparent order parameter is consistently somewhat higher for the fluid component in the membranes than in the extracted lipids. This latter could be the effect of longer range perturbations of the lipids by the protein and/or the effects of exchange between the two lipid components. The apparent order parameters display a species dif-

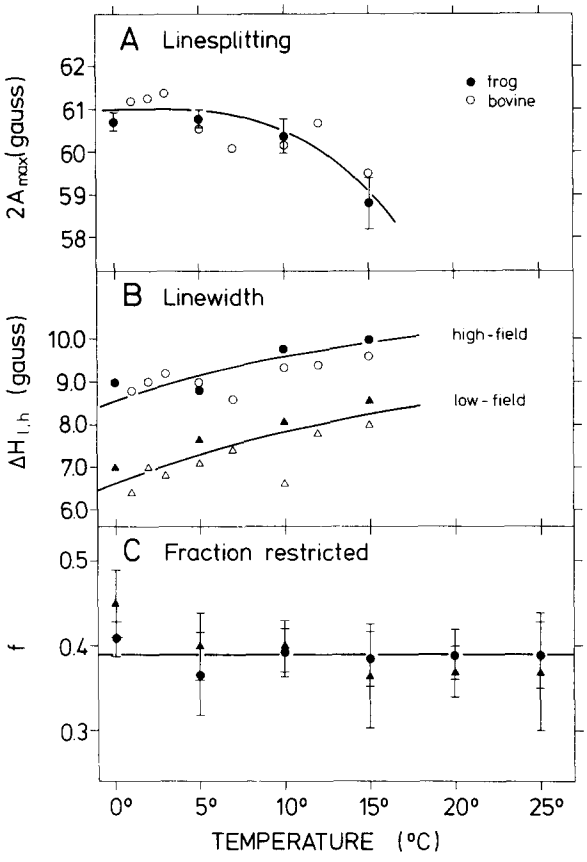

Fig. 4. Temperature dependence of the spectral parameters of the motionally restricted component of the 14-PSSL spin label in frog rod outer segment membranes (filled symbols). Some data are also included for bovine rod outer segment membranes (open symbols). (A) Outer hyperfine splitting, $2A_{max}$. (B) Linewidth (half-full width at half-height) of the low-field line (▲), ΔH_l, and of the high-field line (●), ΔH_h. (C) Fraction, f, of total intensity in the motionally restricted component, deduced from the fluid difference spectra (●) and from the motionally restricted difference spectra (▲).

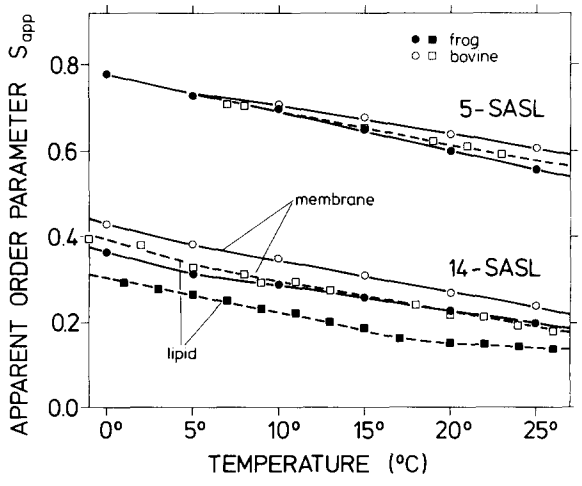

Fig. 5. Temperature dependence of the apparent order parameter, S_{app}, of the stearic acid spin labels 5-SASL and 14-SASL in frog rod outer segment membranes (●———●) and extracted lipids (■- - -■), and in bovine rod outer segment membranes (○———○) and extracted lipids (□- - -□).

ference in that the values for the bovine system are higher than those for the frog. This difference corresponds to an approximately 10 deg. C temperature increase for the bovine system.

Discussion

This work demonstrates the existence of a population of lipids which are motionally-restricted on the ESR timescale over a wide physiological temperature range in the frog rod outer segment membrane. This motional restriction is specifically induced by membrane protein (of which 80–90% is rhodopsin [2]), since it is not detected in dispersions of the extracted rod outer segment lipid. The spectral properties of this labelled lipid component indicate that it has a lifetime of longer than 10^{-8} s, and a motional correlation time of the order of $2 \cdot 10^{-8}$ s. Thus the present data remove any doubt concerning the existence of the motionally-restricted lipid component in rod-outer segment membranes at physiological temperatures [25,26], hence confirming our previous results on the bovine system [1].

The origin of this motionally restricted lipid could in principle be due to protein aggregation which creates pools of 'trapped' lipid, a protein-induced lipid lateral phase separation, or the direct interaction between the lipid molecules and the hydrophobic surface of the membrane protein, as has been suggested for the boundary layer lipid in reconstituted cytochrome oxidase membranes [16,17].

As discussed elsewhere [6,7], the available evidence indicates that rhodopsin rotates relatively fast in the disc membrane, with a correlation time of $20 \cdot 10^{-6}$ s at 20°C [6], and it is thus unlikely that the protein is aggregated. Also, the temperature dependence of neither the fluid nor the motionally restricted lipid components in the frog rod outer segment membrane shows any evidence of phase transitions or lateral phase separations. The lipid composition of these membranes reveals a highly unsaturated and heterogeneous lipid mixture [13], suggesting that lipid phase separations are unlikely. Thus, the best explanation for the origin of the immobilized component is the direct association of the lipid molecules with the hydrophobic surface of rhodopsin [28].

If the lipid-to-protein ratio (n_t) in the sample is known it is possible to calculate the number of lipid molecules N_1 associated with each protein molecule from the fraction of spin labels in the motionally-restricted component given in Table I. If the spin label reflects the unlabelled lipid distribution, and there is no selectivity between the different unlabelled lipids ($K_r = 1$, see Ref. 17), then

$$(n_f^*/n_b^*) = n_t/N_1 - 1 \tag{4}$$

where $n_f^*/n_b^* = (1-f)/f$ is the ratio of the fluid to motionally restricted components in the ESR spectrum. The lipid/protein ratio in our frog rod outer segment was determined to be 61, very similar to the value obtained for bovine rod outer segment of 65, giving rise to a value of N_1 of 22 ± 2. Based on the available data for the structure of frog rhodopsin [18], this figure represents good agreement with the theoretical estimate for the first shell of lipids surrounding to the protein. The low resolution structure obtained from two-dimensional negatively-stained crystals of frog rhodopsin reveals a dimer of cross-sectional dimensions 22–25 Å × 70–80 Å [18]. This is in accord with the radius of gyration measured for bovine rhodopsin, which is consistent with an elongated cylinder of 31.5 Å diameter or ellipsoid of minor axis 34 Å [4]. The number of lipid molecules which can be accommodated around these latter structures has been estimated to be 24 per protein monomer in either case [1]. It is also relevant to note that 25 lipid molecules may be accommodated around the bacteriorhodopsin monomer structure [22], since the dimer of the latter has almost exactly the same projected area as the rhodopsin dimer [18]. Fig. 4C indicates that the fraction of motionally restricted lipid remains essentially constant over a reasonably large temperature range. This is as expected, assuming that the motionally restricted component arises from those lipids in direct contact with the protein surface, and that the exchange rate with the fluid lipids is not so great as to appreciably perturb the quantitation by spectral subtraction [24,27,29]. From the total lipid/protein ratio it can be estimated that the remaining lipids are sufficient for at least one further lipid shell around the protein.

The temperature dependence in Figs. 4A, B indicates mobility of the motionally restricted lipid component on the 20-ns timescale. The origin of this mobility is segmental motion relative to the protein surface and/or exchange on and off the protein. Davoust and Devaux [24] have demonstrated exchange at a rate $\sim 10^7$ s^{-1} for acyl chains covalently linked at the surface of rhodopsin and that spin-labelled lipids approach the protein surface at a diffusion-controlled rate [19]. Previously we have estimated the exchange rate of diffuseable lipids in bovine rod outer segment from lifetime broadening, δH_{pp}, of the fluid lipid component [20]. An upper limit for the off rate constant: $\tau_b^{-1} = (\sqrt{3/2})(g\beta/\hbar) \cdot \delta H_{pp} \times (1-f)/f$ was approx. $4.0 \cdot 10^7$ s^{-1} (see Ref. 27, p. 167), and a somewhat lower value may be estimated from the current data for frog rod outer segment. Thus it seems likely that there is segmental lipid motion at the surface of the protein, in addition to exchange. Recent studies with bovine rod outer segment have revealed rather similar apparent correlation times of the motionally restricted component for different label positions along the lipid chain (Pates and Marsh, in preparation). This, on the other hand, would be consistent with a major contribution to the restricted lipid motion from exchange off the protein.

The data in Fig. 5, comparing the fluid lipid component in membranes with the extracted lipids, suggests some perturbation by rhodopsin of the lipids beyond the first motionally restricted boundary shell. However, part of this perturbation of the subsequent shells may be attributable to exchange with the first shell, as discussed above. Comparing the results on frog rod outer segment lipids with those of bovine rod outer segment in Fig. 5 indicates that at any given temperature the frog lipids are more 'fluid' (in the sense that the lower lipid chain segments display larger motional amplitudes and/or rates). This feature may be attributed to the higher content of polyunsaturated fatty acids found in the latter [14]. However, the difference (which corresponds to a 10 deg. C temperature shift) is smaller than that which might be expected from the difference in the respective physiological temperatures. This may in part be due to the necessity for the frog to adapt to a varying range of ambient temperatures. As mentioned above there is little detectable difference in the dynamic properties of the motionally restricted lipids between the two systems.

The work described above uses the power of the spin label technique to resolve a motionally-distinct population of lipids in the frog rod outer segment disc membrane, the presence of which can be explained by an exchange frequency of lipids on and off the protein surface which is in the range 10^5–10^7 s^{-1}. Further questions regarding the dynamics and order of these lipids in the protein-associated environment will be addressed in a forthcoming publication.

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