Collisions between Nitrogen-14 and Nitrogen-15 Spin-Labels. 2. Investigations on the Specificity of the Lipid Environment of Rhodopsin†

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ABSTRACT: The method of spin–spin interactions between ^{15}N and ^{14}N spin-labels was used to investigate lipid–protein collision rates in reconstituted vesicles containing rhodopsin from bovine disk membranes and an equimolar mixture of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. In each sample, a fraction of one of the three phospholipids was labeled with ^{14}N spin-label while a ^{15}N spin-labeled fatty acid was covalently linked to rhodopsin. The extent of spin–spin interaction between ^{15}N and ^{14}N labels was either calculated by complete spectral simulation or evaluated from the line broadening as deduced from the intensity decrease of the low-field ^{15}N line. It was found that all three spin-labeled phospholipids utilized for these experiments can interact magnetically with the spin-labeled rhodopsin. Above 35 °C little difference between the three species can be detected. Calculation of the diffusion constant of the phospholipids at the boundary of rhodopsin proves that the lifetime of the phospholipids at the protein boundary is short and that no long-lived annular lipids are segregated. At temperatures below approximately 30 °C the spectra of the samples containing spin-labeled phosphatidylserine depend upon the presence or absence of calcium. The extent of ^{15}N line broadening was found weaker in the presence of Ca^{2+} than in the presence of ethylenediaminetetraacetate. Thus Ca^{2+} tends to exclude phosphatidylserine from the lipid environment of rhodopsin. This observation can be attributed to the formation of specific lipid domains within the membrane, induced by Ca^{2+}.

A heterogeneous lipid composition is found in all natural membranes. The lateral distribution of these lipids, within a membrane, is still a rather speculative matter. As a working hypothesis, it is often proposed that intrinsic membrane proteins may be surrounded by specific lipids. The direct experimental proofs of such associations are limited, in spite of the fact that several biophysical techniques have been used for such investigations. Among them are fluorescence quenching (London & Feigenson, 1981a,b; East & Lee, 1982), NMR [see, for example, Zumbulyadis & O'Brien (1979)], and ESR.[1]

ESR has been perhaps the most successful technique in this field. The general approach has been to estimate the amount of "strongly immobilized component" obtained with spin-labeled lipids which were allowed to interact with a specific protein. By this method, Watts et al. (1979) found a very small preference of rhodopsin for phosphatidylserine; Cable & Powell (1980) and Knowles et al. (1981) showed that cytochrome oxidase interacts preferentially with cardiolipin; Brotherus et al. (1980) and Marsh et al. (1982) proved that Na,K-ATPase interacts with negatively charged lipids. Finally, it was shown that free spin-labeled fatty acids bind to the cholinergic receptor protein in *Torpedo* membranes (Rousselet et al., 1979; Mc Namee et al., 1982), while spin-labeled long-chain acyl-CoA binds to a single site of the ADP carrier in mitochondria (Devaux et al., 1975). The assumption underlying these experiments is that spin-labeled lipids at the boundary of an intrinsic protein give rise at all temperatures to a broad ESR spectrum because of the rigidity of the protein boundary. This assumption was shown by several authors to be only partially correct (Chapman et al., 1979; Davoust et al., 1979; Watts et al., 1981). Indeed if a probe linked near the methyl terminal of a C_{16} fatty acid chain exchanges freely between a "boundary" and "bulk" lipid environment, the resulting ESR spectrum is only broad at low temperatures (slow exchange). The splitting of this "motionally restricted component" is 55–60 G in the temperature range 30–0 °C (Watts et al., 1981). At higher temperatures (i.e., at physiological temperature), the spectrum resembles more a fluid bilayer type spectrum. On the other hand if proteins are aggregated or oligomeric, a probe at the boundary can no longer be free to exchange and gives rise at all temperatures to a strongly immobilized component (splitting 60–65 G) (Andersen et al., 1981). In conclusion the quantitation of the amount of broadened ESR component, although it gives valuable comparative results, may lead to controversial interpretation. Also it should be recalled that extrinsic proteins do not immobilize boundary lipids, and thus the classical ESR method cannot be used to study the specificity of the lipid environment of extrinsic proteins.

In the present paper we show that the above limitations are overcome if the proteins are labeled as well as the lipids and if the broadening of the narrow lines due to spin–spin interactions is used to monitor lipid–protein contacts. In 1978, we carried out an experiment using a mixture of spin-labeled rhodopsin and spin-labeled phosphatidylcholine (Rousselet & Devaux, 1978). We were able to show evidence of magnetic interactions between the labeled proteins and the labeled lipids. However, a quantitative approach was found to be difficult, because of the impossibility to separate interactions due to spin–spin exchange between phospholipids and that between lipids and proteins. The latter difficulty can be overcome by the use of different nitrogen isotopes for the nitroxides on the proteins and those on the lipids.

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[1] Abbreviations: ESR, electron spin resonance; $^{15}N(1,14)MSL$, *N*-(2-hydroxyethyl)maleimide ester of 16-[^{15}N]doxylstearic acid (see formula in text); $^{14}N(1,14)PC$, 1-palmitoyl-2-(16-[^{14}N]doxylstearoyl)-phosphatidylcholine; $^{14}N(1,14)PE$, 1-palmitoyl-2-(16-[^{14}N]doxylstearoyl)phosphatidylethanolamine; $^{14}N(1,14)PS$, 1-palmitoyl-2-(16-[^{14}N]doxylstearoyl)phosphatidylserine; $^{15}N(1,14)FA$, 16-[^{15}N]doxylstearic acid; PDPC, 1-palmitoyl-2-dihydrosterculoylphosphatidylcholine; PDPE, 1-palmitoyl-2-dihydrosterculoylphosphatidylethanolamine; PDPS, 1-palmitoyl-2-dihydrosterculoylphosphatidylserine; EDTA, ethylenediaminetetraacetate; ATPase, adenosinetriphosphatase; Tris, tris(hydroxymethyl)aminomethane.

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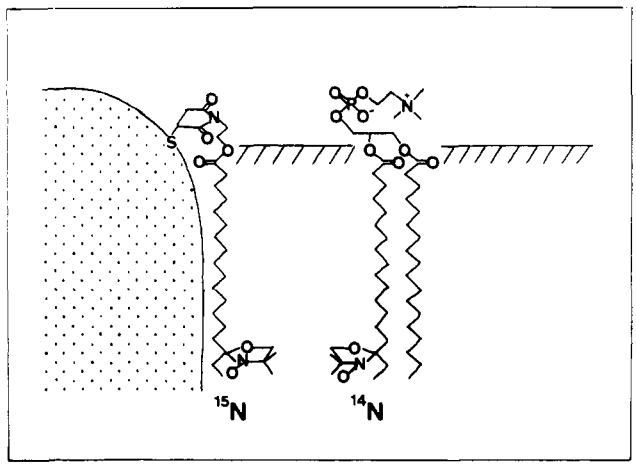


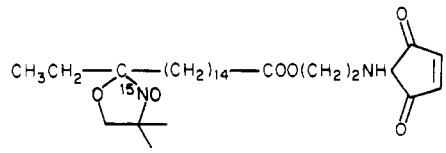
FIGURE 1: Diagrammatic representation of the double-labeling scheme used in this study. The protein label is $^{15}N(1,14)MSL$ bound to a sulfhydryl group of rhodopsin. The phosphatidylcholine label is $^{14}N(1,14)PC$. Spin-labeled PS and PE have also been used (see text for details).

The theoretical background which has been developed in Davoust et al. (1983) is applicable to these lipid–protein complexes. However, a prerequisite for quantitative analysis, which was stressed in the preceding paper, is that both spectra (associated respectively with the ^{14}N and the ^{15}N derivatives) correspond, in the absence of spin–spin interaction, to narrow components. It is possible to obtain such narrow lines with freely diffusing lipids and spin-labeled fatty acid linked to a protein, providing the following conditions are fulfilled (Davoust et al., 1979): (i) the nitroxide is near the ω terminal of a long-chain fatty acid; (ii) the temperature is sufficiently high, which in practice means above room temperature, i.e., at physiological temperature for most systems; (iii) proteins are not in an aggregated form or oligomeric form.

Figure 1 schematizes the type of labeling that can be achieved with rhodopsin reconstituted in lipid vesicles. A preliminary report of double-labeling experiments with rhodopsin incorporated into phosphatidylcholine vesicles has been given (Seigneuret et al., 1981). It will be shown here that in recombinants containing equimolar mixtures of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, rhodopsin appears to experience little preference between these three lipids in the absence of Ca^{2+} but the Ca^{2+} tends to segregate out phosphatidylserine from a rhodopsin environment at low temperatures.

Materials and Methods

Spin-Label and Lipid Synthesis. The synthesis of ^{15}N-(1,14)FA has been described by Bienvenüe et al. (1978) and was adapted to the use of high-pressure liquid chromatography as explained in the preceding paper (Davoust et al., 1983). Its esterification with *N*-(2-hydroxyethyl)maleimide yielding $^{15}N(1,14)MSL$



was performed according to Favre et al. (1979). An additional step of purification by high-pressure liquid chromatography using a preparative silica gel column eluted with chloroform–cyclohexane (50:50) was introduced.

Spin-labeled phospholipids $^{14}N(1,14)PC$ were synthesized as described previously (Hubbell & McConnell, 1971). 1-Palmitoyl-2-dihydrosterculoylphosphatidylcholine (PDPC) was synthesized by the procedure of Kornberg & McConnell (1971). Phosphatidylcholine from egg yolk was purified according to Singleton et al. (1965). The enzymatic exchange of the choline head group of the $^{14}N(1,14)PC$ and the PDPC was catalyzed by phospholipase D (Ito & Ohnishi, 1974). The purification of the corresponding phosphatidylserine and phosphatidylethanolamine derivatives was carried out by thick-layer chromatography (silica gel Merck 60F 254, 2 mm) with chloroform–methanol–water (14:6:1).

Rhodopsin Purification, Labeling, and Reconstitution. Rod outer segment membranes were isolated from fresh cattle retina by the method of De Gripp et al. (1979). Rhodopsin purification was performed as described by Davoust et al. (1979) with the following modification: the disk membranes were solubilized with a solution of 50 mM octyl glucoside, 100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ (buffer A). The concentration of rhodopsin is deduced from the absorbance at 500 nm of an aliquot before and after illumination; 80 nmol of rhodopsin/mL of concanavalin A–Sepharose 4B (Pharmacia) was applied to the affinity column and further washed with 10 mL of buffer A/mL of gel. The protein was then labeled on the affinity column by a 24-h closed circulation of the same buffer containing a 2-fold molar excess of $^{15}N(1,14)MSL$ with respect to the rhodopsin and again extensively washed. The elution was performed with buffer A containing 250 mM of α-methylmannose. The reconstitution was carried out by mixing in chloroform PDPC, PDPS, PDPE, and octyl glucoside (1:1:1:10) and then adding to an aliquot of this chloroformic solution the desired molar fraction of spin-labeled ^{14}N phospholipid with respect to the total phospholipid content. Each aliquot was then dried under argon and resuspended with a fraction of the eluted rhodopsin (100 nmol of rhodopsin for 8 μmol of total lipids). Afterward each sample was dialyzed 3 times for 8 h against a 200-fold excess of a 50 mM Tris, pH 7.4, buffer except the $^{14}N(1,14)PS$ sample which was divided into two parts: one was dialyzed against a 2.5 mM $CaCl_2$–50 mM Tris, pH 7.4, buffer while the other was dialyzed against a 2.5 mM EDTA–50 mM Tris, pH 7.4, buffer. After pelleting, the ESR spectrum of each membranous sample was recorded at various temperatures.

Chemical and Spectral Titration of the Samples. After the ESR experiments, the membrane samples were solubilized with a solution of 100 mM octyl glucoside and 50 mM Tris, pH 7.4. Determination of lipid phosphorus was performed as described by Rouser et al. (1969). Protein concentration was determined from rhodopsin absorbance at 500 nm or by the method of Lowry et al. (1951).

The ESR spectrum of the solubilized sample was recorded and yielded the concentration of ^{15}N spin-label and ^{14}N spin-label as described in Davoust et al. (1983). The spectra of control samples consisting of labeled rhodopsin in detergent or labeled phospholipid in detergent were recorded. From these various titrations we determined the labeling ratio of the rhodopsin after the 24-h dialyzing step, the final lipid to protein ratio, the molar fraction of spin-labeled ^{14}N phospholipid, and the molar ratio of the two spin-labels, $^{15}N/^{14}N$.

The ESR experiments and the data analyses were performed according to the method explained in the preceding paper (Davoust et al., 1983). The spectra were recorded every 10 °C from 5 to 45 °C. No modification in line shape or intensity could be detected after heating to 45 °C.

Results

After dialysis, the vesicles contained one rhodopsin molecule for 80 phospholipids with only a small variation from one

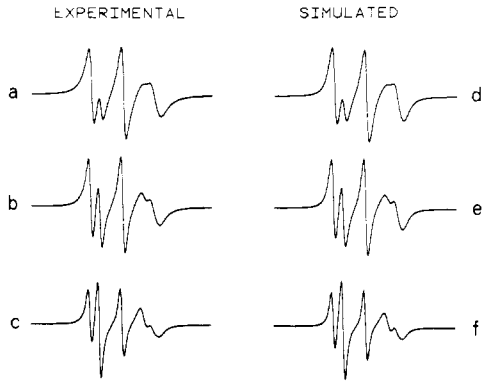


FIGURE 2: (Left) Experimental ESR spectra of rhodopsin-bound ^{15}N(1,14)MSL (~0.7 label/protein) and ^{14}N(1,14)PC in rhodopsin–PDPC–PDPE–PDPS vesicles (1:27:27:27) at 45 °C. Mole fraction of ^{14}N(1,14)PC: (a) 0.029, (b) 0.01, and (c) 0.005. Spectra are normalized in amplitude at the same amount of ^{15}N label. (Right) Examples of simulated ESR spectra. Spectral parameters (in G): $T'_{\parallel}$ = 15.81, $T'_{\perp}$ = 13.52, ΔH = 1.24, LW(1) = 1.53, LW(0) = 1.39, and LW(−1) = 2.38 for ^{14}N (1,14) PC; $T'_{\parallel}$ = 21.22, $T'_{\perp}$ = 19.00, ΔH = 0.77, LW($^1/_2$) = 1.74, and LW($-^1/_2$) = 3.42. Exchange frequencies $\tau^{-1}_{14,14}$ and $\tau^{-1}_{15,14}$ (in MHz): (d) 3.36 and 2.34, (e) 1.35 and 1.52, and (f) 0 and 0.

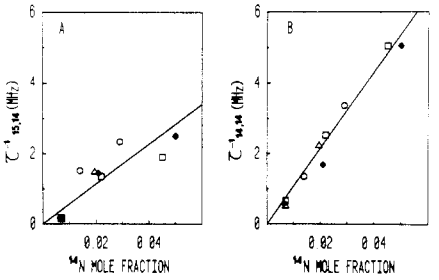


FIGURE 3: ^{15}N–^{14}N (A) and ^{14}N–^{14}N (B) exchange frequencies vs. ^{14}N label mole fraction for rhodopsin-bound ^{15}N(1,14)MSL (~0.7 label/protein) with ^{14}N(1,14)PC(○), ^{14}N(1,14)PE (△), or ^{14}N(1,14)PS in the presence of 2.5 mM EDTA (♦) or 2.5 mM $CaCl_2$ (□) in rhodopsin–PDPC–PDPE–PDPS (1:27:27:27) vesicles at 45 °C. Exchange frequencies were obtained from simulation of the corresponding ESR spectra as described in the preceding paper (Davoust et al., 1983). Error bars of the same order as those of Figure 7 of Davoust et al. (1983) have been omitted for clarity.

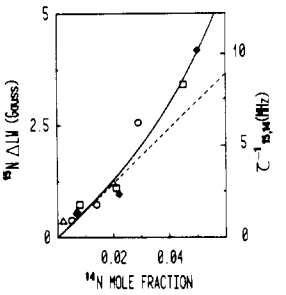


FIGURE 4: ^{15}N label low-field line broadening vs. ^{14}N label mole fraction for rhodopsin-bound ^{15}N(1,14)MSL (~0.7 label/protein) with various spin-labeled phospholipids (symbols as in Fig. 3, left scale) in rhodopsin–PDPC–PDPE–PDPS (1:27:27:27) vesicles at 45 °C. Line broadening expressed in gauss (left scale) were calculated by using eq 3 of Davoust et al. (1983) with an intrinsic line width LW_0 = 1.74 G. Error bars ranging from 0.2 to 1 G have been omitted for clarity. Data were fitted to a fourth-order polynomial by using a nonlinear least-squares program (solid curve). The tangent at origin gives a rough estimate of the ^{15}N–^{14}N exchange frequency expressed in megahertz (right scale) (see also Figure 3A).

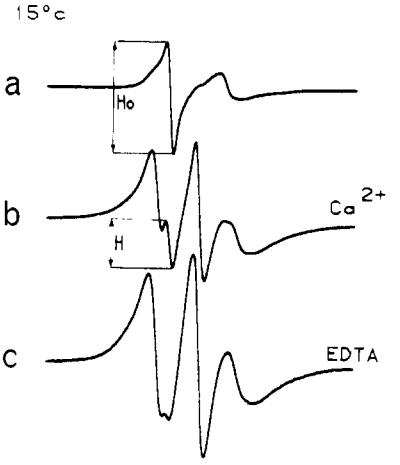


FIGURE 5: Experimental ESR spectra of rhodopsin-bound ^{15}N-(1,14)MSL (~0.7 label/protein) in rhodopsin–PDPC–PDPE–PDPS (1:27:27:27) vesicles at 15 °C alone (a) and with ^{14}N(1,14)PS (mole fraction 0.065) in the presence of 2.5 mM $CaCl_2$ (b) or 2.5 mM EDTA (c). Spectra are normalized in amplitude to the same amount of ^{15}N label.

sample to another. The unlabeled phospholipids were all derived from dihydrosterculoylphosphatidylcholine and thus contained a cyclopropane ring on the β chain. This allowed the phospholipid transition temperature to be lowered, while maintaining a high chemical stability (no spin reduction). The final ratio of spin-labeled rhodopsin per rhodopsin molecule was slightly below 1. Phospholipids contained various fractions of ^{14}N spin-labeled lipid. The ^{14}N label was alternatively on phosphatidylcholine, phosphatidylethanolamine, or phosphatidylserine. Thus all samples had essentially the same composition in protein and lipids except that the ^{14}N label was distributed differently. This equimolar mixture of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine is close to the endogenous lipid composition in disk membranes (Daemen, 1973; Miljanich & Dratz, 1982).

Spectra obtained at 45 °C with three concentrations of ^{14}N(1,14)PC in the presence of spin-labeled rhodopsin and normalized to the amount of ^{15}N label are displayed in the left part of Figure 2. The decrease in intensity of the low-field ^{15}N line reveals an increased spin–spin interaction taking place between ^{14}N and ^{15}N labels. These spectra as well as the corresponding spectra obtained with ^{14}N(1,14)PE and ^{14}N-(1,14)PS in the presence or absence of Ca^{2+} have been computer simulated. The right part of Figure 2 shows the results of the simulation for the PC label, while panels A and B of Figure 3 indicate the exchange frequencies $\tau^{-1}_{15,14}$ and $\tau^{-1}_{14,14}$ calculated for the various samples at 45 °C. The straight line in each figure takes into account the data from all four samples.

Spectral analysis by ^{15}N peak height measurement, as introduced in the preceding paper (Davoust et al., 1983), was also carried out. The results expressed in terms of ^{15}N low-field line broadening at 45 °C are displayed in Figure 4. The full line drawn in the figure fits all data to a fourth-order polynomial using a nonlinear least-squares program. The same treatment was applied to results obtained at 35 and 25 °C. Decreasing temperature had only a small effect on the curve representing ^{15}N line broadening vs. ^{14}N label mole fraction.

We have found that at all temperatures the line shapes recorded with ^{14}N(1,14)PC, ^{14}N(1,14)PE, and ^{14}N(1,14)PS in the presence of spin-labeled rhodopsin were practically identical as long as the concentration of spin-labels were alike. However, if Ca^{2+} (2.5 mM) was added to the samples containing labeled phosphatidylserine, the intensity of ^{15}N line was enhanced, particularly at low temperature, indicating a reduced spin–spin interaction between the labeled lipids and the labeled proteins. Figure 5 compares the 15 °C spectra obtained with spin-labeled PS respectively in the presence of

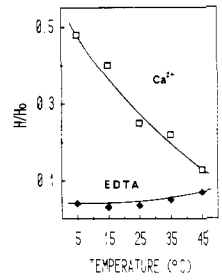


FIGURE 6: ^{15}N low-field line relative intensity decrease vs. temperature for rhodopsin-bound $^{15}N(1,14)MSL$ (~0.7 label/protein) with ^{14}N-(1,14)PS in rhodopsin–PDPC–PDPE–PDPS (1:27:27:27) vesicles at 15 °C in the presence of 2.5 mM $CaCl_2$ (□) or 2.5 mM EDTA (♦). H and H_0 are defined in Figure 5.

Ca^{2+} (2.5 mM) or EDTA. The top spectrum in Figure 5 is the spectrum recorded with $^{15}N(1,14)MSL$ bound to rhodopsin in reconstituted vesicles in the absence of any spin-labeled phospholipids. The peak height H_0 of the low-field ^{15}N line in spectrum a is adjusted so as to correspond to the same concentration of ^{15}N label as in spectra b and c. Thus the ratio H/H_0 gives an empirical measurement of the extent of spin–spin interaction in mixed samples. Note that at low temperatures, it is impossible to express the peak ratio in terms of a line broadening, since obviously the lines are no longer Lorentzian (see spectrum a in Figure 5). Figure 6 indicates ^{15}N peak height ratios measured with 6% $^{14}N(1,14)PC$ present, with or without Ca^{2+}, as a function of temperature.

Finally it is interesting to notice in Figure 5 that while ^{15}N–^{14}N interactions *increase* when going from spectra b to c, on the contrary ^{14}N–^{14}N interactions *decrease* from spectra b to c. This is apparent from peak heights ratios of the low- and mid-field peaks of ^{14}N peaks of spectra b and c.

Discussion

We have shown elsewhere that when (1,14)MSL binds to rhodopsin, the probe is buried in the hydrophobic milieu; reduction rates by ascorbate suggest that the position of the probe in the bilayer is very close to that of the corresponding fatty acid freely diffusing in the membrane (Davoust et al., 1980). This hypothesis is further confirmed in the present paper by evidence of spin–spin interactions which take place between $^{15}N(1,14)MSL$ and $^{14}N(1,14)PC$. If the ^{15}N probe after binding to rhodopsin was pulled to a region close to the phospholipid head groups, as for example the probe on (0,2)PC (Davoust et al., 1983), then no spin–spin interactions would be detected between the heteroisotopic labels. Thus the schematic drawing of Figure 1 is justified.

In former studies, we have also shown that one molecule of rhodopsin can bind a maximum of two molecules of (1,14)MSL (Favre et al., 1979). Here, after the dialysis step, we have obtained a labeling ratio slightly below one spin-label per protein. It is an important fact because it allows us in a first approximation to neglect in the calculation electron spin–electron spin interactions between ^{15}N labels.

In this paper we have used the theoretical framework developed in Davoust et al. (1983) in order to simulate the high-temperature spectra of the mixture of ^{15}N and ^{14}N spin-labels in the presence of rhodopsin. One assumption, which is not rigorous for protein-linked labels, is that the ^{15}N spectrum in the absence of spin–spin interactions corresponds to a pure bilayer type spectrum. We have shown (Davoust & Devaux, 1982) that the spectrum of a spin-labeled fatty acid covalently linked to rhodopsin can be simulated by assuming a rapid chemical exchange between two states of different order parameter. At 37 °C or above the spectrum is in fact quasi-identical with a bilayer spectrum; thus at 45 °C the fit can be very good although the simulation does not need to take into account the actual physical motion experienced by the fatty acid linked to the protein. At low temperature this approach would not be valid (see spectrum a of Figure 5). In addition we have mentioned that, for high concentrations and low temperature, the simulation would have to include the dipole–dipole interaction. In conclusion, the theoretical analysis can only be carried out in the 35–45 °C range. But this range of temperature contains the physiological temperature of bovine retina. The replacement of the natural polyunsaturated lipids by phospholipids containing a cyclopropane ring means that the lipid viscosity above 35 °C is indeed comparable to that of the natural membrane.[2]

The conclusions that can be drawn from the quantitative analysis of the experiments described under Results are the following:

(i) There is little difference between exchange frequencies measured with spin-labeled phosphatidylcholine, phosphatidylethanolamine, or phosphatidylserine at high temperature. Hence rhodopsin, in the model systems studied here, does not select a specific phospholipid for its surrounding. Nevertheless we must realize that having a single spin-label per mole of rhodopsin (see above) limits the efficiency of the investigation. For example, if the protein has a single specific site for a particular phospholipid, it is possible (although not certain) that we might miss it.

(ii) The exchange frequencies are consistent with the hypothesis of a diffusion-controlled phenomenon. Indeed the plot of $\tau^{-1}_{15,14}$ and $\tau^{-1}_{14,14}$ as a function of ^{14}N label mole fraction (Figure 3) is very close to the plot obtained for the mixture of spin-labeled lipids in egg lecithin, in the absence of rhodopsin [see Figure 5 of Davoust et al. (1983)]. Thus the lateral diffusion rate of spin-labeled phospholipids is not markedly affected by the presence of rhodopsin. However, Figure 3 indicates that $\tau^{-1}_{15,14} < \tau^{-1}_{14,14}$; the ratio of the two frequencies is about 0.56. $\tau^{-1}_{15,14}$ is proportional to the probability for a given ^{15}N label to collide with any ^{14}N label, while $\tau^{-1}_{14,14}$ is proportional to the probability for a ^{14}N label to collide with any ^{14}N label. If the two isotopes were on equivalent molecules, the two frequencies would be equal [see Davoust et al. (1983)]. The fact that here $\tau^{-1}_{15,14} < \tau^{-1}_{14,14}$ can be explained by the binding of the ^{15}N label to the protein: the protein-linked label collides less often with freely diffusing ^{14}N labels than if it was itself free to diffuse. As explained under Discussion of Davoust et al. (1983) (see eq 4), one anticipates an exchange rate between ^{15}N and ^{14}N labels equal to half the value obtained between freely diffusing ^{14}N labels. As a consequence the decrease of collision frequency cannot be explained by a different transverse position within the membrane of the ^{15}N labels and the ^{14}N labels. Finally if the ^{15}N labels were attached in a cleft of the protein, the number of surrounding phospholipids (i.e., of ^{14}N labels) would be diminished. A priori this latter hypothesis is very unlikely because it would be accompanied by a strong immobilization of the ^{15}N spin-label. In summary our results clearly indicate that lipid chain collision rates at the protein boundary are of the same order of magnitude as in the bulk lipid phase ($f \simeq 5 \times 10^7\ s^{-1}$ at 45 °C). This finding is in agreement with NMR results obtained with rhodopsin (Brown et al., 1977; Deese et al., 1981) or with other intrinsic proteins (Oldfield et al., 1978; Seelig & Seelig, 1978).

[2] Lipid replacement was necessitated by the chemical instability of polyunsaturated chains.

(iii) We have shown qualitatively that Ca^{2+} modifies the lateral distribution of phosphatidylserine at low temperature (see Figures 5 and 6). The decrease in the interaction between spin-labeled phosphatidylserine and spin-labeled rhodopsin is accompanied by an increased interaction within spin-labeled phosphatidylserine molecules. Thus the interpretation of these experiments is unambiguous: a segregation of phosphatidylserine is induced by Ca^{2+}, at low temperature. It is in agreement with observations from other laboratories working on different membranous systems (Ohnishi & Tokutumi, 1981; London & Feigenson, 1981b; East & Lee, 1982). The fact that we can detect phosphatidylserine segregation proves that the technique of double spin-labeling is indeed sensitive to the specificity of the protein environment, whenever it occurs.

How do our results compare to former investigations concerning the lipid environment of rhodopsin? Watts et al. (1979), in a study mentioned in the introduction, concluded that phosphatidylserine has a slightly higher affinity for rhodopsin than phosphatidylcholine or phosphatidylethanolamine. However, the difference was extremely small (a few percent) and based on the estimation of the amount of immobilized component. This is a very unreliable procedure for spectra recorded at 37 °C. From reconstitution experiments as well as from ^{13}C NMR investigations O'Brien and collaborators have concluded that polyunsaturated chains were preferentially located around rhodopsin molecules; these authors have not claimed any head-group specificity (Zumbulyadis & O'Brien, 1979). Thus it appears that several independent techniques give evidence for the *absence* of head-group specificity in the direct lipid environment of rhodopsin. The various head groups in disk membranes are likely to be important for the binding of the extrinsic proteins, which play a significant role in disks (Kühn, 1982). ESR, NMR, and fluorescence techniques as well as binding studies have shown indeed that the interaction of extrinsic proteins such as myelin basic protein, cytochrome *c*, β-hydroxybutyrate dehydrogenase, and spectrin depends very much upon the nature and charge of the phospholipid head groups.

In this study, we have used spin-labeled phospholipids which were allowed to interact with spin-labeled proteins. The technique is comparable to that employed by London & Feigenson (1981a,b) and East & Lee (1982). These authors used either spin-labeled phospholipids or brominated phospholipids to quench the fluorescence from a variety of membrane-bound molecules. In particular both groups have investigated the head-group specificity of the phospholipids surrounding the Ca^{2+}-ATPase. In the experiments involving fluorescence quenching, only the lipids are labeled, and the intrinsic tryptophan fluorescence is used as a protein marker. In both types of experiments, a basic assumption is that direct contact is required for interaction. However, there are two differences between the fluorescence studies and the present magnetic resonance study: (i) Due to the short lifetime of the excited state of the fluorescent chromophores, the fluorescence experiments use a static quenching process. Exchange rates cannot be inferred. (ii) The mole fraction of modified lipids in the experiments of London and Feigenson or East and Lee (0.1 to 1) is much larger than in the ESR experiments. This could be a limitation if one wants to investigate fragments of biological membranes.

Acknowledgments

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Registry No. PDPC, 81004-53-7; PDPE, 85506-89-4; PDPS, 85506-90-7; N_2, 7727-37-9; ^{15}N, 14390-96-6; Ca, 7440-70-2; octyl glucoside, 29836-26-8.

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Mode of Transcription and Maturation of Ribosomal Ribonucleic Acid in Vitro in Mitochondria from Ehrlich Ascites Cells†

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ABSTRACT: An in vitro system using mitoplasts from Ehrlich ascites mouse tumor cells was shown to be highly active in in vitro protein synthesis [Bhat, N. K., Niranjan, B. G., & Avadhani, N. G. (1982) *Biochemistry 21*, 2452–2460]. In the present studies, this system was used to investigate the mode of transcription and maturation of mitochondrial 12S and 16S rRNA. The in vitro labeled RNA hybridizes to mitochondrial DNA restriction fragments corresponding to both ribosomal and nonribosomal coding sequences. The hybridization pattern suggests that the entire mitochondrial genome is transcribed under these in vitro conditions. The extent of hybridization to various restriction fragments suggests that the rDNA region is transcribed at 20–40 times higher rates than the rest of the genome. Over 60% of the in vitro labeled RNA is adsorbed to cellulose-linked DNA restriction fragments containing rRNA coding sequences and resolves as characteristic 12S and 16S species on denaturing agarose gels. Electrophoretic analysis of in vitro pulse-labeled RNA and Northern blot analysis of steady-state mitochondrial RNA have failed to detect significant levels of common rRNA precursors, suggesting that the major pathway for mitochondrial rRNA maturation may involve endonucleolytic cleavage of nascent transcripts. Our results also indicate that the "D" loop area does not contribute to stable transcripts in the mouse mitochondrial system.

Mitchondria from a variety of animal cells contain a 16 kilobase pair (kbp)[1] ciruclar DNA genome (Borst, 1972; Dawid et al., 1976) which contains information for 2 mt rRNAs, 22 different mt tRNAs, and potentially 13 different polypeptides (Barrell et al., 1980; Anderson et al., 1981; Bibb et al., 1981; Montoya et al., 1981; Ojala et al., 1981). Recent DNA sequence analyses have revealed a remarkable constancy between bovine, mouse, and human mitochondrial systems with respect to size of various rRNA, tRNA, and mRNA genes and also the order in which they are organized on the genome (Anderson et al., 1981, 1982; Bibb et al., 1981). Available information in rat (Parker & Watson, 1977; Saccone et al., 1980) and *Xenopus* (Rastl & Dawid, 1979) suggests that a similar orgainzational scheme might exist in other animal mt systems as well.

Detailed studies in HeLa cell system (Aloni & Attardi, 1971; Murphy et al., 1975) have provided evidence on the symmetrical and complete transcription of both H and L strands of mtDNA. It is also suggested that the transcription of both strands may be initiated at single promoter sites located near the origin of replication (Montoya et al., 1981; Ojala et al., 1981). These results, along with the DNA and RNA sequence data (Anderson et al., 1981; Bibb et al., 1981; Montoya et al., 1981; Ojala et al., 1981) showing the absence of 3′- and 5′-untranslated regions on the mt mRNAs, and close proximal arrangement of genes with no in between spacers suggest that various RNA species such as rRNA, tRNA, and mRNAs in animal cell mt are derived through an intricate transcription and maturation pathway different from known pro- and eukaryotic systems.

Since mtRNA represents a very small fraction of total cell RNA (Avadhani et al., 1975; Lewis et al., 1976; Batty & Clayton, 1978), studies on the transcription and maturation of mtRNAs have been difficult particularly because most animal cells under tissue culture conditions present considerable barriers in the efficient labeling of mt transcription products. Also, specific inhibitors like camptothecin, which have been successfully used in some cells for preferential inhibition of nuclear transcription, are not effective in other cell types (B. G. Niranjan and N. G. Avadhani, unpublished results) either due to their inefficient transport across the cell membranes or due to their nonspecific effects. We have therefore attempted to develop a subcellular system for efficient labeling of mt transcription products. In a previous study from

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[1] Abbreviations: kb, kilobase; kbp, kilobase pair; mt, mitochondria; mtDNA, mitochondrial DNA; rRNA, ribosomal RNA; tRNA, transfer RNA; mRNA, messenger RNA; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); EDTA, disodium ethylenediaminetetraacetate; NaDodSO$_4$, sodium dodecyl sulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; URF, unidentified reading frame; H strand, heavy strand; L strand, light strand.

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