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Molecular Exchange at the Lipid–Rhodopsin Interface: Spin-Label Electron Spin Resonance Studies of Rhodopsin–Dimyristoylphosphatidylcholine Recombinants†

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ABSTRACT: The photoreceptor protein rhodopsin has been reconstituted with a single phospholipid species, dimyristoylphosphatidylcholine, at a range of different lipid/protein ratios, and the exchange rate at the lipid–protein interface has been determined from the electron spin resonance spectra of spin-labeled phosphatidylcholine. For recombinants with lipid/protein ratios in the range 41:1 to 102:1 (mol/mol), the electron spin resonance spectra of 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine consist of a fluid component similar to that found in pure lipid bilayers and a motionally restricted component corresponding to lipids whose motion is reduced by interaction with the intramembranous surface of rhodopsin. The relative proportion of the motionally restricted component increases with increasing protein content in the complex. Spectral subtraction with fluid and motionally restricted components (from fluid- and gel-phase lipid, respectively), which best fit the apparent components in the complex, reveals that 22 ± 2 lipids per 39 000-dalton protein are motionally restricted, independent of lipid/protein ratio and of temperature. Simulation of the two-component spectra with the exchanged-coupled Bloch equations gives values for both the fraction of motionally restricted component and the exchange rate between the two components. Using fixed motionally restricted and fluid component line shapes at a given temperature, it is possible to obtain a consistent description of the lipid/protein ratio dependence of the spectra at each temperature. The number of motionally restricted lipids obtained by simulation, allowing for exchange, is 23 ± 3 per 39 000-dalton protein, again independent of temperature and of lipid/protein ratio. The rate of lipid exchange off the protein is independent of lipid/protein ratio, as expected, and increases from 1.3 × 10^7 s^{-1} at 25 °C to 1.9 × 10^7 s^{-1} at 40 °C in the fluid phase of dimyristoylphosphatidylcholine.

The rod outer segment disc has an archetypal fluid membrane, most probably as a result of the preponderance of unsaturated chains in the membrane lipids. A high degree of mobility has been demonstrated for all its molecular components [see, e.g., Watts (1982)]. Rhodopsin, the principal protein in the membrane, has been found both to rotate rapidly (Cone, 1972) and to undergo fast translational motion (Poo & Cone, 1974) within the plane of the membrane. Both ESR[1] and NMR experiments (Watts et al., 1979, 1981; Pates et al., 1985; Brown et al., 1977; Zumbulyadis & O'Brien, 1979) have revealed rapid motions of the lipid component of the membranes, although qualitatively different spectral effects are observed because of the difference in characteristic time scale of the two spectroscopies (Bienvenue et al., 1982; Deese et al., 1981; Deese & Dratz, 1986).

A point of considerable interest is the coupling of the protein motions to those of the fluid lipids. This is necessary for a detailed description of the mechanism of the protein rotation and translation and to describe the way in which the relatively rigid helical backbone of the protein is interfaced to its fluid lipid environment. For this aspect of the lipid/protein interaction, the exchange of lipids on and off the intramembranous surface of the protein is likely to play a decisive role.

Previously Davoust and Devaux (1982) have demonstrated that the distal ends of hydrocarbon chains covalently anchored to rhodopsin rapidly lift off the protein surface with a frequency of ~10^7 s^{-1}. Davoust et al. (1983) have further demonstrated that, as expected, freely diffusible lipids approach the rhodopsin surface with a rate that is diffusion-controlled. In this work we take advantage of the two-component nature of the ESR spectra of spin-labeled lipids in lipid/protein systems [see e.g., Marsh and Watts (1982)] to estimate the exchange rate of freely diffusible lipids at the surface of rhodopsin. A consistent description is obtained of the dependence of the exchange rates on both temperature and lipid/protein ratio. The values of the measured exchange rates

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[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; 14-PCSL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)-stearoyl]-*sn*-glycero-3-phosphocholine; ROS, rod outer segment; OG, octyl glucoside (*n*-octyl β-D-glucopyranoside); ESR, electron spin resonance; Tris, tris(hydroxymethyl)aminomethane.

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also predict, in agreement with experiment, that the two lipid populations should be in fast exchange on the NMR time scale (Deese & Dratz, 1986).

In contrast to the previous addition and subtraction methods of spin-label spectral analysis, which provide a steady-state description of the lipid/protein stoichiometry (Watts et al., 1979), the exchange simulations therefore can give a quantitative dynamic picture of the lipid/protein interaction. East et al. (1985) have already demonstrated by spectral simulation that freely diffusible spin-labeled phospholipids exchange with a frequency of $\sim 10^7$ s^{-1} at the intramembranous interface with the Ca^{2+}-ATPase.

Materials and Methods

Isolation of Rhodopsin. Bovine rod outer segment (ROS) disc membranes were isolated from fresh, dark-adapted ox eyes obtained from the local slaughterhouse. Retina were suspended in a physiological buffer (Uhl et al., 1979) and homogenized by passage through nylon gauze. The homogenate was centrifuged on top of a discontinuous (1.11 and 1.13 g/mL) sucrose density gradient (Beckman Ti60, 30 K, 30 min), and purified membranes were isolated from the 1.11/1.13 g/mL density interface. ROS were washed with 50 mM Tris–acetate pH 7 buffer containing 1 mM Ca^{2+} and 1 mM Mn^{2+} and were solubilized in 60 mM *n*-octyl β-D-glucopyranoside, OG (Sigma Chemical Co., St. Louis, MO), in this buffer. Rhodopsin was purified by affinity chromatography over concanavalin A–Sepharose (Pharmacia P-L Biochemicals, Freiburg, West Germany) (Albert & Litman, 1978). Rhodopsin was washed free of endogeneous lipid with 50 mM OG and was eluted with 0.2 M methyl α-D-mannopyranoside in buffer containing 30 mM OG. Isolated rhodopsin was used immediately for reconstitution. The spectral absorbance ratio of rhodopsin (A_{280}/A_{500}) was below 1.7. All manipulations involving rhodospin were carried out in the dark or in dim red light.

Reconstitution of Rhodopsin with DMPC and Incorporation of Spin-Label. The mixed cholate–OG detergent system described elsewhere for reconstituting rhodopsin into lipid bilayers (Ryba et al., 1986) was used to obtain samples with a range of different lipid/protein ratios. Spin-label incorporation was achieved by adding ca. 1 mol % of spin-label, with respect to lipid, to the reconstitution immediately prior to passage through the critical micelle concentration of the detergent/lipid/protein mixture.

Lipid and Protein Analysis. Chemical analyses of phospholipid concentrations were carried out by the method of Eibl and Lands (1969), following sulfuric acid digestion. Protein was assayed from the absorption of rhodopsin at 500 nm (molecular weight for rhodopsin of 40000 and molar extinction coefficient of 40×10^6 mol/cm^2) and with a modified Lowry assay (Markwell et al., 1981). After the experiments, rhodopsin was shown to run as a single band, corresponding to an effective M_r of 40000, by polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

Spin-Label Synthesis. Spin-labeled phosphatidylcholine, 14-PCSL, was synthesized from 14-spin-labeled stearic acid by the method of Boss et al. (1975). The 14-spin-labeled stearic acid was produced essentially according to Hubbell and McConnell (1971). For further details, see Marsh and Watts (1982).

Electron Spin Resonance. Electron spin resonance (ESR) experiments were carried out at 9 GHz on a Varian E-12 ESR spectrometer equipped with a nitrogen-gas-flow variable-temperature unit accurate to approximately ±0.1 °C. ESR spectra were recorded and stored digitally on disc via a PDP 11/10 computer. Spectral subtraction was carried out as described previously (Watts et al., 1979; Marsh, 1982).

Spectral Simulations. Spectral simulations were carried out with a two-site chemical exchange model for the fluid and motionally restricted spin-label components, with summation over all angular orientations of the lipid spin-label, corresponding to an unoriented membrane (L. I. Horváth, P. J. Brophy, and D. Marsh, unpublished results). The simulation programme used corresponds to model I of Davoust and Devaux (1982).[2] Because a simplified model had to be used for the motional anisotropy, greatest reliance was placed on the simulations in the low-field region of the spectrum, which is least sensitive to spectral anisotropy and optimally sensitive to exchange. The simulation strategy was to optimize the fluid and motionally restricted components for the 41:1 lipid/protein ratio sample and then to use these components in the simulations for all other lipid/protein ratios. This was done according to the following procedure. (1) The individual fluid and motionally restricted spectral components were simulated, which gave the best fit to the experimental spectrum of the 41:1 lipid/protein ratio complex at 30 °C. These were obtained by matching to the experimental spectra of sonicated DMPC vesicles in the fluid and gel phases, respectively, with subsequent slight adjustments to the hyperfine and *g* tensor components, when necessary. (2) These two components were then used to determine the fraction of the motionally restricted component f in the samples at all lipid/protein ratios by obtaining the best fits to the experimental spectra at 30 °C. At any one lipid/protein ratio, a range of values of motionally restricted fraction and exchange rate could account for the observed spectrum at the best level of spectral fit. However, these values could then be correlated to provide a consistent description of the lipid/protein ratio dependence of both parameters. With this restriction, the values of f, the fraction of motionally restricted component, at a particular lipid/protein ratio were then maintained constant throughout the temperature range. (3) At each temperature a best fit fluid component spectrum was obtained by simulating the experimental spectra of the 41:1 lipid/protein ratio sample. These fluid components were then used in the simulations for the samples at all other lipid/protein ratios. (4) At each temperature the same procedure was repeated for the motionally restricted component, allowing for the progressive decrease in the outer hyperfine splitting with increasing temperature. (5) With the spectral components obtained in (3) and (4) and the fractions of motionally restricted component obtained in (2), the best fits to the experimental spectra at all other lipid/protein ratios and temperatures were obtained by varying the exchange rate in the simulations. In this way it was found possible to obtain a consistent fit of both the lipid/protein ratio dependence and the temperature dependence of the spectra, in terms of exchange at the lipid/protein interface.

Results

The ESR spectra of 14-PCSL in rhodopsin/DMPC recombinants of various lipid/protein ratios are given in Figure 1.

[2] For slow exchange, as is the case here, model II of Davoust and Devaux (1982), which allows exchange between different spin-label orientations in the two components, provides very similar results to the simpler model I (Davoust & Devaux, 1982; East et al., 1985; Horvath et al., 1987). Therefore, model I was chosen in order to minimize the time required for simulation. In particular, model II would not be capable of improving the agreement between simulated and experimental spectra substantially, because the differences between the two are dominated by the increased anisotropy in the experimental fluid component, which is most probably due to longer range effects of the lipid–protein interactions not included in either model (see later and Discussion).

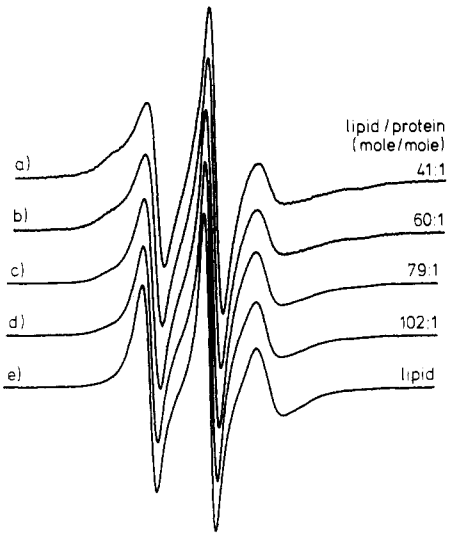

FIGURE 1: ESR spectra of the 14-PCSL phosphatidylcholine spin-label in rhodopsin/dimyristoylphosphatidylcholine recombinants as a function of lipid/protein ratio (mol/mol): (a) 41:1, (b) 60:1, (c) 79:1, (d) 102:1, (e) lipid alone (sonicated vesicles). T = 25 °C; scan range = 100 G.

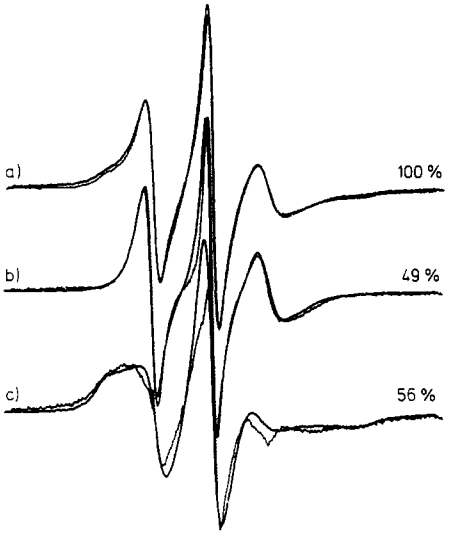

FIGURE 2: Spectral subtraction and addition with a rhodopsin/dimyristoylphosphatidylcholine recombinant labeled with the 14-PCSL spin-label. Full lines are experimental spectra: (a) recombinant of lipid/protein ratio 41:1 (mol/mol) at 30 °C, (b) dimyristoylphosphatidylcholine sonicated vesicles at 25 °C, and (c) dimyristoylphosphatidylcholine sonicated vesicles at 12 °C. Dotted lines are summed or difference spectra: (a) 46% of mobile spectrum b plus 54% of the motionally restricted spectrum c, (b) recombinant spectrum minus 51% of the motionally restricted spectrum c, and (c) recombinant spectrum minus 44% of the mobile spectrum b.

The spectra are recorded in the fluid phase at 25 °C and, like those of ROS disc membranes (Watts et al., 1979) and other lipid/protein recombinant systems [see, e.g., Marsh (1985)], consist apparently of two components. The resolution of the two components is not perfect because they are coupled by slow exchange, as will be seen below. One component of the two has a spectrum very similar to that of DMPC alone, and the other has a larger hyperfine splitting corresponding to spin-labeled lipids whose motion is restricted by interaction with rhodopsin [see, e.g., Watts et al. (1979, 1981) and Pates et al. (1985)]. The proportion of the motionally restricted component decreases with decreasing protein content and can be quantitated by spectral subtraction, as indicated in Figure 2. In order to allow for the broadening effects of exchange

Table I: Temperature and Lipid/Protein Ratio Dependence of the Fraction, f, of Motionally Restricted Phosphatidylcholine Spin-Label, 14-PCSL, in Rhodopsin/Dimyristoylphosphatidylcholine Recombinants, Obtained by Spectral Subtraction (cf. Figure 2)

lipid/protein (mol/mol)	25 °C	30 °C	35 °C	40 °C
41:1	0.53[a]	0.51	0.52	0.50
	0.56[b]	0.56	0.55	0.56
60:1	0.38[a]	0.36	0.37	0.34
	0.44[b]	0.40	0.43	0.37
79:1	0.27[a]	0.26	0.26	0.27
	0.32[b]	0.34	0.31	0.31
102:1	0.15[a]	0.18	0.17	0.17
	0.22[b]	0.25	0.26	0.20

[a] Obtained from the subtraction end point in subtracting a motionally restricted component from the spectrum of the recombinant. [b] Obtained from the subtraction end point in subtracting a fluid component from the spectrum of the recombinant.

Table II: Lipid/Protein Ratio Dependence of the Fraction of Motionally Restricted Spin-Labeled Phosphatidylcholine, 14-PCSL, in Rhodopsin/Dimyristoylphosphatidylcholine Recombinants, Derived from Spectral Subtraction, f_{sub}, and from Simulation, f_{sim}, and Effective Total Numbers of Motionally Restricted Lipids, N_1(sub) and N_1(sim), Respectively[a]

lipid/protein (mol/mol)	f_{sub}	N_1(sub) (mol/mol)	f_{sim}	N_1(sim) (mol/mol)
41:1	0.54	22	0.55	22
60:1	0.39	23	0.43	26
79:1	0.29	23	0.30	24
102:1	0.20	20	0.21	21
		mean 22		mean 23

[a] The values for f_{sub} are the means over the temperature range 25–40 °C and over the complementary subtractions. The values for f_{sim} are the means over the temperature range 25–40 °C.

on the spectral line shapes, fluid- and gel-phase spectra from sonicated DMPC that best fit the apparent fluid and restricted components in the recombinant spectrum were chosen for the subtraction. For the fluid component (Figure 2b), this corresponds to a spectrum from sonicated DMPC vesicles recorded at a temperature lower than that at which the recombinant spectrum (Figure 2a) was recorded. Values for the fraction of motionally restricted lipid, f, obtained from the various subtractions are given in Table I. It is seen that consistent values are obtained from the two complementary methods of subtraction corresponding to Figure 2b,c and that for a given lipid/protein ratio the fraction of motionally restricted lipid is essentially independent of temperature.

Knowing the lipid-to-protein ratios (n_t) of the recombinants, it is possible to calculate the effective number of motionally restricted lipid molecules N_1 associated with each rhodopsin molecule. If the 14-PCSL spin-label reflects the unlabeled DMPC distribution in a 1:1 fashion, as found for other reconstituted systems [K_r(PCSL:DMPC) = 1; Knowles al., 1979; Brophy et al., 1984], then

$$n_f^*/n_b^* = n_t/N_1 - 1 \quad (1)$$

where $n_f^*/n_b^* = (1-f)/f$ is the ratio of fluid to motionally restricted components in the ESR spectrum. The resulting values, N_1(sub), obtained from the subtraction factors, f_{sub}, are given in Table II. It is found that 22 ± 2 lipids per 39 000-dalton protein are motionally restricted, independent of lipid/protein ratio and of temperature.

Simulation of the two-component recombinant spectra with the exchange-coupled Bloch equations (McConnell, 1958) not only gives an independent estimate of the numbers of motionally restricted lipids but, more importantly, yields values for the exchange rate of the spin-labeled lipids between the

Table III: Temperature Dependence and Lipid/Protein Ratio Dependence of the Fraction of Motionally Restricted Lipid, f, and the Exchange Off-Rate Constant, τ_b^{-1}, from Simulations of the ESR Spectra of the 14-PCSL Spin-Label in Rhodopsin/Dimyristoylphosphatidylcholine Recombinants

lipid/protein (mol/mol)	25 °C		30 °C		35 °C		40 °C	
	f	τ_b^{-1} (s^{-1})	f	τ_b^{-1} (s^{-1})	f	τ_b^{-1} (s^{-1})	f	τ_b^{-1} (s^{-1})
41:1	0.56	1.3×10^7	0.56	1.5×10^7	0.56	1.7×10^7	0.5	1.8×10^7
60:1	0.44	1.2×10^7	0.44	1.5×10^7	0.44	1.8×10^7	0.4	1.9×10^7
79:1	0.29	1.3×10^7	0.31	1.6×10^7	0.30	1.8×10^7	0.3	2.0×10^7
102:1	0.22	1.4×10^7	0.22	1.8×10^7	0.22	2.0×10^7	0.18	2.0×10^7
mean		$1.3(0) \times 10^7$		$1.6(0) \times 10^7$		$1.8(1) \times 10^7$		$1.9(2) \times 10^7$

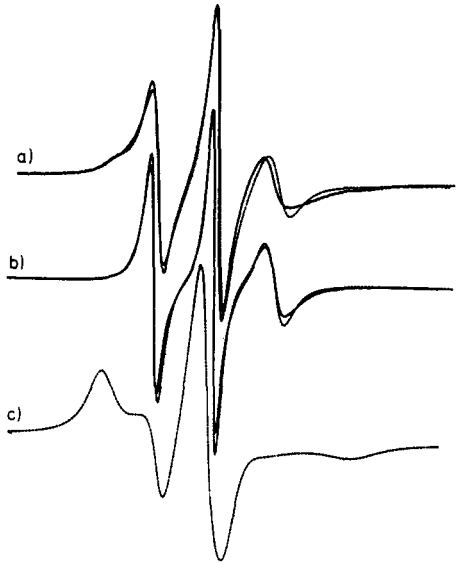

FIGURE 3: Single-component and composite spectra of the 14-PCSL spin-label (full lines) and their simulations (dotted lines): (a) rhodopsin/dimyristoylphosphatidylcholine recombinant of lipid/protein ratio of 41:1 (mol/mol) at 30 °C and simulation using exchanged-coupled single components; (b) dimyristoylphosphatidylcholine sonicated vesicles at 30 °C and simulated single-component spectrum used for the exchange-coupled simulation in (a); (c) simulated motionally restricted single component used for the exchange-coupled simulation in (a).

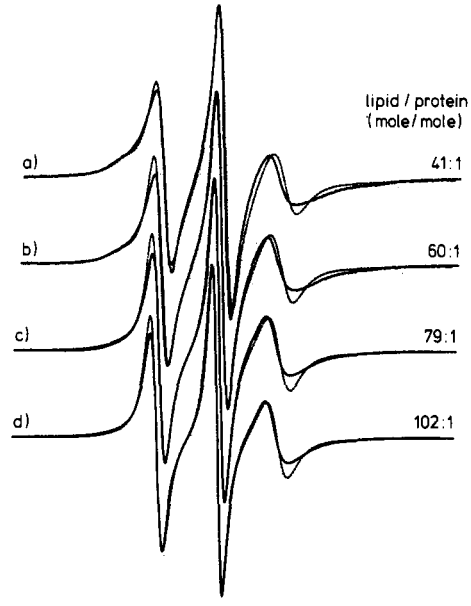

FIGURE 4: Lipid/protein (mol/mol) ratio dependence of experimental (full lines) and simulated (dotted lines) spectra of rhodopsin/dimyristoylphosphatidylcholine recombinants with the 14-PCSL spin-label at 30 °C. Lipid/protein ratio (mol/mol): (a) 41:1, (b) 60:1, (c) 79:1, and (d) 102:1. Simulation parameters are given in Table III.

two components. The simulation program described by Horváth et al. [L. I. Horváth, P. J. Brophy, and D. Marsh, unpublished results] has been used, which corresponds to model I of Davoust and Devaux (1982). The procedure used for the simulation is illustrated in Figure 3. The two individual components are simulated with a simplified model described by Horváth et al. [L. I. Horváth, P. J. Brophy, and D. Marsh, unpublished results] that contains the explicit angular dependence of the different membrane fragments with respect to the magnetic field orientation. For the fluid component (Figure 3b), this is taken to be the same as the spectrum of DMPC sonicated vesicles alone, recorded at the same temperature as that of the recombinant. For the motionally restricted component (Figure 3c), this is taken to be that which best fits the exchanged-coupled spectrum of the 41:1 lipid/protein ratio recombinant (Figure 3a). The choice of this latter component is found not to be absolutely critical, provided that the spectrum has the correct outer hyperfine splitting. This is presumably because this component lies close to the limits of motional sensitivity of conventional ESR spectroscopy.

The simulated spectra are compared with the spectra of the different recombinants at 30 °C in Figure 4. A consistent fit to the different lipid/protein ratios is obtained with the same two individual spectral components given in spectra b and c of Figure 3. Some discrepancies are seen between the experimental and simulated spectra, especially in the high-field region of the spectrum. These can most probably be attributed to perturbations of the lipid beyond the first motionally restricted shell, possibly due to enhancement of slow motional modes of these lipids. Such distortions of the more distant shells of lipid are expected to have their greatest effect in the high-field region of the spectrum. Therefore, agreement between experimental and simulated spectra in the low-field region can be better used to estimate the exchange rates. The simulation procedure has been repeated in the same way as illustrated in Figures 3 and 4 for the different temperatures of measurement, giving fits to the experimental spectra of the recombinants that are of similar quality to those of Figure 4. As an example, the simulations of the spectra of the 41:1 lipid/protein ratio complex at the various temperatures are given in Figure 5. Again, reasonably good fits are obtained throughout the temperature range, apart from some uncertainty in the high-field spectral region, which can be attributed to direct perturbations of the more distant lipid shells.

The fractions of motionally restricted lipids and the exchange rates deduced from the simulations are given in Table III,[3] for all lipid/protein ratios and temperatures studied. The

[3] Although the fit between simulated and experimental spectra is not perfect, the values of f and τ_b^{-1} correspond to a minimum in the sum of squares of the differences between the two spectra, for the choice of the exchanging components described in the text. Changes of either parameter by 5% leads to a visually worse fit between simulated and experimental spectra and to a significant increase in the sum of squares of the differences.

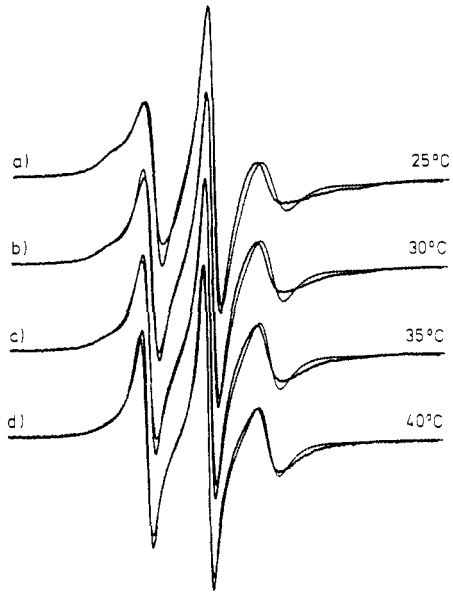

FIGURE 5: Temperature dependence of experimental (full lines) and simulated (dotted lines) spectra of rhodopsin/dimyristoylphosphatidylcholine recombinants of lipid/protein ratio of 41:1 (mol/mol) with the 14-PCSL spin-label: (a) at 25, (b) 30, (c) 35, and (d) 40 °C. Simulation parameters are given in Table III.

exchange is characterized in terms of the off-rate constant τ_b^{-1}, where τ_b is the residence time of the spin-labeled lipid at the protein surface (Marsh, 1985; L. I. Horváth, P. J. Brophy, and D. Marsh, unpublished results). A consistent fit is obtained with values for the fraction of motionally restricted lipid that do not vary with temperature over the range studied. The resulting values of the numbers of motionally restricted lipids, N_1(sim), are given in Table II[3] and in agreement with the results from spectral subtraction are found not to vary appreciably with lipid/protein ratio. Effectively 23 ± 3 lipids per 39 000-dalton protein are found to be motionally restricted, also in good accord with the number deduced from spectral subtractions. The values for the off-rate constants (τ_b^{-1}) are found to be independent of lipid/protein ratio, as expected to be the case, and increase with increasing temperature, also as expected.

Discussion

The first significant feature of this work is the very good agreement between the results of spectral subtraction and the results of the spectral simulations based on an exchange-coupled, two-component mode. The simulation results demonstrate that there are essentially two distinct states of mobility of the spin-labeled lipid chains, although the two populations are strongly coupled by exchange. As found previously (L. I. Horváth, P. J. Brophy, and D. Marsh, unpublished results; Marsh, 1985), the effects of exchange can be allowed for satisfactorily in the spectral subtractions by choosing individual spectral components that best match the apparent components in the exchange-coupled spectrum. Typically, a fluid component is taken from the pure lipids that is recorded at a somewhat lower temperature than that of the lipid/protein recombinant or membrane (cf. Figure 2). In agreement with the findings of Brotherus et al. (1980), this result validates the subtraction protocol used previously for a wide range of different lipid–protein systems [see, e.g., Marsh and Watts (1982) and Marsh (1985)]. Failure to observe this standard protocol of course leads to incorrect estimates of the fraction of exchanging motionally restricted lipid component, as demonstrated by Davoust and Devaux (1982).

The effective number of motionally restricted lipids per protein, N_1, obtained from both spectral subtraction and simulation, is found to be independent of the lipid/protein ratio of the recombinant (see Table II). This result both substantiates the assumption that there is no selectivity between the labeled and the unlabeled phosphatidylcholine [K_r(PCSL:DMPC) = 1] and demonstrates that there is no progressive protein aggregation in the recombinants with changing lipid/protein ratio.[4] Approximately 22 ± 3 lipids per 39 000-dalton protein are motionally restricted, as determined both by spectral subtraction and by simulation. This is in good agreement with determinations in ROS disc membranes (Watts et al., 1979; Pates et al., 1985; Pates & Marsh, 1987), where the rhodopsin is known to be present as a monomer (Brett & Findlay, 1979; Ebrey, 1971). As reviewed by Pates et al. (1985), this number is also in good agreement with estimates of the total number of lipid molecules that may be accommodated around the intramembranous surface of rhodopsin. Together with the constant stoichiometry with lipid/protein ratio, this gives yet further support to the original suggestion that the motionally restricted lipids constitute the first shell or boundary layer surrounding rhodopsin (Watts et al., 1979, 1981). Thus, from the above results, it appears that rhodopsin is present as a monomer in the DMPC recombinants and the gross features of the lipid–protein interactions do not differ significantly from those in ROS membranes. The similarity in behavior with dimyristoylphosphatidylcholine and the natural lipid mixture is in agreement with the lack of selectivity of different lipids for the interaction with rhodopsin (Watts et al., 1979; Watts, 1982; Davoust et al., 1983).

The spectra of the motionally restricted spin-label component lie in the slow motion regime of ESR spectroscopy, indicating that the effective rotational correlation times are in the range $\tau_R \geq 10^{-8}$ s. A somewhat more detailed analysis of the motional properties in ROS membranes has indicated that the effective correlation times lie in the range $\tau_R \approx (1\text{–}2) \times 10^{-8}$ s (Pates & Marsh, 1987). These values are somewhat shorter than the residence times on the protein in DMPC bilayers, which lie in the range $\tau_b \approx (5\text{–}7) \times 10^{-8}$ s (see Table III), suggesting that there may be some segmental motion of the lipids on the protein surface. (However, it should be noted that these estimates are based on quite different motional models, and therefore, there may be inconsistencies in the absolute values.) As pointed out under Results, there are systematic deviations from the experimental spectra in the high-field region of the simulations. These discrepancies are greater at the lower lipid/protein ratios (cf. Figure 4) and most probably are due to a direct perturbation of the mobility of the lipids beyond the first boundary layer shell, as suggested originally for cytochrome oxidase–DMPC recombinants (Knowles et al., 1979). It is very difficult to estimate the magnitude of this effect, since the spectra almost certainly contain contributions from both the fast and slow motional regimes of conventional spin-label ESR spectroscopy (Lange et al., 1985). Possibly, the motional rates of the lipids at the rhodopsin surface are not so very different from those of the first shell lipids, and the good resolution of the two components arises because the motions of both components lie very close to (but on opposite sides of) the border of motional sensitivity

[4] On the basis of measurements of protein rotational rate, Kusumi and Hyde (1982) have proposed that there is a *transient* association of monomers in phosphatidylcholine bilayers that is least for the myristoyl and palmitoyl chain lengths. It would appear that such a transient dimerization does not have a large effect on the number of lipids associated with the intramembranous surface of rhodopsin detected here.

in conventional ESR spectroscopy. In any case, the results of the present simulations indicate that the various lipid populations are continuous with respect to exchange and therefore must have a continuous, even if rather steep, mobility gradient between the different shells surrounding the protein.

The off-rate constants for spin-labels leaving the surface of the protein are found to be constant, independent of the total number of lipids per protein in the recombinant, for all temperatures of measurement (see Table III). This is to be expected, provided that the number of second-shell lipids available for exchange is not limiting and that there is rapid equilibration by lateral diffusion within the pool of fluid, exchangeable lipids. Together with the expected increase in exchange rate with increasing temperature, this lack of lipid/protein ratio dependence provides strong support for the simulation method used to determine the exchange rates and further substantiates the two-component, exchange-coupled model used to interpret the spectra.

The measured off-rate constants are of the same order of magnitude, although significantly slower than the exchange rates for lateral diffusion in fluid lipid bilayers. This demonstrates, in agreement with ^{2}H NMR experiments (Deese et al., 1981; Bienvenue et al., 1982), that there is no long-lived binding of the lipids to the intramembranous surface of rhodopsin. The lipid exchange rates due to lateral diffusion in dimyristoylphosphatidylcholine bilayers have been determined to be $\tau_{dif}^{-1} = 4D_T/\langle x^2 \rangle \sim (2.5\text{–}5.6) \times 10^7$ s^{-1} by photobleaching (Vaz et al., 1985) and $(6.9\text{–}9.4) \times 10^7$ s^{-1} for spin-labeled phosphatidylcholine by ESR spectroscopy (Sachse et al., 1987), over the temperature range 25–40 °C. These rates are appreciably faster than the one-to-one lipid exchange on and off the protein, which is characterized by the values of τ_b^{-1} in Table III. The reason for this could be purely geometrical, in that the lipid–protein interface forces the exchange to be essentially unidirectional. Nevertheless, the relative rates suggest that the residence time of the lipid at the protein interface is 2–5 times greater than that in the fluid lipid milieu. Some uncertainty is, however, involved in the absolute comparisons with lateral diffusion rates, since the interpretation of the measurements involves rather different models.

Experiments on the collision rates between diffusible ^{14}N-labeled lipids and ^{15}N-labeled chains covalently linked to rhodopsin have indicated values that are not appreciably different from those arising from free lipid diffusion in fluid bilayers (Davoust et al., 1983). These experiments, however, preferentially measure the collision rate with the protein surface rather than the residence time at the lipid–protein interface, since the covalently linked chain is inherently nonexchangeable. Comparison of the exchange rates for diffusible and bound labels therefore suggests that the lipids approach the protein surface with a diffusion-controlled rate but that the one-to-one exchange at the protein interface is limited by steric and possibly thermodynamic effects and by the reduced segmental motion of the first-shell lipids, to give a 2–5 times slower rate. Other experiments with covalently linked spin-labeled chains (Davoust & Devaux, 1982) have shown that the chain ends exchange between fluid and motionally restricted environments, even though the whole chain cannot exchange. This raises the question as to whether the present measurements with diffusible labels truly measure the exchange rate of the lipid molecule as a whole. The answer lies in the different lipid/protein ratio dependences of the exchange rate. Whereas the experiments with covalently linked chains measured an on-rate that had little dependence on lipid/protein ratio (Davoust & Devaux, 1982), the present results yield an on-rate, $\tau_f^{-1} = f\tau_b^{-1}$, that has the dependence expected for a true exchange, together with rapid equilibration by lateral diffusion within the fluid pool of lipids. Thus, the values of τ_b^{-1} in Table III provide a true reflection of the lipid exchange rates at the protein interface and, together with recent studies on the ordering and dynamics of spin-labeled lipid chains in ROS membranes (Pates and Marsh, 1987), provide a rather detailed description of the lipid–protein interaction with rhodopsin.

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Quenching of the Zinc–Protoporphyrin Triplet State as a Measure of Small-Molecule Diffusion through the Structure of Myoglobin†

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ABSTRACT: The diffusion of small molecules through the myoglobin structure was studied. It has been shown that the fluorescent Zn–protoporphyrin substitutes easily for the native nonfluorescent Fe–protoporphyrin in myoglobin. The quenching rate of the E-type delayed fluorescence of Zn–protoporphyrin in a substituted myoglobin by the quenchers oxygen and anthraquinonesulfonate was used to measure their diffusion from the ambient solution through the protein to the ligand binding site. The quenching rate constant (at 21 °C) for oxygen is $k_q = (9.6 \pm 0.9) \times 10^7$ M^{-1} s^{-1}, only 1 order of magnitude less than that for Zn–hematoporphyrin quenching in aqueous solution. The activation energy in the range between 2 and 40 °C is $E_a = 6.0 \pm 0.6$ kcal/mol. The corresponding data for anthraquinonesulfonate are $k_q = (2.1 \pm 0.3) \times 10^8$ M^{-1} s^{-1} and $E_a = 5.8 \pm 0.6$ kcal/mol. Taking into account the statistical factor involved in the oxygen quenching of the Zn–porphyrin triplet, the quenching rates are very similar. The data are discussed in terms of the "gated reaction" theory of Northrup and McCammon. The similar rate constants and activation energies indicate that the diffusion rate in the protein is determined by the frequency of the conformational changes that open "gates" for the passage of the quencher through the protein.

Myoglobin (Mb) is one of the most intensively studied proteins with respect to structural implications for its biological functions. Most notable are the studies on ligand binding following photodissociation of the Mb–CO and the Mb–O_2 complexes over a wide range of temperatures (Austin et al., 1975; Debrunner & Frauenfelder, 1982) and the resonance Raman studies on Mb and on hemoglobin that describe in detail the conformational changes at the ligand binding site which accompany the above binding process (Spiro, 1985; Friedman, 1985). The most important conclusions drawn from these binding studies are the following: The protein exists in a number of conformational states, each of which binds the ligand at its specific rate. At room temperature these substrates are in rapid equilibrium with each other via conformational fluctuations while at low temperatures ($T < 180$ K) each protein molecule remains in its given substate. Similar conclusions about structural fluctuations have been drawn from X-ray diffraction studies (Artymiuk et al., 1979; Frauenfelder et al., 1979) and from molecular dynamics calculations (Karplus & McCammon, 1981). Moreover, the above binding studies indicate that, in order for a ligand molecule in the ambient solution to be bound to the myoglobin porphyrin, it must overcome a number of potential barriers on its way through the protein structure to the binding site.

The question being addressed here concerns one part of the above overall ligand binding process, namely, the diffusional movement of the small molecule from the solution through the protein. This movement has not yet been studied extensively in myoglobin. A rate constant of $k = 2 \times 10^8$ M^{-1} S^{-1} has been assigned to the diffusion in de-Fe myoglobin (Jameson et al., 1984). Here we describe the measurements of small-molecule diffusion through Mb without the process being influenced by the actual binding step. To this end, we use Mb whose Fe–protoporphyrin has been exchanged for Zn–protoporphyrin (ZnPP). The spectral properties of the latter are easy to follow, and the quenching rate of its long-lived excited triplet state yields a direct measure for the rate of diffusion of the quencher molecule from the external solution to the (ZnPP-substituted) binding site.

Experimental Procedures

The detailed procedure used for the preparation of ZnPP-substituted myoglobin (ZnPP-Mb) has been described previously (Feitelson & Spiro, 1986). Since apomyoglobin is very heat sensitive, its preparation and subsequent reconstitution were carried out in the cold room at approximately 3 °C. All solutions were precooled in an ice bath. Briefly, about 200 mg of sperm whale myoglobin (Sigma) were dissolved in 10 mL of water, acidified to pH 4, and shaken in a separating funnel with 15 mL of cold 2-butanone (Teale, 1959). The light-colored aqueous apomyoglobin phase was dialyzed overnight against water at 3 °C. The protein was reconstituted

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