Spin-Label Studies of Lipid–Protein Interactions in Retinal Rod Outer Segment Membranes. Fluidity of the Boundary Layer†

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ABSTRACT: In order to fix spin-labeled acids at the boundary layer of membrane-bound proteins, spin-labeled long-chain derivatives (m,n)MSL (general formula, $CH_3(CH_2)_mR-(CH_2)_nCOO(CH_2)_2-M$, where R is an oxazolidine ring containing a nitroxide and M is a maleimide residue) were synthesized. The spin-labeled molecules bind covalently to at least two different classes of sulfhydryl groups on rhodopsin in disc membrane fragments from bovine retina. One class of sites is hydrophilic and corresponds to the two SH groups labeled readily by *N*-ethylmaleimide; the second class of sites is only reached by hydrophobic probes. (10,3)MSL binds equally well to the two classes of sites on rhodopsin, whereas (1,14)MSL, more hydrophobic, binds preferentially to the hydrophobic sites. Apparently a third class of SH groups can be labeled if a very large excess of (m,n)MSL is employed, but proteins may be denatured in this latter case. Labels not covalently bound are removed from the membranes by incubation with fatty acid free bovine serum albumin. However, it is found that the probes do not bind only to rhodopsin in the disc membranes. (m,n)MSL also binds covalently to phosphatidylethanolamine in the rod outer segments or in liposomes. This covalent binding to phospholipids is demonstrated by lipid extraction and thin-layer chromatographic analysis. In order to obtain the pure EPR spectra of the spin-labeled fatty acids bound to the protein, the spectra corresponding to phospholipid-bound spin labels have been subtracted. (1,14)MSL corresponds to the spin label with the nitroxide near the ω-2 carbon of the acyl chain. When this spin label is bound to rhodopsin in the disc membranes, it gives rise to an EPR spectrum not very different from the spectrum of the corresponding fatty acid diffusing freely in the lipid phase. This result suggests that, in native membranes, a high degree of fluidity exists in the boundary layer of phospholipids and therefore indicates that the lipid phase of the rod outer segment membranes is largely homogeneous. If membranes are illuminated at 37 °C for an hour, an immobilized component appears, superimposed on the former spectrum of (1,14)MSL. Similarly if membranes are partially delipidated with phospholipase A_2, a strongly immobilized component is always seen. The (10,3)MSL, which has a probe closer to the maleimide residue, is more immobilized than the corresponding free fatty acid. However, saturation transfer spectroscopy demonstrates that, in this latter case, the motion of the probe still does not reflect the rotation of the protein; thus, it is not rigidly fixed to the protein. Only when membranes are highly delipidated is it possible to liken the protein motion to the remaining hydrocarbon chain motion. However, in this latter case the apparent correlation time describing the motion is increased by more than two orders of magnitude, showing that lipid-depleted membranes cannot be used to characterize the viscosity of the boundary layer of native membranes.

It is often admitted that intrinsic membrane proteins are surrounded by a boundary layer or "annulus" of rigidly bound lipid. The immobilization of this shell of lipid has been deduced essentially from EPR experiments involving spin-labeled fatty acids incorporated into reconstituted systems containing variable lipid to protein ratios. Jost et al. (1973) were the first to propose from spin-label experiments the model of a boundary layer of lipid surrounding an intrinsic membrane protein, namely cytochrome oxidase. Later, Hesketh et al. (1976) reported similar experiments with Ca^{2+}–ATPase, while Chapman et al. (1977) showed that gramcidin A can lead to the same EPR results, if this polypeptide is dissolved in a small amount of lipid.

Rhodopsin was also tested with spin-labeled fatty acids. Pontus and Delmelle (1975) found evidence of a rigid boundary layer around this hydrophobic protein. However, previously, Hong & Hubbell (1972) had reached a different conclusion from spin-label experiments with rhodopsin reincorporated into phospholipid; they suggested that the *average* viscosity of the membranes was dependent on the lipid to protein ratio. This is in good agreement with recent results put foward by Cherry et al. (1977) from very different experiments involving bacteriorhodopsin. Finally, using proton NMR and rod outer segment membranes, Brown et al. (1977) concluded that, although the segmental motion of hydrocarbon chains was affected by the proximity of the proteins, all phospholipids can diffuse rapidly in the plane of the membrane and that boundary and free lipids exchange rapidly.

Concurrently, using spin-labeled fatty acid derivatives containing specific polar head groups, we were able to explore the hydrophobic environment of different membrane-bound proteins in native membranes (Devaux et al., 1975; Lauquin et al., 1977; Bienvenue et al., 1977). Our general conclusion was that, although evidence of hindrance in the motion of spin-labeled chains accompanies the binding to the proteins, fluidity can be found in the direct environment of such proteins.

In the present article our aim is to probe the direct lipid environment of rhodopsin in the disc membranes, without changing the lipid/protein ratio. However, we have also delipidated the disc membrane fragments with phospholipase A_2 to show the modifications induced on the boundary layer of the protein by an artificial decrease of the lipid to protein ratio. A subsequent paper will deal with reconstituted rhodopsin–lipid vesicles.

Since rhodopsin does not have a specific site to which one can anchor a fatty acid chain, we took advantage of the SH groups of the proteins to link covalently, via a maleimide residue, the fatty acid spin labels. In a typical rod outer segment preparation, rhodopsin represents about 85% of the protein and SH groups on other proteins can be neglected (Daemen, 1973). It will be shown that, with some precautions, it is possible to label selectively only one class of SH groups.

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0006-2960/79/0418-1156$01.00/0

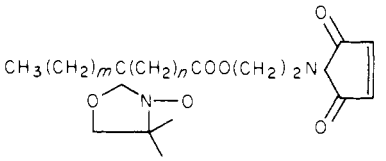
However, it is necessary in our experiments to take into account the covalent binding of the spin labels to phosphatidylethanolamine.

Conventional EPR[1] has been used and, in some experiments, it has been found helpful to also employ the saturation transfer technique, in order to discriminate between spectra associated with different types of strongly immobilized nitroxide probes.

Materials and Methods

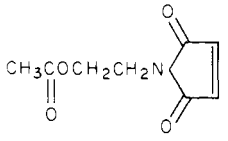
Membrane preparation, assay, and delipidation procedures have been described (Baroin et al., 1979). The method for studying the kinetics of reduction of the EPR signal, in the presence of sodium ascorbate, was taken from Rousselet et al. (1976).

Synthesis of (10,3)MSL and (1,14)MSL. Two long-chain spin-labeled maleimide derivatives were synthesized by using labeled fatty acids and *N*-(2-hydroxyethyl)maleimide.



(*m*,*n*)MSL
(10,3), *m*, *n* = 10, 3
(1,14), *m*, *n* = 1, 14

The spin-labeled fatty acids were prepared according to Hubbell & McConnell (1971). *N*-(2-Hydroxyethyl)maleimide was prepared according to Yamada et al. (1965) and Miyadera et al. (1971) and was checked by NMR and IR spectroscopy. In order to synthesize (10,3) and (1,14) derivatives, a mixed carboxylic carbonic anhydride of the spin-labeled fatty acid was made by adding slowly, at 0 °C, 50 μmol of ethyl chloroformate into a solution of spin-labeled fatty acid (50 μmol) in 1 mL of CCl_4 containing 50 μmol of triethylamine. The reaction was followed by infrared spectroscopy. Two hours at room temperature led to a complete reaction. After filtration and evaporation of the solvent, the mixed anhydride was dissolved in 200 μL of toluene; 50 μmol of *N*-(2-hydroxyethyl)maleimide and 15 μmol of triethylamine were then added and the mixture was stirred overnight at 60 °C. Toluene was removed from the reaction mixture, and the products were dissolved in chloroform. The solution was chromatographed on a preparative silica gel plate (2-mm thick, Merck) with a 50/50 chloroform–acetone mixture as eluant. The band at $R_f = 0.6$ was scraped off and thoroughly washed with acetone. After evaporation of the acetone, the (*m*,*n*)MSL gave a single spot by TLC with the same solvent mixture as before and was identified by its IR spectrum in CCl_4. It presented two well-resolved bands: one at 1720 cm^{-1} (maleimide cycle) and a weaker one at 1740 cm^{-1} (ester bond) exactly as the following reference compound:



This latter compound was prepared by the classical method: reflux of the corresponding hydroxamic acid in acetic anhydride. It was unambiguously identified by NMR in $CDCl_3$: 6.85 ppm (s, 2 H); between 3.8 and 4.35 ppm (m, 4 H, A_2B_2 pattern); 2.1 ppm (s, 3 H). It was pure as shown by TLC on silica gel with the same solvent mixture. As expected, its R_f was slightly smaller than with the long-chain analogue.

(*m*,*n*)MSL spin labels were stored at −20 °C in ethanol. All labels used in this work were shown to be free of isomaleimide.

Spin Labeling of the Rod Outer Segment Membranes. The long-chain maleimide derivatives ((10,3)MSL and (1,14)-MSL) were incubated with the membranes in such a way that an appreciable fraction of the label was in water. Typically 4×10^{-8} mol of spin label was added to 2×10^{-8} mol of membrane-bound rhodopsin in a total volume of 1 mL. The incubation was carried out at room temperature for 3 h in complete darkness under argon. Unreacted label was washed by centrifugation of the membranes (100000*g*, 30 min), at least twice in a large volume (8 mL for 10^{-8} mol of rhodopsin) of buffer containing 2% fatty acid free BSA (Sigma). After a final wash in buffer alone, membranes were suspended in a minimum volume and transferred to a quartz cell for EPR spectroscopy. Typical concentration of spin label in the cell was 100 μM. In a few experiments, the incubation was conducted with ten times more of the spin label, corresponding to approximately 20 molecules of spin label per rhodopsin molecule.

Quantitative Determination of the Binding Distribution. Systematic binding to phosphatidylethanolamine and maybe to phosphatidylserine takes place as a side effect of the labeling of the proteins. In order to determine the distribution of the probes in the membrane, a special procedure has to be employed. After incubation, membranes were washed several times with BSA and the total amount of signal remaining in the membranes was determined by double integration of the conventional EPR spectrum. The lipids were then extracted by the method of Schmid et al. (1973) and chromatographed on silica gel G plates (hexane–ethyl ether, 70:40). The phospholipids stay at $R_f = 0$, while free (*m*,*n*)MSL migrates to $R_f = 0.7$. The two bands are scraped off the plates and eluted with chloroform/methanol, and the concentration of spin label is determined. Protein aggregates are also recovered and the corresponding amount of spin is determined.

Lipid-Binding Experiments. Egg lecithin (10 mM) or an equimolar mixture of egg lecithin (5mM) and dipalmitoylphosphatidylethanolamine (5 mM) and 0.3 mM (*m*,*n*)MSL were suspended in 100 mM phosphate buffer, pH 7.4, and allowed to incubate at 20 °C for 15 h. Lipids were extracted and chromatographed by the method described above. The spin-label concentration was determined for each band.

EPR Experiments. The EPR apparatus has been described (Baroin et al., 1979). However, in order to make the subtraction more accurately, the cells were usually provided with a small capillary containing powdered manganese sulfide. This compound gives two narrow lines centered at about $g = 2$ with a splitting of 78 G. The spectra were then stored on tape with this field reference position.

Results

We will start by presenting data not directly relevant to lipid–protein interactions in the rod outer segments, but necessary to the understanding of subsequent experiments.

(m,n)MSL Binding to Phosphatidylethanolamine in Rod Outer Segment Membranes or in Liposomes. As explained in the introductory section, the spin labels were designed to react covalently with membrane-bound proteins. However, when incorporated into real membranes containing a mixture of phospholipids including phosphatidylethanolamine, it is found that, even at neutral pH, a nonnegligible fraction of the

[1] Abbreviations used: (1,14)MSL and (10,3)MSL, spin-labeled fatty acid derivatives whose formulas are presented in Materials and Methods; NEM, *N*-ethylmaleimide; EPR, electron paramagnetic resonance; BSA, bovine serum albumin.

Table I: Percentage of Binding of (1,14)MSL to Liposomes or Membranes Determined by Lipid Extraction[a]

	liposomes		
	PC	PC + PE, 1/1	rod outer segment membranes
$R_f = 0$ (phospholipids)	<5	35[b]	47[b]
$R_f = 0.7$–1 ((*m*,*n*)MSL)	>95	65	15
proteins			38

[a] Means of three experiments, ±5%. [b] The EPR spectra of these fractions, in an aqueous suspension (phosphate buffer, pH 7.4) are identical with the spectra of (1,14)fatty acid in the same host lipid system. This remark holds true for (10,3)MSL as compared with (10,3)fatty acid.

molecules reacts with phospholipid and therefore cannot be removed from the membranes by BSA. This phenomenon was found also in liposomes containing a mixture of egg lecithin and phosphatidylethanolamine (50/50), while no binding takes place with pure egg lecithin. The first two columns of Table I summarize the results. Incubation at acidic pH decreases the binding. However, it was found that long periods of incubation at pH 5, followed by periods of centrifugation at the same low pH, lead to severe decreases in the signal. Furthermore, when lipids are extracted with organic solvent, partial reoxidation takes place, making any reliable quantitative estimation of the binding distribution impossible. For that reason, incubation of the rod outer segment membranes was routinely performed at pH 7.4.

The procedure described in Materials and Methods enables us (see Table I, last column) to determine reasonably well how the spin labels are distributed in the rod outer segment membranes. The results show unambiguously that (a) a substantial fraction of the spin label binds covalently to the phospholipid (between 40 and 50% of the total label in the membrane); (b) approximately the same fraction of the spin label binds to the protein (30–40%); and (c) only a small fraction of the spin label is neither covalently bound to any membrane component nor removed by BSA (<15%).

(1,14)MSL Binds Preferentially to One Class of Hydrophobic SH Groups on Rhodopsin. Long-chain maleimide spin labels were incubated systematically at a concentration comparable to the rhodopsin concentration. This concentration was chosen because such amphiphilic molecules are likely to be slightly detergent and a large excess of spin label during the incubation period could result in the denaturation of proteins. If (1,14)MSL is incubated in such a way that two spin labels per rhodopsin are present in the medium, the amount of membrane-bound spin label found after BSA treatment is of the order of one per rhodopsin. After correction for the amount of binding to lipid, one finds less than one bound spin label per protein. If rhodopsin is preincubated first with a large excess of NEM, only a small decrease in the binding of (1,14)MSL is found, which means that normally only 10–20% of the (1,14)MSL bound to protein is present on sulfhydryl sites of rhodopsin readily accessible to hydrophilic maleimides. The rest of the label must bind to the SH groups only accessible to hydrophobic markers (De Grip et al., 1975).

Finally, if (1,14)MSL is incubated in a large excess (more than 10 molecules of spin label per rhodopsin), it will be shown that a new component appears in the EPR spectrum of the labeled membranes, suggesting binding at a third class of SH groups on rhodopsin (see below), but denaturation of the proteins may accompany this binding.

(10,3)MSL Binds at Two Classes of SH Groups on Rhodopsin. (10,3)MSL does not bind to rhodopsin in exactly the same way as (1,14)MSL. If membranes are preincubated with NEM, the shape and the amplitude of the EPR signal are modified. A reduction of about 50% of the binding takes place. If one takes into account the number of phospholipid-bound spin labels, it appears that (10,3)MSL binds preferentially to the hydrophilic SH groups. The rest of the protein-bound (10,3)MSL binds probably to the same sites to which (1,14)MSL is attached. Finally, it is difficult to decide whether (10,3)MSL binds to a third class of SH, when incubated in large excess, because no obvious new component appears in the spectrum.

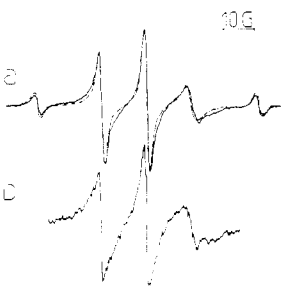


FIGURE 1: EPR spectra of (1,14)MSL in intact membranes at 20 °C. (a) The full line spectrum is obtained after preincubation with NEM, incubation with (1,14)MSL, and several washes with BSA. The dotted line spectrum corresponds to (1,14)fatty acid in the same membranes. The two extreme peaks with a splitting of 78 G are obtained with manganese sulfide powder and are used as field references. (b) This spectrum is obtained by a combination of the two former spectra. It corresponds to (1,14)MSL bound to rhodopsin (see text).

The EPR Spectrum of (1,14)MSL Bound to Rhodopsin Indicates That the Probe Is Essentially Mobile at Room Temperature. Figure 1 shows the conventional EPR spectrum (first harmonic in phase) of (1,14)MSL in rod outer segment membranes under different conditions.

Figure 1a shows the spectrum obtained after preincubation with NEM and incubation with a low concentration of spin label and several washes with BSA. Superimposed on this spectrum is shown that of the (1,14)fatty acid in the same membranes. This dotted-line spectrum can also be obtained with (1,14)MSL if a vey low concentration of spin label is added to the membranes and no incubation performed. Figure 1b is obtained by computer subtraction of two former spectra, the relative weight of each spectrum being determined from the estimate of the amount of protein-bound and lipid-bound label obtained by the method explained before. We have mentioned already that a certain inaccuracy exists in such quantitative determinations: namely, the amount of protein-bound label is, for example, 50 ± 10%. However, it is very important to realize that, whatever one chooses for the relative weight of each spectrum, it is never possible to generate by subtraction a typical "strongly immobilized spectrum". This, however, does not exclude the possibility of such a component being present as a minor fraction of the signal corresponding to the protein-bound label.

Figure 2a is the spectrum obtained after incubation with a large excess of (1,14)MSL in rod outer segment membranes. The membranes have been washed with BSA, but no computer subtraction has been performed. This is because spin–spin interactions exist between the phospholipid-bound (1,14)MSL and therefore a subtraction would not be meaningful. The spectrum shows clearly, however, that a new component exists and apparently corresponds to what is usually called a strongly immobilized spectrum (see the arrow in Figure 2a). Figure 2b corresponds to the same sample as that giving rise to the full-line spectrum of Figure 1a, except that the sample was illuminated for 1 h at 37 °C before recording the spectrum

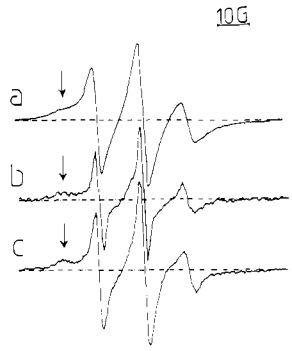


FIGURE 2: Different conditions giving rise to an immobilized component with (1,14)MSL in membranes at 20 °C. (a) Incubation with a large excess of spin label (20 spin labels per rhodopsin); (b) membranes are allowed to stand 1 h at 37 °C, under continuous illumination; (c) partial delipidation (about 30% lipid removed).

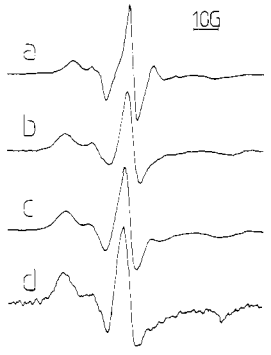


FIGURE 3: Spectra of (10,3)MSL in intact membranes at 20 °C. (a) (10,3)Fatty acid; (b) (10,3)MSL without any preincubation with NEM but with several washes with BSA; (c) preincubation of the membranes with NEM and incubation with (10,3)MSL followed by BSA treatment; (d) combination of c and a so as to obtain the spectrum of the pure rhodopsin-bound label in the case of membranes preincubated with NEM. In order to do this subtraction, the references used in Figure 1 were employed but have been computer-subtracted from the displayed spectra. The technique used to determine the amount of protein-bound label is described in Materials and Methods.

at 20 °C. Again the arrow indicates the presence of a new strongly immobilized component. Finally, Figure 1c shows the spectrum of (1,14)MSL in membranes partially delipidated with phospholipase A_2 (about 30% delipidation). The overall shape of this two-component spectrum is very similar to that obtained by incorporation of (1,14)fatty acid in the same membrane fragments. Yet in the displayed spectrum, about 60–70% of the spin label is protein bound.

First Harmonic Spectra of (10,3)MSL in the Rod Outer Segment Membranes. Figure 3 shows the first harmonic in-phase (conventional) spectra of (10,3)MSL: Figure 3a corresponds to the fatty acid spin label, i.e., free to diffuse in the lipids. The same spectrum is obtained with (10,3)MSL if the spin labels are not incubated. Figure 3b is the spectrum one obtains after incubation and treatment of the membranes with BSA but without any preincubation with NEM. Figure 3c is obtained in a similar manner except that membranes were preincubated with NEM before adding the spin label. Figure 3d is obtained with the help of the computer by combining spectrum c and spectrum a, in the ratio of protein vs. lipid binding determined by lipid extraction. The spectrum shown represents the signal of (10,3)MSL bound to rhodopsin on the more hydrophobic site, very likely the one to which (1,14)MSL binds preferentially; this latter spectrum should be compared with spectrum b of Figure 1.

First Harmonic Spectra and Second Harmonic, 90° Out-of-Phase Spectra of (10,3)MSL in Intact Rod Outer Segment Membranes and in Partially Delipidated Membranes. Classical spectroscopy of (10,3)MSL bound to rhodopsin indicates a strong immobilization of the probe. It is therefore interesting to use saturation transfer spectroscopy to investigate the actual motion experienced and particularly to see if this motion is further reduced by a controlled delipidation of the membranes. Furthermore, by this technique, whenever two components are present in roughly the same amounts, the signal due to the more immobilized probe is favored (i.e., the amplitude of the more immobilized component is larger). This fact allows one, to a first approximation, to neglect the contribution of the lipid-bound spin label in saturation transfer spectroscopy (see legend of Figure 4). Figure 4 shows a set of spectra, corresponding to first harmonic and second harmonic displays of (10,3)MSL in disc membranes under variable conditions. Spectra a and b correspond in fact to the (10,3)fatty acid (without the maleimide residue). The other spectra were all obtained after preincubation of the membranes with NEM, in order to direct the binding with the hydrophobic SH groups of rhodopsin. Spectra c and d correspond to intact membranes, while e and f correspond to membranes with a lipid to protein ratio decreased by 30%; g and h correspond to 70% delipidation.

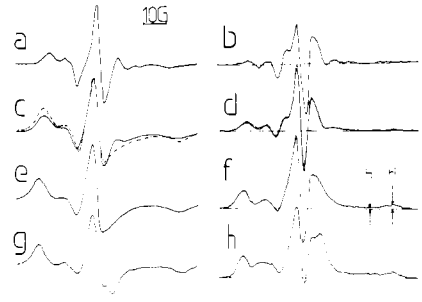


FIGURE 4: Comparison of the conventional EPR and saturation transfer EPR with (10,3)MSL in membranes progressively delipidated at 20 °C. Spectra on the left correspond to the first harmonic, in phase, 10 mW, 2-G modulation. Spectra on the right correspond to the second harmonic, 90° out of phase, 5-G modulation, 32 mW. Spectra a and b: (10,3)fatty acid in intact membranes. Spectra c and d: (10,3)MSL in intact membranes. The dotted line spectra superimposed correspond to the signal due to the protein-bound label only (see text). Spectra e and f are obtained with partially delipidated membranes (30% delipidation); spectra g and h correspond to highly delipidated membranes (over 70% delipidation). H and H″ correspond to the signal amplitude at the indicated positions (for a more detailed, theoretical explanation, see Thomas et al., 1976).

Figure 4 shows clearly that saturation transfer spectroscopy can differentiate between various states of the boundary layer, unlike conventional EPR. It is interesting to remark that inspection of the high-field region shows clearly that the spectrum d cannot be a linear combination of b and h. This is important because it means that, whatever the corrections made to d by subtraction of b, the result will always indicate that (10,3)MSL bound to rhodopsin experiences more motion than in a highly delipidated sample. Similarly, if one pays attention to the high-field region of spectrum f, it appears that f cannot be generated by a combination of d and h. Indeed d has essentially no signal in the high-field region, while $H''/H \simeq 0.33$ for f and $H''/H \simeq 0.90$ for h (H''/H is a parameter which increases when the movement of the probe decreases: Thomas et al., 1976). Thus an intermediate viscosity state must exist between the original viscosity of the discs and the viscosity of highly delipidated membranes. Finally we want to point out that in the case of highly delipidated membranes (g and h), there is no significant difference between (10,3)fatty acid and (10,3)MSL, and therefore there is no point in making any correction to spectrum g or h.

Ascorbate Assay. Disc membrane fragments labeled with (1,14)MSL were incubated at 0 °C with 5 mM sodium ascorbate, and the amplitude of the EPR signal was monitored. After 2 h, practically no decrease of the signal had taken place. Yet when a nitroxide is fixed at the 5th position of the chain of a phospholipid incorporated into lecithin vesicles or into biological membranes, it can be reduced slowly at 0 °C (Rousselet et al., 1976). Consequently when (1,14)MSL is bound to rhodopsin in the disc membranes, the nitroxide moiety must be deeply burried into the hydrophobic core of the membrane.

Discussion

Where Does (m,n)MSL Bind on Rhodopsin? In this article rhodopsin is studied with covalently attached hydrophobic spin labels. The spin labels used contain a maleimide residue which forms a covalent bond with sulfhydryl groups and, less efficiently, with amino groups. The advantage of using a specialized membrane such as the disc membranes from bovine retina is that pratically only one protein is present in large quantity: about 85% of the protein is rhodopsin (Daemen, 1973). Therefore only one protein is likely to be labeled in spite of the nonspecificity of the maleimide reacting group. If hydrophilic spin-labeled derivatives of NEM are used, two SH groups can be labeled readily (Delmelle & Virmaux, 1977; Baroin et al., 1977). Similarly, if membrane-bound rhodopsin is labeled in the dark with tritiated NEM, a rather hydrophilic compound, only two sulfhydryl groups are able to react (De Grip et al., 1975). According to these authors, two other SH groups are labeled readily if slightly detergent probes are used. Hubbell et al. (1977) showed also that, if the first two SH groups of rhodopsin are blocked, a second class of sulfhydryl groups is still accessible to organomercuric spin labels. Finally, it has been shown that a third class of SH group becomes accessible when proteins are extensively denatured by strong detergents (De Grip et al., 1975; Daemen et al., 1976).

The long-chain spin-labeled maleimide derivatives used in the present work are likely to be site directed by the hydrophobic chain. In this regard, it is remarkable that (1,14)MSL and (10,3)MSL do not behave in exactly the same way. This is consistent with the fact that the hydrophobicity of the two molecules is different, the first being a stearic acid analogue, while the second is a palmitic acid analogue. We have shown elsewhere (Bienvenue et al., 1977) that the affinity for membrane-bound proteins of long-chain spin-labeled derivatives is highly dependent upon the partition coefficient between water and lipids for these amphiphilic molecules. Therefore the more hydrophilic (10,3)MSL reacts significantly with the sites of rhodopsin exposed to the aqueous phase and competes with NEM. On the other hand, (1,14)MSL, more hydrophobic, seems to react preferentially with the hydrophobic sulfhydryl groups of rhodopsin. The fact that both spin labels also react with phosphatidylethanolamine to a very considerable extent demonstrates that the long chain positions the maleimide residue at the level of the polar head groups of phospholipids.[2] Therefore, the hydrophobic sulfhydryl residues of rhodopsin may very well be not deeply buried in the membrane. Only when the long-chain maleimide derivatives are incubated in a large excess can one reach more deeply buried SH groups; this, however, may occur at the expense of the integrity of the proteins.

Consequently, it is possible to incubate (*m,n*)MSL in such a way that the spin-labeled fatty acyl chains that bind to rhodopsin are positioned into the membrane in the same way as the fatty acid residues of the phospholipids in contact with rhodopsin. This latter conclusion implies that, whenever the carboxylic group of the labeled fatty acid is positioned near the phospholipid head groups, automatically the alkyl chain will orient itself perpendicularly to the plane of the membrane, thus allowing the nitroxide, even at the 16th position of the chain, to probe the lipid–protein interface. This assumption is based on the following arguments.

(a) If the attached chains were lying parallel to the plane of the membranes or were bent in such a way as to allow the nitroxide groups to approach the water interface, they would be reached by ascorbate and chemically reduced. Yet, as indicated in the Results section, such reduction does not take place.

(b) If the labeled chains were exploring randomly the hydrophobic environment of the proteins, without any preferential orientation of the chains, the probe motion on the (1,14)MSL would be restricted by contact with neighboring rhodopsin molecules. Indeed in the native membranes, the average distance between boundary layers of two neighboring proteins is quite comparable to the length of (1,14)MSL in its fully extended form ($\sim$25 Å).

(c) The final argument is a more theoretical one: one should not consider that the lipid phase is an isotropic medium such as the water surrounding the membrane. Due to the average alignment of the phospholipid chains, there is no doubt that it would require more energy for an extended fatty acid chain to stay parallel to the plane of the membrane rather than perpendicular to it. Therefore the labeled fatty acid chains linked to the rhodopsin molecules are very likely to be, on the average, oriented perpendicularly to the plane of the membranes and, hence, the probes explore the lipid–protein interface.

Mobility of the Hydrocarbon Chains in Direct Contact with Rhodopsin as Deduced from Experiments with (1,14)MLS. The mobility of the hydrocarbon chains in close contact with an intrinsic membrane-bound protein is usually studied with spin-labeled fatty acids or phospholipids, i.e., with probes having no specific affinity for proteins. In order to detect the signal from the protein-bound spin label, it is therefore necessary to use reconstituted systems with a low level of lipids. One may then question the significance of the motion detected in such conditions since protein aggregation and/or a general increase of lipid viscosity can be expected under such conditions. This point was stressed by Chapman et al. (1977), who studied the interaction of gramicidin A, considered as a model of intrinsic protein, with lipids by using spin-labeled fatty acid. These authors showed that the immobilized component, usually attributed to protein-bound fatty acids, totally disappear when the ratio of lipid to gramicidin A is higher than 5 (mole to mole). Our approach to the problem has been to artificially link the spin-labeled fatty acids to intrinsic proteins in native membranes (Devaux et al., 1975; Bienvenue et al., 1977). In the present paper, a maleimide group added at the carboxylic end of the spin-labeled fatty acid provides the affinity for rhodopsin. The important finding is that the probe undergoes considerable motion in spite of the vicinity of an intrinsic protein, which spans the whole lipid bilayer in which it is embedded (Saibil et al., 1976). If one tries to associate a correlation time τ with the spectrum of the

[2] It should be pointed out that the short chain maleimide spin label, designated as MSL in our previous article (Baroin et al., 1979), binds phospholipid, in disc membrane fragments, with very little efficiency. When MSL is incubated in a large excess with the disc membranes, only about 10% of the final signal corresponds to a weakly immobilized probe and may be attributed to phosphatidylethanolamine-bound spin labels.

protein-bound label, and similarly with that of the spin label in the bulk lipids,[3] it appears that $\Delta\tau/\tau \simeq 0.5$. This clearly illustrates the fact that the chains are not "strongly immobilized" by the protein in spite of the covalent binding existing in our case. In current work, we are using reconstituted systems with a high ratio of lipid to rhodopsin and find directly the same spectrum as in Figure 1b. It is possible, however, to generate strongly immobilized signals with (1,14)MSL in disc membranes, but only if they are seriously modified. For example, if 30% or more of the phospholipid is removed, this broad component appears superimposed on the narrow lines. Prolonged illumination at 37 °C or incubation with a very large excess of these detergent-type spin labels will also produce the same result. We have already shown (Baroin et al., 1979) that under such conditions the rotational mobility of the proteins is also reduced and that protein aggregation is very likely the reason for the observed phenomena.

Further Information on the Viscosity of the Boundary Layer as Deduced from Experiments with (10,3)MSL. As already indicated in the Results section, conventional EPR shows that the motion of (10,3)MSL is strongly affected by binding to rhodopsin. It is not surprising that the order parameter of (10,3)MSL increases more than that of (1,14)MSL when the molecule is attached to rhodopsin, because the distance between the probe and the point of fixation is smaller in the former case than in the latter. This result should not be taken as proof of the increase of the local viscosity of the lipid phase in the boundary layer of the protein. It only reflects the binding of the maleimide residue.[4]

The conventional EPR spectrum of (10,3)MSL bound to rhodopsin is very similar to the spectrum one obtains with a tightly bound probe designed to report on the motion of the protein itself (see, for example, Baroin et al., 1979). To find out if (10,3)MSL also reports on the motion of the protein or, alternatively, if the probe in this latter case has a supplementary degree of freedom, saturation transfer spectroscopy appears to be an appropriate technique. However, no straightforward conclusion can be reached by comparing spectrum g of Figure 4 with the saturation transfer spectrum of MSL tightly bound to rhodopsin (Baroin et al., 1979, Figure 1). Although the overall shapes are comparable, the positions of the low-field peaks do not correspond at all. This discrepancy is probably due to the anisotropy of the motion and is likely to reflect the particular average orientations of the two types of probe. However, the high-field region of the saturation transfer spectra, according to Thomas (Thomas et al., 1976) is not very anisotropy sensitive and the amplitude in that region may be taken as indicative of the correlation time. The most striking feature in the high-field region of the saturation transfer spectrum of (10,3)MSL in intact membranes is that practically no signal exists, suggesting a relatively fast motion, probably faster than what is measured with a tightly bound probe such as that used in Baroin et al. (1979). Thus, a probe on a fatty acid chain, even covalently attached to the protein, does not properly reflect the protein motion. This confirms that a boundary layer of lipids is not rigidly attached to rhodopsin.

If disc membranes are progressively delipidated, the conventional EPR spectra of protein-bound (10,3)MSL are practically unchanged but the saturation transfer spectra indicate a regular increase in the immobilization of the probe. Finally the spectrum obtained with (10,3)MSL (spectrum h) becomes very close, although not identical, to that of MSL bound to rhodopsin in highly delipidated membranes (spectrum c, Figure 4 of Baroin et al., 1979). A comparison of spectra d and h of Figure 4, based on a crude theoretical interpretation, suggests that τ changes from $\tau < 10^{-7}$ s to $\tau \sim 10^{-4}$ s. Thus, apparently a difference of three orders of magnitude exists between the correlation times associated with the motion of the probe situated in the boundary layer of intact disc membranes and in membranes with a very low lipid content.

What Can We Say about Intermediate States of Delipidation? Conventional EPR of (1,14)MSL shows clearly that partially delipidated samples (30%) are not homogeneous (see Figure 2c). However, saturation transfer EPR, as already discussed, emphasizes the more immobilized component whenever two signals are superimposed. As a result, analysis of the saturation transfer EPR of (10,3)MSL (spectrum f, Figure 4) enables one to see that the immobilization of the probe in partially delipidated membranes is not identical with its immobilization in highly delipidated membranes (see Results). In other words, intermediate states of viscosity must exist. Thus, to summarize, two phenomena accompany the delipidation of membranes, as had already been suggested by looking at the protein motion (Baroin et al., 1979): (a) increased protein aggregation probability, resulting in complete immobilization of some of the protein together with surrounding trapped lipid; and (b) a general and progressive increase in the lipid viscosity.

General Conclusions. We concluded that the mobility of the hydrocarbon chains is restrained by the contact with rhodopsin, but only moderately in intact membranes; this suggests a continuity between the bulk lipid phase and the lipid in the boundary layer around rhodopsin. This conclusion is entirely consistent with the proton NMR results of Brown et al. (1977) on the mobility of hydrocarbon chains in rod outer segment membranes. It is also consistent with the results of Hong & Hubbell (1972). These authors have shown that lipid viscosity depends upon the presence of rhodopsin. Indeed, any model assuming a continuity of the whole lipid phase implies a reciprocal interaction between lipid and protein.

The continuity of the phospholipid phase in intact membranes may indicate a rapid exchange by diffusion in the plane of the membrane of all the phospholipid. Our experiments give no idea of the exchange rate. They nevertheless suggest that, if there is tight binding between phospholipid and protein, it is probably not by van der Waals forces at the level of the hydrocarbon chains,[5] but more likely by electrostatic forces involving the head groups particularly in the case of charged phospholipids.

Our final remark is that measurement of the rate of collision between phospholipids and proteins is very important, but definitely should be performed in intact membranes or in artificial membranes containing a normal lipid to protein ratio.

[3] τ was estimated by a formula of the type proposed by Stone et al. (1965). It is clear that in our case the motion of the probe is anisotropic, and that only very crude indications of τ can be expected by this method.

[4] Similarly, our former results with (10,3)CoA, (10,3)atractyloside, or (7,6)choline also did not prove that the viscosity of the boundary layer around the ADP carrier or the cholinergic receptor is high. The results only demonstrated the binding of the spin-labeled molecules to their target (Devaux et al., 1975; Lauquin et al., 1977; Bienvenue et al., 1977).

[5] It should be kept in mind that oxyoxazolidine rings are slightly polar and therefore may disrupt the binding between hydrophobic moieties in the core of the membrane. Consequently hydrophobic interactions are probably slightly underestimated by the spin-label method. Since this remark holds equally well for the interaction between two phospholipid molecules or the interactions between an intrinsic protein and a phospholipid, our general conclusion about the similarity of the physical state of most lipids in the membrane is still meaningful.

Acknowledgments

The expert technical assistance of P. Hervé is gratefully acknowledged. J. Davoust is also thanked for making available preliminary data on spin-labeled rhodopsin reincorporated into lecithin vesicles.

References

Baroin, A., Thomas, D. D., Osborne, B., & Devaux, P. F. (1977) *Biochem. Biophys. Res. Commun. 78*, 442–447.

Baroin, A., Bienvenue, A., & Devaux, P. F. (1979) *Biochemistry 18* (preceding paper in this issue).

Bienvenue, A., Rousselet, A., Kato, G., & Devaux, P. F. (1977) *Biochemistry 16*, 841–850.

Brown, M. F., Miljaniech, G. P., & Dratz, E. A. (1977) *Biochemistry 16*, 2640–2648.

Chapman, D., Cornell, B. A., Eliasz, A. W., & Perry, A. (1977) *J. Mol. Biol. 113*, 517–538.

Cherry, R. J., Müller, U., & Schneider, G. (1977) *FEBS Lett. 80*, 465–469.

Daemen, F. J. M. (1973) *Biochim. Biophys. Acta 300*, 255–288.

Daemen, F. J. M., Van Breugel, P. J. G. M., Jansen, P. A. A., & Boring, S. L. (1976) *Biochim. Biophys. Acta 453*, 374–382.

De Grip, W. J., Bonting, S. L., & Daemen, F. J. M. (1975) *Biochim. Biophys. Acta 396*, 104–115.

Delmelle, M., & Virmaux, N. (1977) *Biochim. Biophys. Acta 464*, 370–377.

Devaux, P. F., Bienvenue, A., Lauquin, G., Brisson, A. D., Vignais, P. M., & Vignais, P. V. (1975) *Biochemistry 14*, 1272–1280.

Hesketh, T. R., Smith, G. A., Houslay, M. D., McGill, K. A., Birdsall, N. J. M., Metcalfe, J. C., & Warren, G. B. (1976) *Biochemistry 15*, 4145–4151.

Hong, K., & Hubbell, W. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2617–2621.

Hubbell, W. L., & McConnell, H. M. (1971) *J. Am. Chem. Soc. 93*, 314–326.

Hubbell, W. L., Fung, K. K., Hong, K., & Chen, Y. S. (1977) *Vertebrates Photoreception* (Barlow, H., & Fatt, P., Eds.) pp 41–59, Academic Press, New York.

Jost, P. C., Griffith, O. H., Capaldi, R. A., & Van Derkooi, G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 480–484.

Lauquin, G. J. M., Devaux, P. F., Bienvenue, A., Villiers, C., & Vignais, P. V. (1977) *Biochemistry 16*, 1202–1208.

Miyadera, T., Kosower, E. M., & Kosower, N. S. (1971) *J. Med. Chem. 14*, 873–878.

Pontus, M., & Delmelle, M. (1975) *Biochim. Biophys. Acta 401*, 221–230.

Rousselet, A., Colbeau, A., Vignais, P., & Devaux P. F. (1976) *Biochim. Biophys. Acta 426*, 372–384.

Saibil, H., Chabre, M., & Worcester, D. D. (1976) *Nature (London) 262*, 266–270.

Schmid, P., Calvert, J., & Steiner, R. (1973) *Physiol. Chem. Phys. 5*, 157–166.

Stone, T., Buckman, T., Nordio, P., & McConnell, H. M. (1965) *Proc. Natl. Acad. Sci U.S.A. 54*, 1010–1017.

Thomas, D. D., Dalton, L. R., & Hyde, J. S. (1976) *J. Chem. Phys. 65*, 3006–3024.

Yamada, M., Takase, I., Hayashi, K., Hashimoto, Y., & Komiya, Y. (1965) *Yuki Gosei Kagaku Kyokai Shi 23*, 166; (1965) *Chem. Abstr. 63*, 489.

Effect of Organic Solvents on the Beef Heart Mitochondrial Adenosine Triphosphatase†

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ABSTRACT: The effect of organic solvents on the beef heart mitochondrial ATPase-catalyzed ATP and ITP hydrolysis was examined. It was observed that numerous organic solvents stimulated ATP hydrolysis while ITP hydrolysis was inhibited. Methanol at 20% (v/v) was found to stimulate ATP hydrolysis by over 300%, while at the same methanol concentration ITP hydrolysis was inhibited approximately 50%. In the presence of 20% methanol, ATP hydrolysis exhibited linear plots of $1/[ATP]$ vs. $1/v$, while in the absence of methanol negative cooperativity was observed. These data can be interpreted to imply that the catalytic and regulatory sites of the mitochondrial ATPase are being dissociated in 20% methanol. The effect of methanol on the hydrolysis of ATP and ITP was examined as a function of pH. It was found that, at high pH in totally aqueous solutions, the hydrolysis of ATP and ITP was inhibited, while the presence of 20% methanol either caused the hydrolytic rate to peak and remain constant above pH 8 (with ATP as substrate) or caused the rate of hydrolysis to continue to increase above pH 8 (when ITP was the substrate). These data are interpreted to indicate that an acidic group in the active site may be ionizing, limiting the ATPase-catalyzed hydrolytic rate, and, with 20% methanol, this ionization was inhibited.

The effects of organic solvent systems on the stability of beef heart mitochondrial ATPase (F_1) preparations have been explored in some detail, while the effects of these systems on F_1 catalytic activity have only been briefly described. Penefsky & Warner (1965) have shown that ethylene glycol, methanol, ethanol, and glycerol in concentrations from 2 to 20% (v/v) dramatically protected F_1 from cold inactivation. In addition, these authors found that all of the solvents except methanol caused an inhibition of F_1 activity. Methanol caused stimulations of F_1-catalyzed ATP hydrolysis activity with up to double the activity found in completely aqueous solutions. Recent work emphasizes the use of cosolvent systems such as 50% glycerol to stabilize liver and heart F_1 preparations

† From the Department of Chemistry and School of Life Sciences, The University of Nebraska, Lincoln, Nebraska 68588. *Received September 13, 1978; revised manuscript received December 29, 1978.* A portion of this work was presented in preliminary form in (1977) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 3329. Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for the support of this research.

0006-2960/79/0418-1162$01.00/0