Biochimica et Biophysica Acta, 596 (1980) 28—42

BBA 78612

BOUNDARY LIPIDS AND PROTEIN MOBILITY IN RHODOPSIN-PHOSPHATIDYLCHOLINE VESICLES

EFFECT OF LIPID PHASE TRANSITIONS

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(Received May 22nd, 1979)

Key words: Rhodopsin; Lipid-protein interaction; Protein-protein-interaction; ESR; Saturation transfer spectroscopy

Summary

Purified rhodopsin from bovine retina has been incorporated into phospholipid bilayers. Dimiristoylphosphatidylcholine, dipalmitoylphosphatidylcholine, dioleylphosphatidylcholine and egg phosphatidylcholine were used as host lipids, with a ratio of lipid to protein of 120 : 1 (mol to mol). In order to probe the lipid-protein interface specifically, a spin-labeled fatty acid was covalently bound to rhodopsin via an isocyanate reacting group. A spin-labeled phospholipid was used to probe the bulk lipidic phase while a tightly bound maleimide spin label was used to obtain the protein rotational correlation time by the saturation transfer technique.

The following results were obtained:

(1) The kinetics of reduction by ascorbate of the spin-labeled fatty acid covalently bound to rhodopsin demonstrate that the alkyl chain attached to the protein is positioned in the membrane in the same way as the alkyl chains of a phospholipid.

(2) The EPR spectra of the latter shows two components: a strongly immobilized component and a weakly immobilized component. The ratio of the two depends upon the temperature and on the nature of the phospholipids.

(3) The signal of the weakly immobilized component is compared to that obtained in the corresponding pure lipids. The latter signal, assumed to represent non-bounded lipids, indicates a sharp transition at the phospholipid phase

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Abbreviations: (*m*, *n*) NCO, spin-labeled isocyanates; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleylphosphatidylcholine.

transition with dimyristoylphosphatidylcholine or dipalmitoylphosphatidylcholine. The former signal (corresponding to the lipid-protein interface) indicates only a broad transition extending over 7° C with dipalmitoylphosphatidylcholine and almost no transition with dimyristoylphosphatidylcholine.

(4) In a similar way, the rotational correlation time of the protein only changes progressively when the phase transition occurs.

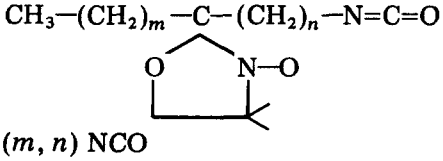
Our interpretation of the data can be summarized as follows:

The immobilized component seen by the EPR technique in the hydrophobic environment of this intrinsic protein very probably reflects protein-protein contacts and thus corresponds to hindrance of the labeled chains, when they are trapped between neighbouring proteins. Below the phase transition lipid segregation should increase the probability of protein contact. However, over a certain range of temperature, the contact with the protein interface probably at the same time prevents the non-segregated phospholipids from freezing. The differences in the results obtained with the various phosphatidylcholines above their transition temperature suggest that the solubility of rhodopsin in bilayers depends not only on the fluidity of the lipids, but also, to some extent, on the phospholipid chain length.

Introduction

In previous reports, we have shown by means of the spin-label technique that a large fraction of the lipid chains which are in direct contact with rhodopsin either in disc membranes [1] or in reconstituted egg phosphatidylcholine-rhodopsin vesicles [2] is highly mobile at physiological temperatures and lipid to protein ratios. Using a long-chain spin-labeled fatty acid covalently linked to rhodopsin, which was then purified and reassociated with phosphatidylcholine, we have shown [2] that, in fact, the EPR spectrum consists of two components. It can be deduced that the percentage of immobilized probe present in a given sample varies with temperature as well as with the lipid to protein ratio. It represents less than 20% of the protein-bound labels at 20° C with a lipid to protein ratio of 100/1 (mol to mol).

In the present report we use a type of covalently bound fatty acid slightly different from that of the former reports. The molecule originally used to bind the SH groups of rhodopsin was a spin-labeled fatty acid linked to a maleimide residue at the carboxylic terminal. The molecules used hereafter contain instead an isocyanate residue. The general formula is:



(m, n) NCO

Secondly, we have now modified the nature of the phospholipids reassociated with rhodopsin in order to take advantage of synthetic phospholipids of defined chain length. Four types of phosphatidylcholine molecules were used: dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine

(DPPC), dioleylphosphatidylcholine (DOPC) and egg phosphatidylcholine.

In parallel experiments, we have measured the rotational mobility of the proteins by using a spin-label analogue of *N*-ethylmaleimide together with the saturation transfer technique. Therefore, it became possible to correlate the mobility of the lipid environment of the proteins and their rotational diffusion rate.

These experiments on rhodopsin which do not use the disc endogenous phospholipids are oriented toward an understanding of the physical interaction between an intrinsic protein and a phospholipid bilayer. In this instance, rhodopsin is considered as a mere representative of hydrophobic proteins.

Materials and Methods

Synthesis of spin labels

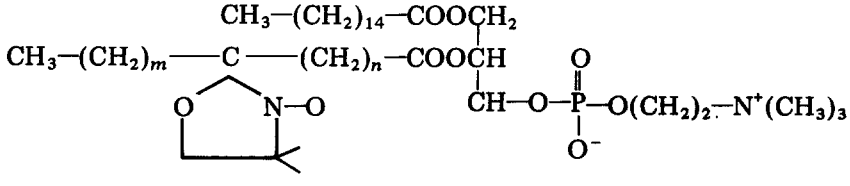
Isocyanates. Spin-labeled isocyanates were synthesized by Curtius rearrangement of corresponding acyl azides which were prepared from mixed anhydrides by sodium azide substitution reaction [3]. A typical experiment is as follows.

Fatty acid (50 μmol) and triethylamine (75 μmol freshly distilled from $LiAlH_4$) were dissolved in 5 ml dry CCl_4. After cooling to 0°C, ethyl chloroformiate (50 μmol) was added in three portions in 0.5 h, the mixture was then stirred for 1 h at room temperature. Triethylamine chlorhydrate was filtered off and CCl_4 was evaporated. By infrared spectroscopy and EPR, it was shown that mixed anhydride was formed without too great a loss of free radicals. Generally about 30% of the EPR signal disappeared, probably by acidic reduction in spite of triethylamine scavenging.

Mixed anhydride was dissolved at 0°C in acetone, and 50 μl of 1 M aqueous sodium azide was added with good stirring. The mixture was kept at 0°C during the next operations: supplementary reaction time of 1 h, addition of 0.5 ml of cold water, extraction of organic azide by cold toluene (three times 2 ml) and drying of this solution on $MgSO_4$.

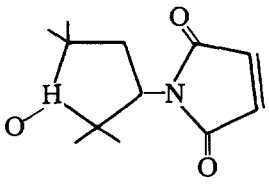
After filtration of the sulfate, the solution was kept at 100°C for 0.5 h and the toluene was evaporated. The resulting spin-labeled isocyanate could be kept for 3 months in dry CCl_4 at −30°C, under N_2, without any trace of decomposition. The product exhibits the strong infrared band at 2270 cm^{-1} characteristic of the isocyanate group, generally with a small trace of absorption at 1750—1700 cm^{-1} (about 10% of the isocyanate absorption intensity). The free radical concentration was checked by EPR. There was no loss of signal after the mixed anhydride step.

Spin-labeled phosphatidylcholine analogues. The following spin-labeled phosphatidylcholine analogues were also used, (10,3) phosphatidylcholine and (1,14) phosphatidylcholine:



(*m*, *n*) phosphatidylcholine

They were synthetized according to Hubbell and McConnell [4]. For the saturation transfer experiments, proteins were labeled with 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidynoxyl purchased from Syva (Palo-Alto):



3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidynoxyl

Sample preparation

Rod outer segment membranes were isolated from cattle retina and checked as described in Ref. 1. Freshly prepared membranes were incubated overnight with a 5-fold excess of *N*-ethylmaleimide (Merck). After centrifugation (30 min at 150 000 × *g*) the membranes were resuspended at a protein concentration of 200 nmol/ml in 10 mM Tris-HCl (pH 7.5) in the presence of a 5-fold molar excess of (*m*, *n*) NCO. After 12 h incubation in the dark at 4° C, rhodopsin was solubilised with β-octylglucoside (Sigma) and purified as described in Ref. 1. Practically all endogenous phospholipids were removed on the column (less than one phosphate group per rhodopsin is left). The lipid-free spin-labeled rhodopsin eluted from the column was mixed with phosphatidylcholine solubilized with the same detergent, in a molar ratio of 120 phospholipids per rhodopsin. Egg phosphatidylcholine was a generous gift of Dr. Rousselet. DMPC, DPPC and DOPC were purchased from Sigma. No trace of lyso derivatives could be detected by thin-layer chromatography. The detergent was removed by dialysis against 10 mM Tris-HCl, pH 7.5, for 48 h at 4° C, with several changes of the buffer which had been carefully deoxygenated. The ratio of lipid to protein was unchanged after dialysis.

For the spin-label experiments, the membranes were concentrated by centrifugation to a final concentration of approximately 350 μM of proteins.

EPR experiments and analysis of the data

EPR experiments were performed in the dark with a Varian E 109 provided with a field frequency lock and a temperature control accessory and connected to a Tektronix 4051 computer for accumulation, storage and manipulation of the spectra. Several spectra corresponded to the superposition of a strongly immobilized component and a weakly immobilized component. In order to determine the ratio of the two components, a typical signal associated with a strongly immobilized probe, was subtracted from the inhomogenous spectra until no evidence of inhomogeneity could be detected, particularly in the low-field region and the high-field region.

This procedure is described in Ref. 2. The strongly immobilized spectrum was, in the present case, obtained at −5° C with (1,14) NCO bound to rhodopsin in DMPC vesicles. Fortunately, the determination of fraction *r* of immobilized component is not very sensitive to small variations in the shape of the

reference spectrum. The proper extreme splitting ($2\,T'_{//}$) is the most important requirement.

The subtraction generates a signal corresponding to a pure mobile component. It is associated with labels in direct contact with rhodopsin. This latter signal was compared systematically to the spectra obtained at the same temperature with a spin-labeled phospholipid incorporated into pure liposomes of the same lipids. In the case of DMPC or DPPC, a molar ratio of unlabeled phospholipid to spin-labeled phospholipid of 1000 : 1 had to be chosen in order to avoid phase separation of the spin-labeled lipids at low temperatures.

Unfortunately, simple analysis of the EPR spectra do not allow us to measure an unique parameter with an unambigous physical meaning (such as the order parameter or the rotational correlation time) over the whole temperature range. Consequently, a rather arbitrary parameter, defined as the ratio of the mid-field peak height over the low-field height (H_{+1}/H_0) was used to describe the variation with temperature of the main spectral features.

With pure lipids, this parameter as shown in Results is sensitive to discontinuities in the viscosity variation of the membrane when it goes through a transition temperature. Conversely, its progressive modification with temperature measured with probes at the boundary layer was considered as being indicative of very different behavior at the lipid-protein interface.

Saturation transfer experiments

Saturation transfer spectra (2nd harmonic, 90° out of phase) were recorded as previously described [5].

Ascorbate assays

In order to localize the probes covalently linked to rhodopsin, sodium ascorbate was added to egg phosphatidylcholine vesicles containing rhodopsin labeled either with (10,3) NCO or (1,14) NCO. For these experiments, the vesicles were previously sonicated for about 10 s, in the dark with a small tip and low power (Ultra-son Annemasse). During the reduction assay, temperature was maintained at 0°C. 5 mM ascorbate was used, by a 2-fold dilution of a 10 mM sodium ascorbate solution at pH 7. Pure egg phosphatidylcholine vesicles containing a small amount of spin-labeled phospholipid were also assayed with ascorbate in parallel experiments. 20 min sonication was used in these latter experiments to obtain the phosphatidylcholine vesicles from hand-shaken liposomes.

Results

Spin-label binding

Under the incubation conditions described above, the amount of (m, n) NCO bound per rhodopsin was found to be less than unity: 0.5— 0.8 (mol to mol). This is slightly above but comparable to the results obtained with the long-chain maleimide spin-label derivative [1]. The binding of the short-chain maleimide derivative is more effective: about 1.2 spin labels per rhodopsin [5].

(10,3) NCO and (1,14) NCO: localization of the probe

(10,3) NCO and (1,14) NCO, when bound to egg phosphatidylcholine-rhodopsin complex, give rise to EPR spectra with overall lineshapes closely comparable to what is obtained in pure phosphatidylcholine with the corresponding free fatty acid or phospholipid spin labels (see Fig. 1, inserts B and A). For example, the spectrum of (10,3) NCO indicates a much higher order parameter than that of (1,14) NCO at the same temperature.

In the present paper, we will only consider the signal obtained with (10,3) NCO as a way of locating the rhodopsin binding site of this class of spin labels. For this purpose, ascorbate was added to rhodopsin-phosphatidylcholine vesicles at 0° C and the low-field peak height was monitored.

Similar experiments were performed with (1,14) NCO bound to rhodopsin in egg phosphatidylcholine and also with (10,3) phosphatidylcholine or (1,14) phosphatidylcholine in pure egg phosphatidylcholine vesicles for the sake of comparison. The results are displayed in Fig. 1; the reduction rate of this five member oxyoxazolidine ring nitroxide radical in water is also shown.

If one pays attention only to gross effect (initial rates), it is striking how similar the kinetics of reduction of the phospholipid and the protein-bound spin labels are. In particular, the absence of reduction of the (1,14) labels indicates that in both instances the probe is deeply buried in the hydrophobic core of the membrane, while (10,3) labels must be closer to the aqueous interface yet not directly accessible to reagents in the aqueous phase. We take these results as a proof that the reacting site involved in rhodopsin (very likely a SH group) is situated near the polar head groups of the phospholipids at the lipid-protein interface. These results also indicate that the spin-labeled acyl chain must adopt an average orientation perpendicular to the plane of the membrane.

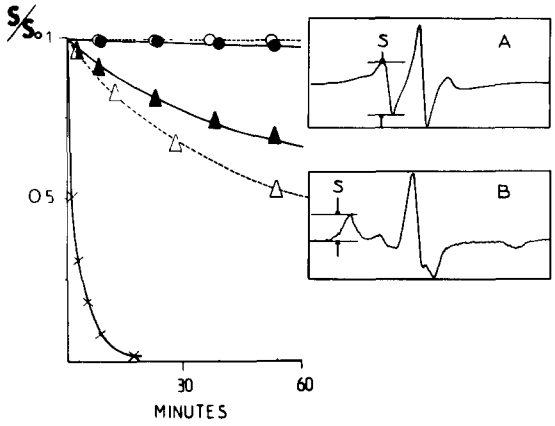


Fig. 1. Reduction rates by 5 mM ascorbate (0° C). In order to locate the binding site of the spin labels (*m*, *n*) NCO the reduction rates of (*m*, *n*) NCO bound to rhodopsin in rhodopsin-egg phosphatidylcholine vesicles are compared to the reduction rates of the corresponding spin-labeled phospholipids, (*m*, *n*) phosphatidylcholine, in sonicated egg phosphatidylcholine vesicles. •, (1,14) NCO; ▲, (10,3) NCO; ○, (1,14) phosphatidylcholine; △, (10,3) phosphatidylcholine; X, (10,3) fatty acid in water. Inserts A and B show the EPR spectra obtained at 0° C with (1,14) NCO and (10,3) NCO, respectively, in egg phosphatidylcholine-rhodopsin complexes. The spectra are monitored in the low-field region [5], to avoid overlap with the characteristic peaks associated with sodium ascorbate.

Hence, (1,14) NCO is a good reporter of the physical state at the rhodopsin-phospholipid interface.

Boundary lipids and protein mobility in DMPC; temperature study through the DMPC phase transition

Fig. 2 shows a comparison of the EPR spectra of (1,14) NCO bound to rhodopsin in DMPC vesicles (A) and (1,14) phosphatidylcholine-DMPC vesicles (B). These latter spectra are not very different from the spectra of (1,14) phosphatidylcholine in DMPC liposomes. They seem to correspond to homogeneous spectra, i.e. the presence of rhodopsin molecules (with a lipid to protein ratio of 120 : 1) does not seem to give rise to a second component but simply to restrict slightly the average motion of the probe. The passage through the transition temperature of DMPC (23°C) produces a marked discontinuity in the evolution of the spectra of the phospholipid spin label. This is clearly indicated on Fig. 3a, where the ratio H_{+1}/H_0 is plotted versus temperature for (1,14) phosphatidylcholine in pure DMPC (open circles). Almost the same curve can be drawn for (1,14) phosphatidylcholine in rhodopsin-containing vesicles, with a high ratio of lipid to protein.

The discontinuity in the viscosity of the membrane is clearly reflected by this arbitrary parameter. Higher values of H_{+1}/H_0 correspond to a lower viscosity. For very high temperatures (above 40°C), it should be pointed out that H_{+1}/H_0 becomes slightly greater than 1. This certainly reflects the fact that (1,14) phosphatidylcholine contains C_{16} chains and therefore, when incorporated in DMPC, near the $\omega - 2$ carbon of the acyl chain reflects a tilt of the terminal part of the chain.

In contrast to the spectra of (1,14) phosphatidylcholine, the spectra of (1,14) NCO (Fig. 2A) exhibit clearly the presence of two components; at least over the temperature range 10—20°C. This happens in spite of the fact that the spin labels are covalently bound to rhodopsin in this latter case. The amount of immobilized component can be estimated quantitatively (see Materials and Methods). The results appear in Fig. 3b. After subtraction of the immobilized

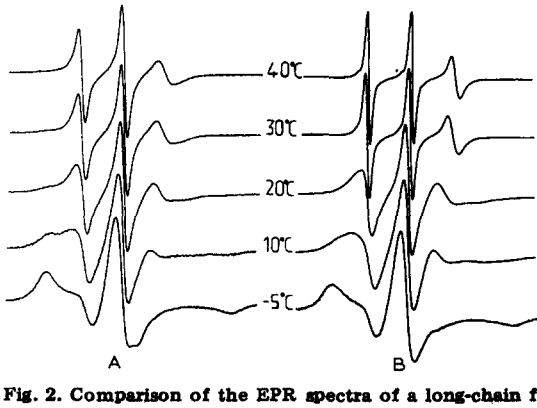


Fig. 2. Comparison of the EPR spectra of a long-chain fatty acid at the boundary layer of rhodopsin (A) or free to diffuse in rhodopsin-DMPC vesicles (B). Spectra A are recorded with (1,14) NCO covalently bound to rhodopsin; spectra B are recorded with (1,14) phosphatidylcholine incorporated into the same type of rhodopsin-DMPC vesicles. The lipid to protein ratio is 120 : 1 mol to mol, in both cases.

component, the shape of the more mobile component can be obtained. In Fig. 3a, the peak ratio H_{+1}/H_0 for such spectra is plotted versus temperature from 0 to 40°C (full circles). Some examples of these spectra are shown in Fig. 4A. Lower temperatures are associated with more noise since the fraction of mobile component at low temperatures is smaller. For comparison, the spectra of (1,14) phosphatidylcholine in pure DMPC at the same temperatures are displayed in Fig. 4B. These latter spectra are assumed to represent the pure non-bounded lipid phase.

These results will be discussed more thoroughly in Discussion (see below); however, Figs. 3 and 4 call for some comment. Firstly, Fig. 3 shows that the mobile component at the rhodopsin boundary layer is almost insensitive to the phospholipid transition temperature. The intersection of two curves of Fig. 3a at approximately 23°C (at the transition temperature) is accompanied by an inversion of the relative mobility of the boundary layer and the bulk lipids.

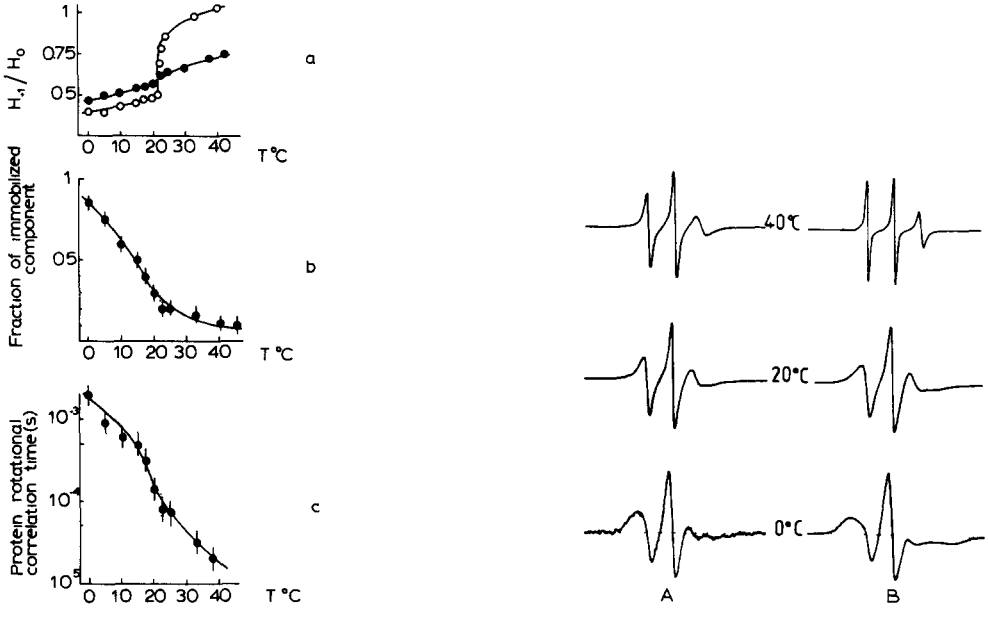


Fig. 3. Comparison of the temperature dependence of various parameters, deduced from the analysis of EPS spectra obtained with spin labels associated either with pure lipids, boundary lipids or proteins in DMPC. (a) Peak ratios (low-field peak over mid-field peak) for (1,14) phosphatidylcholine in pure DMPC (○), or for the more mobile component obtained with (1,14) NCO bound to rhodopsin in rhodopsin-DMPC vesicles (lipid to protein ratio 120 : 1) (●). Note that if (1,14) phosphatidylcholine is incorporated into rhodopsin-DMPC vesicles with a ratio of lipid to protein of 120 : 1 (see spectra B of Fig. 2), the discontinuity of H_{+1}/H_0 is almost identical to what is observed with pure DMPC liposomes. (b) Fraction r of immobilized component in the EPR spectra of (1,14) NCO bound to rhodopsin in rhodopsin-DMPC vesicles (lipid to protein ratio 120 : 1). The procedure employed to obtain this fraction is explained in the text. (c) Rotational correlation time of rhodopsin to DMPC (lipid to protein ratio 120 : 1) as deduced from saturation transfer spectra of maleimide spin label bound to rhodopsin.

Fig. 4. Comparison of the EPR spectra of (1,14) fatty acid at the boundary layer of rhodopsin in DMPC (A) or in pure DMPC (B). On the spectra A, a strongly immobilized component has been subtracted. One can consider that spectra A represent the boundary layer while spectra B represent the 'bulk lipids'. It is remarkable that, below the transition temperature, more motion is seen at the boundary layer while, above the transition temperature, the bulk lipids are more mobile.

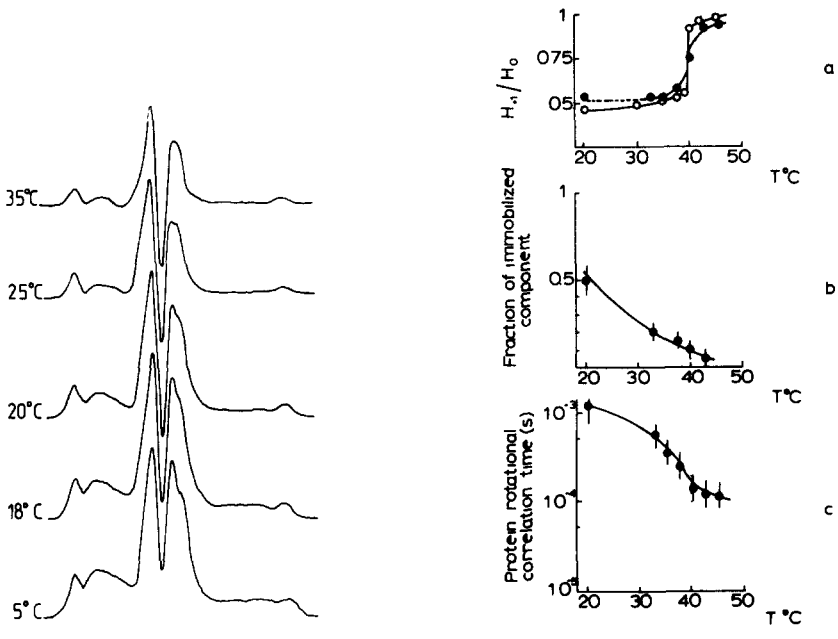


Fig. 5. Saturation transfer spectra (2nd harmonic, 90° out of phase) of spin-labeled rhodpsin in DMPC. Rhodopsin in labeled with maleimide spin label and purified before reincorporation into DMPC vesicles.

Fig. 6. Same spectral parameters as in Fig. 3, but DPPC is used as host lipid.

Above this transition temperature, the boundary layer corresponds to slightly less motion than in bulk lipids (40° C). Below the transition temperature, the situation is reversed. Comparison of the spectra (Fig. 4) with reference spectra in an isotropic medium indicates that the viscosity associated with bulk lipids at 0° C is higher than at the boundary layer at the same temperature.

Secondly, it is interesting to note that the percentage of immobilized component, displayed in Fig. 3b, does not seem to be critically sensitive to the lipid phase transition. Any explanations of the phenomena observed will have to take these facts into account.

The relative insensitiveness of the boundary layer to the bulk lipid transition is also reflected in the temperature dependence of the protein rotational correlation time. Fig. 5 shows a set of saturation transfer spectra for rhodopsin in DMPC. Estimation of the rotational correlation time from an analysis based on the measurement of H''/H and V''/V (see Thomas et al. [6]), is given in Fig. 3c. Again, no sharp discontinuity of correlation time τ appears at the transition temperature. In fact, it is almost impossible to define a transition range in Fig. 3c.

Boundary lipids and rhodopsin mobility in other phosphatidylcholine molecules: DPPC, DOPC and egg phosphatidylcholine

Figs. 6—8 summarize the results of similar spin-label studies undertaken with various phosphatidylcholine molecules used as host lipids for the purified rhodopsin. Endogenous lipids from disc membranes have not been used for the

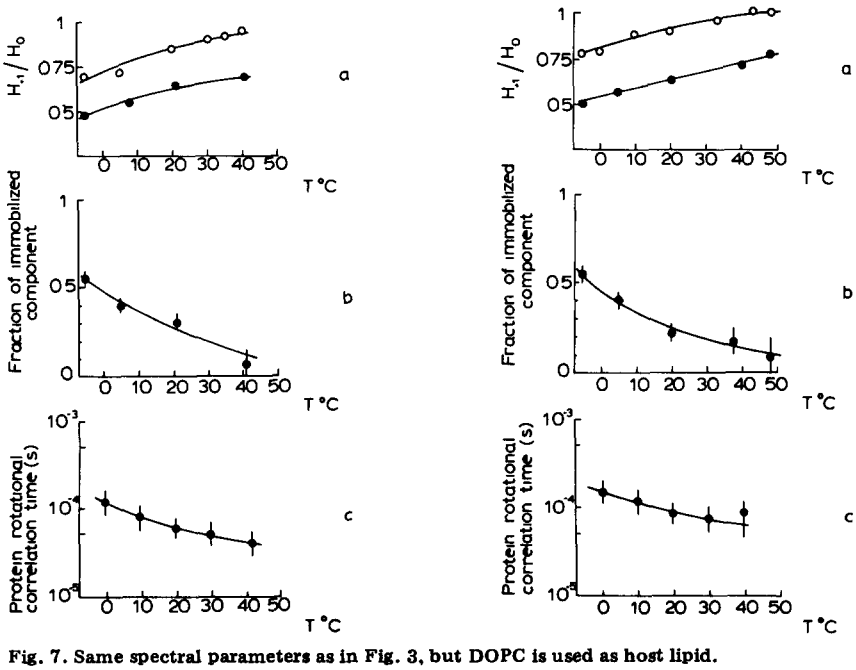


Fig. 7. Same spectral parameters as in Fig. 3, but DOPC is used as host lipid.

Fig. 8. Same spectral parameters as in Fig. 3, but egg phosphatidylcholine is used as host lipid.

moment because of the great instability of these polyunsaturated lipids which make them unsuitable for several days dialysis. Preliminary experiments show that one of the manifestations of the chemical reactivity of these lipids was spin reduction. DPPC was studied at slightly higher temperature than DMPC since its transition temperature is higher.

Plots of H_{+1}/H_0 show the same type of inversion around the transition temperature as for DMPC. However, the mobile component at the boundary layer appears to be more sensitive to the phase transition of the bulk lipids than in DMPC. A second remark is that above the DPPC transition temperature, the amount of immobilized signal detected in the spectrum of (1,14) NCO (Fig. 6b) is of the same order as the sensitivity limit of the method.

This is in contrast not only with DMPC but also with DOPC and egg phosphatidylcholine (Figs. 7 and 8). For all these phospholipids, a remarkable amount of immobilized component is present above the transition temperature.

As expected in the case of DOPC and egg phosphatidylcholine, the parameter H_{+1}/H_0 corresponding to the boundary layer and pure phospholipids, respectively, varies monotonically with temperature. The boundary layer is systematically more viscous than the pure lipid phase consistent with the results obtained for DMPC and DPPC.

Finally, it is interesting to note that the rotational correlation time values for rhodopsin in DOPC (Fig. 7c) are quite close to the values determined by the same method in disc membrane from bovine retina [5]. Therefore, the role of the long chains and the high degree of unsaturation encountered in disc mem-

branes is not simply to provide low viscosity since this requirement can be satisfied with more common lipids, such as DOPC.

Discussion

Our previous results on lipid-protein interactions with rhodopsin

In spite of the frequently accepted idea that lipid chains in close contact with intrinsic proteins should be strongly immobilized (see review by Griffith and Jost [7]), we have shown, in previous papers, that: (a) a long-chain spin-labeled fatty acid in close contact with rhodopsin in disc membranes is highly mobile at 20°C [1]; (b) the same probe linked to rhodopsin in egg phosphatidylcholine-rhodopsin vesicles [2] sees in fact two environments: one corresponds to low viscosity, very close to that of the bulk lipids; the second giving rise to marked probe immobilization on the time scale of conventional EPR. The ratio of the two components depends on the lipid to protein ratio and on the temperature.

Several explanations for the immobilized components were proposed in our previous report. One was based on the possible formation at low temperature of an immobilized boundary layer surrounding isolated proteins. Theoretical bases for this model have been proposed in the literature [8].

However, we proposed another explanation along the lines of Chapman's model for gramicidin A in lipid vesicles [9]. It assumes that the immobilized component reflects protein-protein contact and therefore is due to labeled lipid chains trapped between clustered proteins.

The new data

(a) At high temperature, this new spin label, which is more tightly bound to rhodopsin than the maleimide derivative used originally, confirms that the vicinity of this intrinsic protein is highly fluid. The fact that this result does not depend critically on the exact configuration of the spin probe used suggests that it is not an accidental finding reflecting a very local peculiarity. Four different phospholipids give the same result. Therefore, we can conclude that, for temperatures well above the phase transition, the boundary layer and the bulk lipids form a continuous fluid medium. In all cases, a small restriction in the mobility of the probe reporting on the boundary layer is seen [2].

(b) The present observations also confirm that a strongly immobilized component exists in practically all situations. This component does not correspond to a different site on rhodopsin since the ratio r between strongly immobilized and weakly immobilized signal varies reversibly with temperature. It always decreases when the temperature increases. When we go from one phospholipid to another, the curve $r = f(T)$ is shifted towards higher temperatures if the phospholipid transition temperature is higher. However, the phenomenon is certainly not accounted for by a simple shift of the transition temperature. For example, the curve $r = f(T)$ for DOPC or egg phosphatidylcholine is not superimposable on the curve corresponding to DPPC. In fact, even DMPC and DPPC, which have a sharp transition above room temperature, do not lead to $r = f(T)$ curves which can be superimposed by simple translation. In no case do the

curves $r = f(T)$ show a discontinuity at the phospholipid phase transition.

(c) An interesting new result is clearly illustrated on Figs. 3 and 4: whenever a phospholipid phase transition occurs, the relative mobilities of the boundary layer and of the bulk lipids are inverted. This seems true both for DMPC and DPPC, although it is more spectacular with DMPC.

(d) Finally, simultaneous determination of the protein rotational mobility in the different lipids indicates a rather continuous variation of the rotational correlation time with temperature. This parameter can hardly be used to define the transition temperature of the lipids. However there is no proof that the saturation transfer spectra are homogeneous. They may correspond to a superposition of two very different states. The values of τ_c indicated would correspond to intermediate values. Similar type of measurements have been performed by Kusumi et al. [10].

Interpretation of the results

Before interpreting the data, we must recall the freeze-fracture results obtained some years ago by Chen and Hubbell in rhodopsin-lipid membranes [11]. These authors showed that if unbleached membranes are rapidly frozen from well above the phase transition of the lipids, the particles associated with the proteins are randomly distributed. If the membranes are frozen from below the phase transition, protein-deficient areas are seen. DMPC, in particular, shows characteristic regions with parallel ridges associated with the solid phase of this lipid, but other phospholipids (DPPC for example) exhibit also large protein-poor regions if the sample is quenched from low temperatures. Nevertheless, the formation of these domains does not seem to depend very critically on the transition temperature of the phospholipids. For example, Chen and Hubbell showed that particle-depleted domains occur with di-(18 : 1)t phosphatidylcholine, if it is frozen from 20°C, i.e. above the phospholipid phase transition ($T_c \approx 10°C$). However, if the sample is frozen from 5°C (i.e. below the phase transition temperature), the area of these domains seems larger.

The results of Chen and Hubbell are important for an understanding of our spin-label data: with a great variety of phosphatidylcholine molecules, proteins tend to segregate out as the temperature is reduced. This phenomenon depends to some extent on the transition temperature. However, depending on the lipids, it can anticipate more or less the phospholipid phase transition. The particle-rich regions formed at low temperature are not necessarily without phospholipids. They correspond to regions where the lipid to protein ratio must be lower than the average titration value. This has two consequences: firstly, the lipids remaining in the protein-rich regions form small pools which are unsuitable for the thermodynamic crystallization. Therefore, one expects these lipids to remain more mobile than the segregated lipids. Secondly, in the protein-rich regions, collision or close contact between two or several proteins is statistically favored. In this case, the concentration of entrapped lipid chains whose motion is completely blocked, not by lipid crystallization but because of steric hindrance between two neighbouring proteins, must increase.

The events described above provide a simple and complete framework to explain all our spin-label data. It is only necessary to assume that the appearance of a strongly immobilized spectrum at the lipid-protein interface (except

for very low temperatures) is associated with lipid chains trapped between proteins. It is never necessary to assume the spontaneous immobilization of a lipid chain at the boundary layer of isolated proteins.

Even well above the phase transition, it is conceivable that there is always about 10% of the immobilized signal in our spectra. This would be perfectly explained with a lipid to protein ratio of 120 : 1. Considering the estimated relative surface areas of the proteins and the lipids, one finds that the number of collisions between proteins must be high at this ratio (see Fig. 9).

The protein-protein collisions occur with a higher probability close to or below the phospholipid phase transition temperature due to the local decrease of the lipid to protein ratio accompanying a segregation of lipids (Fig. 9). This phenomenon is accompanied by an increase in the immobilized EPR component, as we have shown both with partially delipidated disc membranes [1] and with vesicles reconstituted with variable ratios of egg phosphatidylcholine per rhodopsin [2]. Therefore protein-lipid segregation at low temperatures would explain the increase in percentage of immobilized signal * seen with our samples.

To explain the relative values of the ratio r of immobilized versus mobile component for different phospholipids and, in particular, to explain the non-negligible value above the transition temperature (50% at 0°C for DOPC), one is forced to make assumptions about the solubility properties of the protein in the different lipids. The solubility certainly depends on how well the phospholipids accommodate the protein. It should depend very much on the chain length, double bonds, etc. DMPC, for example, is probably too short to solubilize rhodopsin properly and therefore there is more immobilization slightly above the phase transition than in the case of DPPC.

Generalisation of the model and comparison with other results and models in the literature

In 1973, Jost et al. [12] proposed a model of an immobilized boundary layer around reconstituted cytochrome oxidase deduced from spin-label experiments. This important experiment was reproduced and refined by many authors working on cytochrome oxidase [13,14], or other hydrophobic proteins such as Ca^{2+}-ATPase [15], lipophilin [16] and also rhodopsin [17]. However, Chapman et al. [9] pointed out how dangerous it was to extrapolate from low-lipid-containing membranes the physical nature of the lipid-protein interactions in normal membranes.

We have shown on various systems such as mitochondria and torpedo membranes that a fatty acid spin label can be mobile in the direct vicinity of hydrophobic proteins such as the ADP carrier [18] or the acetylcholine receptor [19]. At this point, we think that the results concerning the existence of an immobilized boundary layer surrounding intrinsic proteins deduced from low-lipid-containing systems should be considered as irrevelant to physiological membranes.

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* The fraction of immobolized lipids is lower than the fraction of immobilized proteins. Indeed, small aggregates of proteins can be surrounded by mobile lipids. Therefore, a probe at the protein aggregate-lipid interface can be mobile while the proteins are immobilized.

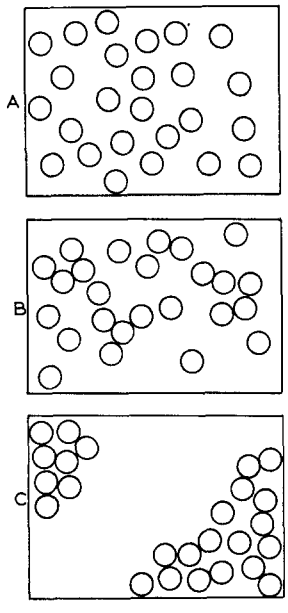

Fig. 9. Schematic representation of the distribution of proteins above and below a phospholipid phase transition. (A) A distribution with no proteins is contact; it is very improbable except if proteins have repulsive interactions. (B) A more probable type of distribution corresponding to the same average density as in (A). Several proteins are in close contact. A spin-labeled fatty acid chain on the surface of the protein has a non-negligible chance of being strongly immobilized. (C) A phase separation has partially segregated the lipids and the proteins. The average density of proteins in the protein-rich region is high; therefore the number of proteins in close contact has increased. As a result the number of immobilized probes at the lipid-protein interface increases also.

Other techniques such as NMR or differential microcalorimetry have provided some support to the immobilized boundary layer. Early results by NMR, in particular, have confirmed the immobilized boundary layer around cytochrome oxidase [20,21]. However, later results by the same technique did not seem to agree [22—24].

It seems that controversy about the relationship between ^{2}H NMR measurements and the lipid-protein interaction has now been settled. It is generally agreed that this technique shows evidence for an homogenous population of lipids for most membrane systems above the phase transition temperature. The proteins appear to be responsible only for the average disordering of the lipids. If any peculiarity exists at the boundary layer, it must be averaged by rapid exchange on NMR time scale. Interestingly enough, it is only below the phase transition of the major phospholipids that an annulus of specific lipids were found by ^{13}C NMR by Brulet and McConnell [25] around reconstituted glycophorin. This was the first evidence of a 'fluid annulus' surrounding a membrane protein.

As for the differential microcalorimetry experiments, we think that they have been largely overinterpreted. There is no doubt that in many reconstituted systems the enthalpy measured for the transition does not account for the total amount of lipid present [26]. However this is, by no means, proof of the existence of an immobilized boundary layer surrounding the proteins. We have

clearly shown in the present paper that a large fraction of the lipids at the boundary layer, in fact, anticipates the phospholipid phase transition. Certainly differential microcalorimetry cannot demonstrate whether lipids lacking in thermodynamic measurements are really surrounding isolated proteins or are trapped between neighbour proteins. We believe that differential microcalorimetry results are perfectly consistent with our model.

Our final remark is to emphasize the obvious fact that none of our results are in contradiction with possible tight binding between proteins and specific phospholipids due, for example, to electrostatic interactions at the level of the phospholipid headgroups. In disc membranes, phosphatidylserine is a good candidate for such interaction. This type of strong interaction between phospholipid headgroups and proteins certainly exists in the case of extrinsic proteins.

Acknowledgements

We would like to thank Miss P. Hervé for helpful technical assistance. Dr. B. Shoot for his contribution to some experiments and Professor M. Bloom for critically reading the manuscript. This investigation was supported by a research grant from the 'Délégation Générale à La Recherche Scientifique et Technique', the 'Centre National de la Recherche Scientifique' and the Université of Paris VII.

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