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Physical modifications of rhodopsin boundary lipids in lecithin–rhodopsin complexes: A spin-label study

(lipid–protein interactions/electron spin resonance)

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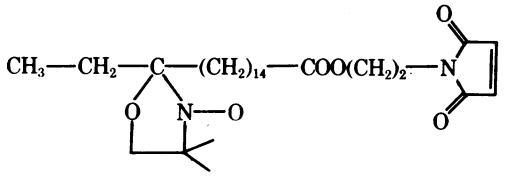
ABSTRACT **The microviscosity of rhodopsin boundary lipids was studied with a spin-labeled fatty acid covalently attached to rhodopsin, in rhodopsin–egg lecithin vesicles. When the lipid-to-protein ratio was high (500:1, mole to mole), only narrow peaks were visible in electron paramagnetic resonance spectrum at 37°C. This enabled us to show that, under these conditions, not more than 10% of the probes have their motion strongly restricted by the proximity of the protein. When the temperature was reduced, a second component characteristic of strong immobilization appeared. It corresponds to 50% of the signal at −5°C. At all temperatures reduction of the lipid-to-protein ratio also resulted in an increase of the amount of immobilized lipid. These results show that the rhodopsin boundary layer under physiological conditions is associated with low microviscosity. However, low temperatures, low lipid-to-protein ratios, or combinations of the two can induce dramatic modifications of the physical state of the boundary lipids, which under these conditions may no longer be representative of the functional biological system. These results are relevant to the general theory of lipid–protein interaction.**

The physical state of the lipid chain in direct contact with intrinsic membrane proteins has been extensively studied by various techniques, including nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR). However, both of these techniques have led to rather controversial results on reconstituted lipid–protein systems. For example, ^2H NMR applied to cytochrome oxidase–lipid complexes led Dahlquist *et al.* (1) to conclude in favor of a temperature-dependent boundary layer, whereas more recent results, of Seelig and Seelig (2) and Oldfield *et al.* (3), suggest that the lipid population is homogeneous at all temperatures. According to the latter authors, the protein decreases the average order parameter of the lipid. From spin-label experiments involving low lipid-to-protein ratios, various authors have come to the conclusion that boundary lipids are strongly immobilized around proteins such as cytochrome oxidase (4–7), Ca^{2+}-ATPase (8), lipophilin (9), and rhodopsin (10). These results, however, were criticized by Chapman *et al.* (11), who tested the interaction of gramicidin A with lipids, using the spin-label method. Concurrently we used spin-labeled acyl derivatives of specific ligands or covalently bound spin-labeled fatty acids. We demonstrated the possibility that spin labels that are in direct contact with intrinsic proteins, such as the ADP carrier in mitochondria (12, 13), the acetylcholine receptor in torpedo membranes (14), or rhodopsin in disc membrane fragments (15), are moving rapidly. We showed that a spin-labeled fatty acid can be anchored to rhodopsin, provided the fatty acid contains a maleimide residue linked to the carboxylic terminal. The fatty acid moiety allows the maleimide to react specifically with sulfhydryl groups of rhodopsin not accessible to hydrophilic reagents. The probe is positioned at the lipid–protein interface (see discussion in ref. 15). The advantage of this technique over the classical use of spin-labeled fatty acid partitioning between the protein environment and the bulk lipid phase is that the signal corresponding to the boundary layer can be directly recorded. However, we showed that these maleimide spin labels react also with phosphatidylethanolamine (15). Consequently, in the disc membranes the protein-bound signal could only be obtained indirectly, a quantitative computer subtraction of the phospholipid signal having been necessary. By means of this procedure we showed (15) that when the probe is near the $\omega - 2$ carbon of the acyl chain, it is highly mobile at 20°C, even in the direct vicinity of the protein. This result is important because it suggests a new description of lipid boundary layers from spin-label experiments. However, we could not rule out the possibility that an immobilized component associated with a minor fraction of the signal is superimposed on narrow lines obtained with disc membranes. We have now purified spin-labeled rhodopsin and recombined it with egg lecithin. It appears that a strongly immobilized component exists, superimposed on the weakly immobilized component. The ratio between these signals depends both on temperature and on lipid-to-protein ratio.

MATERIALS AND METHODS

Membrane Preparation. Rod outer segment membranes were isolated from cattle retina as described by Osborne *et al.* (16). Membranes were suspended in 10 mM Tris·HCl (pH 7.5) and were freshly prepared before use. Experiments were carried out under dim red light. The rhodopsin concentration was determined by the absorbance at 500 nm, after solubilization of an aliquot in 3% Ammonyx; 10% acrylamide/sodium dodecyl sulfate gel electrophoresis experiments were performed according to Weber and Osborn (17). Phosphate was determined after mineralization of an aliquot in $HClO_4$ as described by Rouser *et al.* (18).

Rhodopsin Labeling and Purification. The ester of 16-doxylstearic acid and *N*-(2-hydroxyethyl)maleimide (spin label I) was prepared according to Favre *et al.* (15). Rhodopsin



Spin label I

(100–200 μM) was incubated overnight at 4°C with a 5-fold molar excess of *N*-ethylamaleimide (Merck) in order to alkylate

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Abbreviation: EPR, electron paramagnetic resonance.

the two hydrophilic sulfhydryl groups (19, 20). Excess *N*-ethylaleimide was removed by centrifugation (30 min at 150,000 × *g*), then the membranes were resuspended at a protein concentration of 200 μM in 10 mM Tris·HCl (pH 7.5) in the presence of 2-fold molar excess of spin label **I**. After 10-hr incubation at 4°C, rhodopsin was solubilized in a medium containing 10 mM Tris·HCl (pH 7.5), 100 mM octyl β-glucoside (Sigma), 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ and was diluted in this medium to a final concentration of 10 μM. A small amount of insoluble material was removed by centrifugation (30 min at 150,000 × *g*).

The spin-labeled rhodopsin was freed from phospholipids by affinity chromatography over a concanavalin A-Sepharose column (Pharmacia), according to De Grip *et al.* (21).

After application, phospholipids and unreacted spin label **I** were removed by washing the gel with the application medium except that $MnCl_2$ was omitted in order to allow EPR control of the eluate. The washing procedure was stopped when no EPR signal from nitroxide radicals could be detected in the eluate. Ten column volumes of washing solution were usually required. The lipid-free rhodopsin was eluted by addition of 300 mM methyl α-D-mannopyranoside to the washing buffer (5 column volumes required). In the first 3 column volumes the recovery was about 60% of the applied rhodopsin.

Reconstitution with Lecithin. Dry egg yolk lecithin, prepared according to Singleton *et al.* (22), was dissolved to a final concentration of 3 mM in 10 mM Tris·HCl (pH 7.5)/100 mM octyl β-glucoside at room temperature. Aliquots of this solution were mixed with the purified rhodopsin to give the desired phospholipid-to-protein ratio. The detergent was then removed by dialysis against 10 mM Tris·HCl (pH 7.5) for 48 hr, with several changes of the buffer, which had been deoxygenated with argon. In a few experiments, after the purification step, ^{14}C-labeled octyl β-glucoside [50 mCi/mmol (1 Ci = 3.7×10^{10} becquerels), a gift from G. Lauqin, (Centre d'Etudes Nucléaires, Grenoble, France)] was added to the rhodopsin–detergent complexes and the radioactivity was measured before and after dialysis.

The size of the lecithin–rhodopsin complexes was estimated by electron microscopy from thin sections of vesicles embedded in Vestopal.

EPR Experiments and Analysis of the Data. EPR experiments were performed in the dark with a Varian E109 spectrometer connected to a Tektronix 4051 computer as described in ref. 15. The EPR spectra frequently showed evidence of heterogeneity due to combinations of weakly and strongly immobilized probes.

The fraction of strongly immobilized probes was calculated after computer subtraction of a strongly immobilized spectrum (see ref. 5). The upper limit was determined by the appearance of a negative signal in the *low field* region. The lower limit was determined from the amount of signal that had to be subtracted in order to remove the minimum (indicated in Fig. 1*A* by an arrow) in the *high field* region. The strongly immobilized spectrum used for these subtractions was the spectrum of spin label **I** bound to rhodopsin, which was recorded at 37°C in lipid-free samples. Its extreme splitting agreed with the extreme splitting in lipid-containing samples.

At low temperature (−5°C) or low lipid-to-protein ratios, the fraction of the probes which was immobilized could be confirmed by a different method, described by Marsh *et al.* (23). An arbitrary "weakly immobilized" spectrum was subtracted from the spectra obtained with protein-bound spin labels until a pure strongly immobilized spectrum was generated. The weakly immobilized signal was obtained from spin label **I** in pure lecithin bilayers, at an arbitrary low temperature. However, we were unable to use this technique with rhodopsin whenever the immobilized fraction represented less than 50% of the signal (i.e., high temperature or high lipid-to-protein ratio), because the spectra generated in pure lecithin would not coincide with the narrow lines visible in the presence of proteins.

Finally, the rotational correlation time τ of the nitroxide was estimated whenever the spectrum consisted of narrow peaks by using the formula derived by Keith *et al.* (24). It is obviously a crude estimate for an anisotropic motion of the type experienced by a fatty acid chain in close contact with a protein.

RESULTS AND DISCUSSION

Reconstitution of Rhodopsin–Lecithin Vesicles. Purified rhodopsin gave a single band on the gels corresponding to molecular weight 40,000, whereas, before purification, minor bands were still visible in the region corresponding to lower molecular weight. When the detergent was dialyzed out without any addition of lipids, the amount of phosphate found per rhodopsin was less than one molecule per molecule showing that complete delipidation was reached on the column. This means that after reconstitution the preparation contained only rhodopsin as a protein component and egg lecithin as a phospholipid. Experiments with radioactive detergent showed that for vesicles with a lipid-to-protein molar ratio of 100:1 the amount of detergent remaining after dialysis was less than one molecule of detergent per molecule of rhodopsin. Electron micrographs of the same preparation showed a rather homogeneous distribution of vesicles corresponding to an average size of 2000 Å.

Effect of the Temperature on the Boundary Layer Microviscosity. In order to study the effect of temperature on the physical state of the lipid boundary layer of rhodopsin, we deliberately chose to employ first a large lipid excess (500:1) because we wanted to minimize protein–protein interactions, which may create perturbations of the boundary lipids.

In the case of a large lipid-to-protein ratio the protein-bound spin labels gave rise at all temperatures to a spectrum with a major component corresponding to fast motion (Fig. 1*A*). The overall lineshape of this component (peak ratios and line width) varied with temperature in a manner comparable to that of the spectrum of the corresponding spin-labeled fatty acid in pure lecithin vesicles (Fig. 1*B*). The spectrum of the protein-bound labels, however, always indicated greater restriction in mobility. For example, at 37°C a rough estimation of τ, based on the assumption of isotropic motion, leads to $\tau = 0.7$ ns in pure lecithin and $\tau = 1.4$ ns at the protein boundary layer (Fig. 2). This result is consistent with the result of Brown *et al.* (25) deduced from proton NMR and related to the small restriction in segmental motion of lipid chains in direct contact with rhodopsin.

At low temperatures (−5 and +5°C), the spectrum clearly indicated a superposition of two different components, one of which is a strongly immobilized component and corresponds to about 50% of the signal at −5°C. Fig. 2 shows the temperature dependence of the amount of immobilized spin label **I** in the sample containing 500 lipid molecules per rhodopsin molecule. The method of determination (explained in *Materials and Methods*) is based on the analysis of the spectra shown in Fig. 1*A*. In spite of the uncertainty in the determination of the percentage of the immobilized component, it is clear that the same fraction of label is not immobilized at all temperatures. Yet the probes are always in direct contact with the protein, because we are dealing with covalently bound spin labels. The results suggest that the immobilized boundary layer shrinks when the temperature is increased.

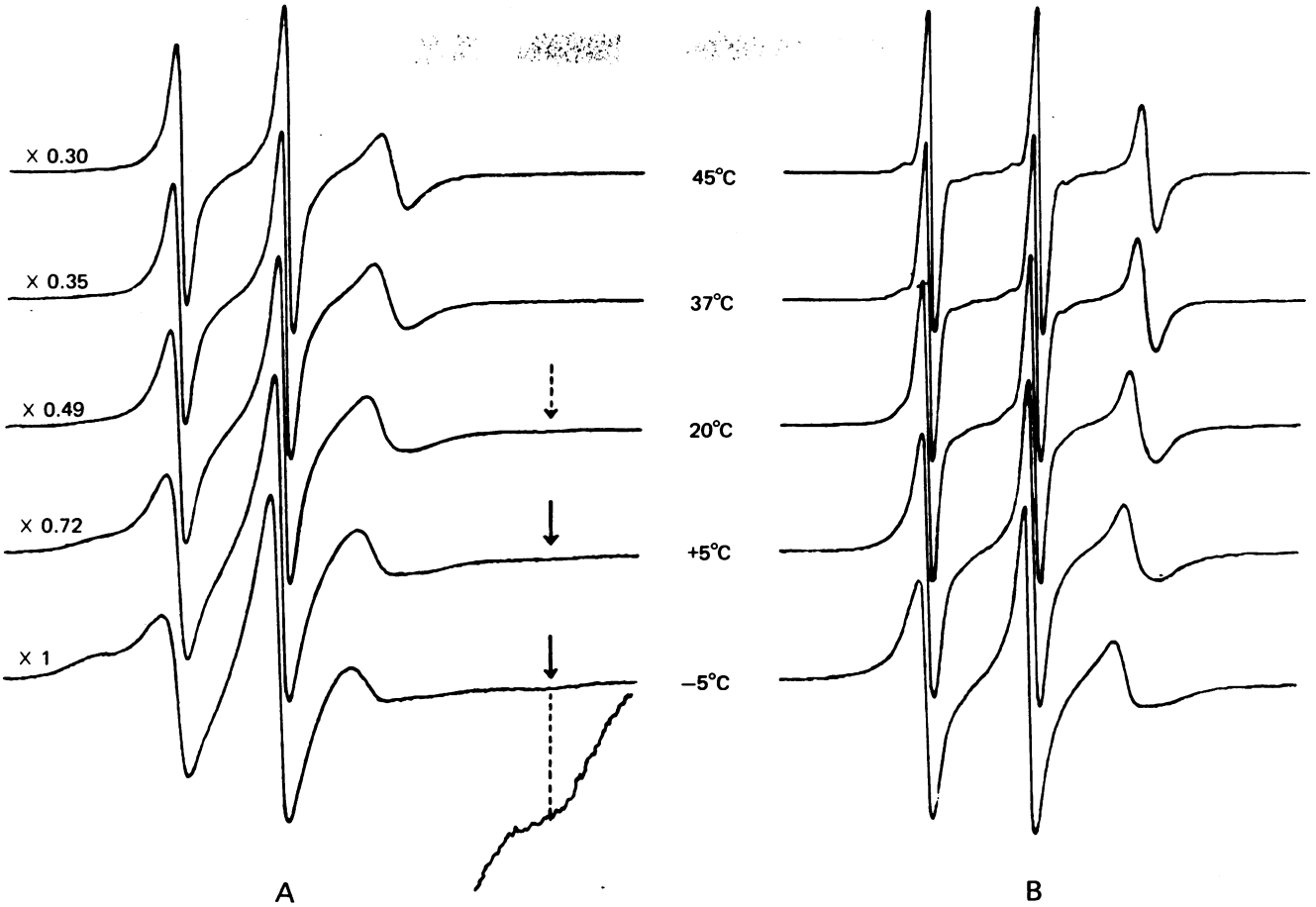

FIG. 1. (*A*) EPR spectra of spin label I covalently bound to rhodopsin in rhodopsin–egg lecithin vesicles (lipid-to-protein ratio 500:1). Numbers on the left hand side correspond to relative gain of the spectrometer. (*B*) Spectra of spin label I in lecithin liposomes.

It should be pointed out that the above analysis assumes that the immobilized spectrum is temperature independent. However, this assumption is not essential. If the "immobilized signal" is less and less immobilized when the temperature rises, the two components will collapse into a single spectrum. It is then impossible to determine their ratio. Nevertheless the conclusion is basically the same and fits into the framework of the former model: the boundary layer microviscosity depends largely on the temperature; at high temperature there is no strongly immobilized lipid.

This finding supports very well the theory developed by Owicki *et al.* (26), if one assumes that the order parameter described in ref. 26 can be related to the modifications of the EPR spectra of spin-labeled fatty acids in membranes, as suggested by these authors. According to Owicki *et al.* it is possible that the amount of "boundary lipids" is very small, provided that the temperature is far above the transition temperature.

Although we probed only the direct environment of rhodopsin, two components were seen with samples at low temperatures; this result requires an explanation: (*i*) It is conceivable that the probe can explore more than a single shell of lipids around the proteins. If the difference between the first and the second shell is very important [which is possible under certain conditions in the model of Owicki *et al.* (26)], then two different superimposed spectra would be anticipated. (*ii*) The two-component system may even correspond to the first shell. If the calculated immobilized boundary layer actually extends over less than one shell of lipids, one would expect inhomogeneities to be seen in the spectra due to local fluctuations between the two extreme states, ordered and disordered. (*iii*) Another explanation of the two environments of rhodopsin, observed at low temperatures, could be that proteins tend to segregate out

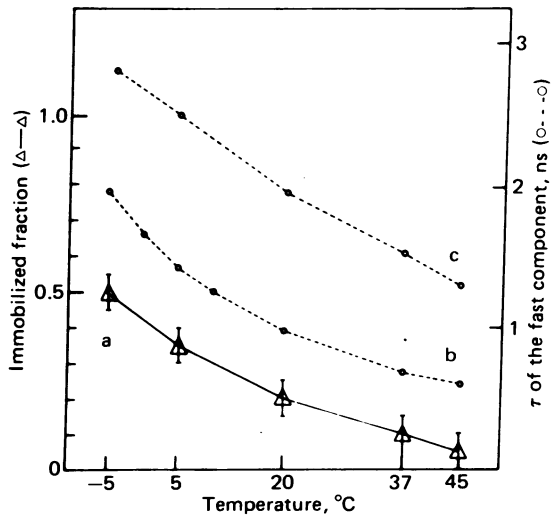

FIG. 2. Curve a, fraction of immobilized probes at the lipid–protein interface, as a function of temperature, for rhodopsin–lecithin vesicles containing 500 moles of lecithin per mole of rhodopsin. Curve b, approximate rotational correlation time, τ, of the fast component associated with the protein-bound spin labels, in the same reconstitution system as for curve a. Curve c, approximate rotational correlation time for spin label I in lecithin liposomes. The remarkable point is how close the values on curves b and c are. For comparison, the rotational correlation time of the protein is in the order of 10,000 ns.

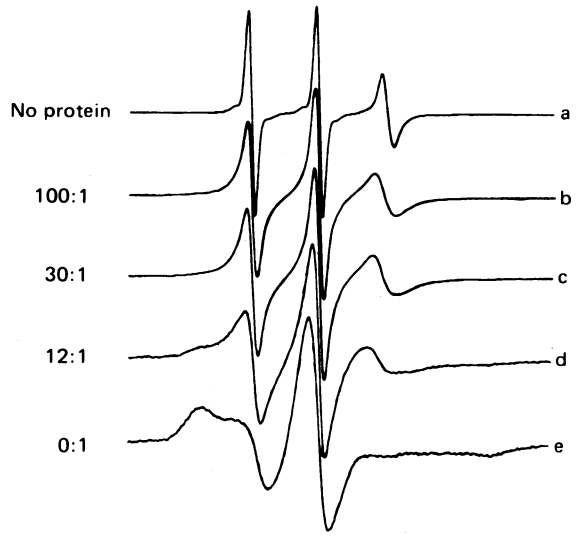

FIG. 3. Influence of the lipid-to-protein molar ratio (numbers on left) on the EPR spectra of spin label I bound to rhodopsin in rhodopsin–lecithin complexes. The temperature was 37°C. Spectrum e corresponds to lipid-free rhodopsin; it is considered as a reference spectrum and used to quantify the amount of immobilized component present in the other spectra.

whenever lipids are crystallizing (see ref. 11). The immobilized fraction of spin label I would reveal these clusters.

Effect of the Lipid-to-Protein Ratio on the Boundary Layer Microviscosity. When the lipid-to-protein ratio was varied, the shape of the spectrum corresponding to the protein-bound labels changed dramatically (Fig. 3). With 12 lipid molecules per protein molecule, which is far below the lipid-to-protein ratio in discs, the spectrum obviously had components even at high temperatures and contained a strongly immobilized signal.

Fig. 4 shows an estimate of the amount of immobilized signal as a function of lipid-to-protein ratio for various temperatures. As before, several explanations of this phenomenon can be proposed: (*i*) It can be explained within the framework of Owicki's model (26), providing we assume a homogeneous distribution of proteins. In this case a low lipid-to-protein ratio corresponds to a short distance between rhodopsin molecules and may result in a dramatic increase in the boundary effects

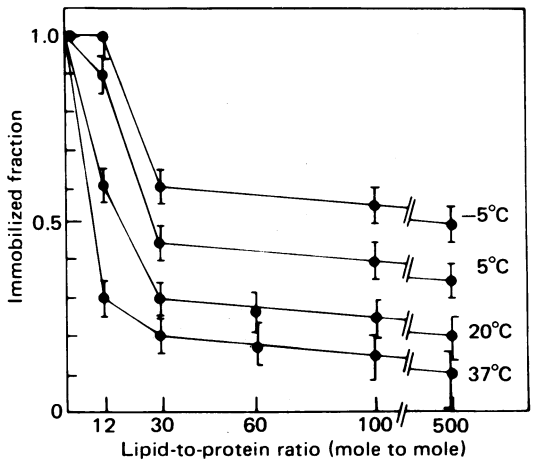

FIG. 4. Fraction of immobilized probes at the lipid–protein interface as a function of the lecithin-to-rhodopsin ratio, in reconstituted systems at various temperatures.

imposed by the proteins. (*ii*) The immobilization of spin label I at low lipid-to-protein ratios can also be due to protein aggregation, which certainly occurs with a higher probability if this ratio is decreased. At low lipid-to-protein ratios the two models become indistinguishable.

General Conclusions. Whatever the interpretation at low temperatures or low lipid-to-protein ratio, one is always faced with the conclusion that the immobilized boundary layer in rhodopsin–lecithin vesicles vanishes at temperatures and lipid-to-protein ratios of physiological relevance (i.e., 37°C and a lipid-to-protein ratio of about 80:1). One may argue that we have replaced the original lipids by lecithin. In fact this means that the lipids used are *less* fluid than the native lipids. It is known that lipids from disc membranes are highly unsaturated, whereas egg lecithin molecules contain on the average only one double bond. Therefore, to imitate the actual situation in the biological membrane one should look at temperatures even higher than 37°C when lecithin is being used. In this case no trace of rigid chains will be detected, even with a lipid-to-protein ratio of 80:1.

The results of the present study lead us to question not only the interpretation of former spin-label studies related to rhodopsin–lipid interactions (10) but also the relevance of many similar studies involving other proteins. It is known that rhodopsin has a very high mobility in disc membranes: no other proteins are found to have such a high rotational diffusion constant. This may signify that rhodopsin experiences an unusual type of lipid–protein interaction. However, our results at least show that new experiments should be done with cytochrome oxidase or Ca^{2+}-ATPase to demonstrate that an immobilized boundary layer (or annulus) exists under physiological conditions (high lipid-to-protein ratio and high temperature).

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