Exchange rates at the lipid-protein interface of the myelin proteolipid protein determined by saturation transfer electron spin resonance and continuous wave saturation studies

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ABSTRACT The microwave saturation properties of various spin-labeled lipids in reconstituted complexes of the myelin proteolipid protein with dimyristoyl phosphatidylcholine have been studied both by conventional and saturation transfer electron spin resonance (ESR) spectroscopy. In the fluid phase, the conventional ESR spectra consist of a fluid and a motionally restricted (i.e., protein-associated) component, whose relative proportions can be determined by spectral subtractions and depend on the selectivity of the particular spin-labeled lipid for the protein. At 4°C when the bulk lipid is in the gel phase, the integrated intensity of the saturation transfer ESR spectra displays a linear dependence on the fraction of motionally restricted lipid that is deduced from the conventional ESR spectra in the fluid phase, indicating the presence of distinct populations of free and protein-interacting lipid with no exchange between them on the saturation transfer ESR time scale in the gel phase. At 30°C when the bulk lipid is in the fluid phase, the saturation transfer integral displays a nonlinear dependence on the fraction of motionally restricted lipid, consistent with exchange between the two lipid populations on the saturation transfer ESR time scale in the fluid phase. For lipid spin labels with different selectivities for the protein in complexes of fixed lipid/protein ratio, the data in the fluid phase are consistent with a constant (diffusion-controlled) on-rate for exchange at the lipid-protein interface. Values ranging between 1 and $9 \times 10^6\ s^{-1}$ are estimated for the intrinsic off-rates for exchange of spin-labeled stearic acid and phosphatidylcholine, respectively, at 30°C. Conventional continuous wave saturation experiments lead to similar conclusions regarding the lipid exchange rates in the fluid and gel phases of the lipid/protein recombinants. The ESR saturation studies therefore demonstrate exchange on the time scale of the nitroxide spin-lattice relaxation at the lipid-protein interface of myelin proteolipid/dimyristoyl phosphatidylcholine complexes in the fluid phase but not in the gel phase.

INTRODUCTION

Lipid-protein interactions are important determinants of both structure and function in biological membranes. Spin-label electron spin resonance (ESR)[1] spectroscopy, as introduced by McConnell (1), has proved to be an extremely useful means of investigating such interactions in natural membranes and reconstituted systems, because of the favorable timescale of conventional nitroxide ESR spectroscopy (2). The conventional ESR spectra of lipid spin labels in these systems are found to contain a resolved component corresponding to a lipid population whose chains are restricted in their mobility by direct interaction with the intramembranous surface of the integral membrane proteins (see, e.g., reference 3). The size of the motionally restricted population relative to that of the bulk fluid membrane lipids can be determined by difference spectroscopy and used to determine both the stoichiometry and specificity of the lipid-protein interaction. In addition, simulation of the conventional ESR lineshapes using the exchange-coupled Bloch equations (4) allows determination of the lipid exchange rates at the protein interface, provided that these are nonvanishing relative to the intrinsic spin-spin relaxation rates, $1/T_2^0$, (or linewidths) (5).

The intrinsic exchange rates measured in the above way reflect the selectivity of the different lipids for interaction with the membrane protein and are found to lie in the range of $\sim 10^6$ to $10^7\ s^{-1}$ (see, e.g., reference 6), which is close to the limits of motional sensitivity of conventional spin label ESR spectroscopy (7). Exchange at these rates is comparable with, or faster than, the intrinsic spin-lattice relaxation ($1/T_1^0$) of the motionally restricted spin labels (see, e.g., references 8–10) and hence should have a more pronounced effect on the saturation properties of the lipid spin label ESR spectra than on their lineshapes or linewidths. Investigations of the saturation behavior of spin-labeled lipids in the presence of integral proteins therefore constitute a valuable extension to the previous studies of lipid-protein interactions by ESR spectroscopy and are the subject of the present article. A prerequisite for such studies is that the protein-interacting and the bulk spin-labeled lipid components should differ in their saturation properties. This has been demonstrated already to be the case for the lipids interacting with the Ca^{2+}-ATPase in sarcoplasmic reticulum (11).

Lipid interactions with the integral proteolipid protein (PLP) from nerve myelin have been characterized extensively by ESR spectroscopy of spin-labeled lipids (12–15), particularly with regard to the exchange rates at the

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[1] *Abbreviations used in this article:* CW, continuous wave; DMPC, dimyristoylphosphatidylcholine; ESR, electron spin resonance; 14-PCSL, -PGSL, -PSSL, and -PASL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine, -phosphoglycerol, -phosphoserine, and -phosphoric acid; PLP, myelin proteolipid apoprotein; 14-SASL, 14-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; STESR, saturation transfer ESR.

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lipid-protein interface. These measurements were confined to conventional ESR lineshape studies and to protein complexes in the fluid lipid phase. In the present article, we investigate the spin-lattice relaxation of different spin-labeled lipids in reconstituted complexes of the myelin proteolipid protein with dimyristoyl phosphatidylcholine both by conventional continuous wave (CW) saturation ESR and by saturation transfer ESR spectroscopy (16). Measurements are made at temperatures both in the gel phase and in the fluid phase of the bulk lipid population. It is found that a protein-interacting lipid population can be distinguished by its saturation properties, also in the lipid gel phase, but that the exchange rates with the bulk lipid population are too slow in the gel phase to affect the spin-lattice relaxation. In contrast, the saturation properties of spin-labeled lipids in lipid/protein complexes in the fluid phase are strongly influenced by the exchange at the lipid-protein interface and are found to depend on the lipid selectivity in a manner that is consistent with the results deduced previously from the conventional ESR lineshapes.

MATERIALS AND METHODS

Materials

Dimyristoylphosphatidylcholine (DMPC) was obtained from Fluka (Buchs, Switzerland). The C-14 positional isomer of spin-labeled 14-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid (14-SASL) was synthesized according to Hubbell and McConnell (17). Spin-labeled phosphatidylcholine (1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholine [14-PCSL]) was synthesized by acylation of lyso-phosphatidylcholine from egg yolk with 14-SASL, and other C-14 labeled phospholipids, 14-PASL (phosphatidic acid), 14-PSSL (phosphatidylserine), and 14-PGSL (phosphatidylglycerol), were synthesized from 14-PCSL by transphosphatidylation catalyzed with phospholipase D (for details see reference 18). The purity of the spin-labeled lipids was checked by thin-layer chromatography.

Isolation and reconstitution of myelin proteolipid protein

Myelin was extracted from bovine spinal cord and delipidated by gel filtration on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) as described by Brophy (19). Chromatography was repeated to ensure the complete removal of endogenous lipids; delipidation was verified by thin-layer chromatography and phosphate analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli (20).

The delipidated myelin proteolipid protein was reconstituted with DMPC by dissolving both components in freshly distilled 2-chloroethanol (Fluka) and dialyzing against three changes of reconstitution buffer (100 mM NaCl, 1 mM ethylenediaminetetraacetate, 2 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, pH 7.4) as described previously (19). The homogeneity of protein incorporation was verified by sucrose density gradient centrifugation for 3 h at 40,000 rpm (10–55% sucrose in reconstitution buffer; SW 40 Ti rotor; Beckman Instruments, Inc., Palo Alto, CA), and the position of the single band was consistent with the lipid/protein ratio of the sample. A similar procedure was used for the delipidated protein to produce the protein-alone samples. Lipid and protein contents were determined by the methods of Eibl and Lands (21) and Lowry et al. (22), respectively.

Sample preparation

Samples were spin labeled at a level of ca. 0.5 mol% by adding a concentrated ethanolic solution of the lipid spin label, with vortex mixing. Unincorporated label was removed by washing. Spin-labeled pure lipid samples were prepared by hydrating the DMPC/spin label mixture dried down from dichloromethane. ESR samples were packed as pellets of 5-mm length in 1-mm-ID glass capillaries. All ESR samples were deoxygenated by saturating the buffers with argon and flushing the sample capillaries also with argon. For further details see references 12–15.

ESR measurements

ESR spectra were recorded on a E-12 Century Line 9 GHz spectrometer (Varian Associates, Inc., Palo Alto, CA) interfaced to an IBM PC via a Labmaster interface card (Tekmar Co., Cleveland, OH) using software written by Dr. M. D. King (Max-Planck-Institut, Göttingen, Germany). The 1-mm-ID glass capillaries were centered in a standard 4-mm quartz ESR tube containing light silicone oil for thermal stability, the temperature being regulated by a nitrogen gas flow system. Conventional, in-phase, absorption (V_1) spectra were recorded at a modulation amplitude of 1.6 G p-p and various microwave powers. Spectral subtractions of the ESR spectra recorded at low ($\langle H_1^2 \rangle^{1/2}$ = 0.02 G) microwave power were performed with an interactive graphics display terminal (VT-11) using a dedicated PDP 11/10 computer and software written by Dr. W. Möller (Max-Planck-Institut, Göttingen, Germany).

A standardized sample configuration was used in all CW saturation and saturation transfer ESR (STESR) experiments (23, 24), and all measurements were performed under critical coupling conditions. The microwave field was calibrated by measuring the CW saturation properties of deoxygenated aqueous peroxylamine disulphonate, as described by Kooser et al. (25) and Fajer and Marsh (23). STESR spectra were recorded in the second harmonic, 90° out-of-phase, absorption (V_2') mode at a modulation frequency of 50 kHz and a modulation amplitude of 5 G p-p. All STESR measurements were performed at a microwave power giving an average $\langle H_1^2 \rangle^{1/2}$ field over the sample of 0.25 G (8). For details of corrections for changes in cavity Q, see Fajer and Marsh (23) and Hemminga et al. (24). Phase shifts were adjusted by the self-null method at subsaturating microwave power.

CW saturation experiments were analyzed by evaluating the second integral of the conventional (V_1) spectrum, as a function of the average microwave power, P, at the sample. From these measurements, the mean microwave power at half-saturation, $P_{1/2}$, was determined by fitting the saturation curves, which are displayed by normalization of the spectral integral relative to the linear dependence on $\sqrt{P}$ in the absence of saturation (cf. reference 26). STESR spectra were analyzed using the integrated intensity (I_{ST}) that is defined as the first integral of the STESR (V_2') spectrum, normalized with respect to the second integral of the conventional (V_1) spectrum, with both recorded according to a standardized protocol (24, 27):

$$I_{ST} = \int V_2'(H) \cdot dH \Big/ \iint V_1(H) \cdot d^2H. \qquad (1)$$

Spectral subtractions with the conventional (V_1) ESR spectra were performed as described by Marsh (28).

RESULTS

CW saturation ESR of lipid/protein complexes

The conventional ESR spectrum (V_1-display) of the 14-PCSL phosphatidylcholine spin label in a complex of the myelin proteolipid apoprotein with dimyristoyl phos-

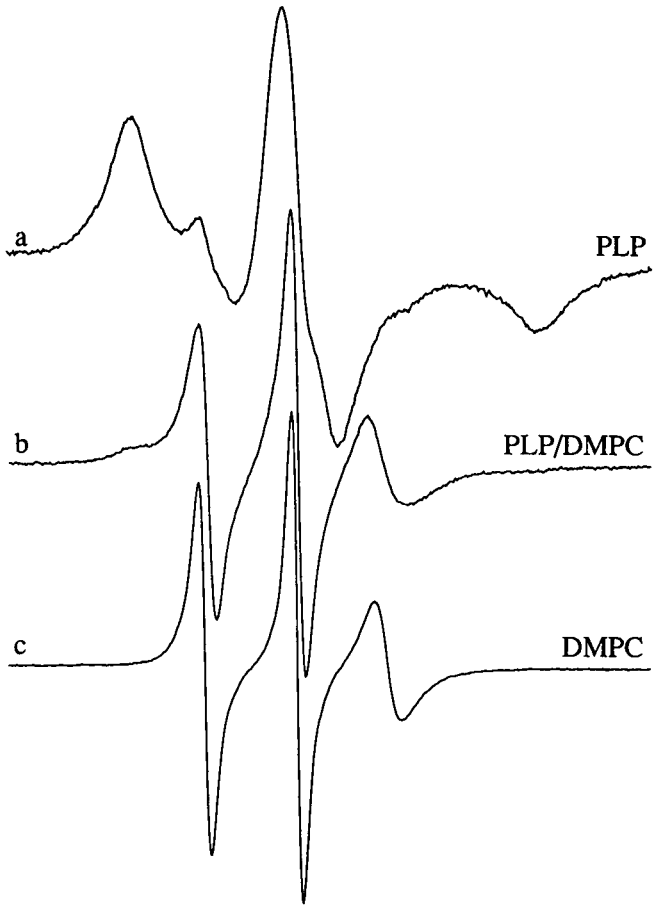

FIGURE 1 Conventional ESR spectra (V_1-display) of the 14-PCSL phosphatidylcholine spin label in (*a*) myelin proteolipid apoprotein alone, (*b*) myelin proteolipid protein/dimyristoyl phosphatidylcholine complex of lipid/protein ratio 24:1 mol/mol, (*c*) dimyristoyl phosphatidylcholine bilayers alone. T = 30°C; scan range = 100 G.

phatidylcholine is compared with those of the same spin label in bilayers of the pure lipid and in association with the delipidated protein in Fig. 1. The ESR spectra are recorded at a temperature in the fluid phase of the lipid and indicate clearly that the spectrum of the lipid/protein complex (Fig. 1 *b*) is composed of two components: one corresponding to the fluid bilayer regions of the lipid/protein complex (cf. Fig. 1 *c*) and the other corresponding to the lipids that are in direct contact with the intramembranous section of the protein (cf. Fig. 1 *a*, and see reference 12). The spectra of the delipidated protein are found to correspond rather well with the motionally restricted, protein-interacting component in the two-component spectra of the lipid/protein complex; the spectrum of the delipidated protein recorded, at most, 2° higher in temperature than that of the lipid/protein complex gives optimum matching of the lineshapes (12). For optimum matching with the fluid component in the lipid/protein complex, a spectrum of the pure lipid recorded 3–5° lower in temperature is required, principally because of broadening due to exchange between the two components in the complex (13). Subtraction of single-component spectra of the pure lipid, or of the delipidated protein samples, from the two-component spectrum of the lipid/protein complex (12, 28) reveals that the fraction, f, of spin-labeled phosphatidylcholine interacting directly with the protein is $f = 0.49$ for the sample with lipid/protein ratio 24:1 mol/mol. More extensive spectral subtraction data on complexes with different lipid/protein ratios have revealed that there is little or no selectivity of the spin-labeled phosphatidylcholine relative to the DMPC host lipid in interaction with the myelin proteolipid protein (12). Therefore, any effects of the differences in acyl chain composition between the spin-labeled and host lipid on the thermodynamics of interaction (and correspondingly on the equilibrium exchange kinetics) are small.

The progressive saturation with increasing microwave power, P, of the ESR spectra from the three samples of Fig. 1 is shown in Fig. 2 for spectra recorded both in the gel phase at 4°C (left-hand side) and in the fluid phase at 30°C (right-hand side) of the DMPC bilayer lipids. In this figure, the saturation curves are displayed as the double-integrated ESR intensity at a given power, S_{CW}, normalized relative to the value, S^0_{CW}, that would be obtained at the same power in the absence of saturation. At both temperatures, a very marked difference is found in the saturation behavior of the lipid spin label in the pure lipid and delipidated protein environments. A similar observation has been made previously by Squier and Thomas (11) in comparing the saturation behavior of spin-labeled lipids in sarcoplasmic reticulum membranes with those in the extracted membrane lipids. The saturation curves of the double integral from the first-derivative spectra of the single components in Fig. 2 have been fitted to the expression for the integrated ESR intensity of component i (29):

$$S_{CW,i} = S^0_{CW,i}/[1 + \sigma^S_{1/2}(P/P^0_{1/2,i})]^{1/2}, \qquad (2)$$

where $P^0_{1/2,i}$ is the microwave power at half-saturation (for component i) and $\sigma^S_{1/2} = 3$ is the saturation parameter of the integral at half-saturation. These nonlinear least-squares fits are given by the full lines in Fig. 2 and represent the saturation behavior of the single components reasonably well, with deviations principally at low powers where the intensities are small and the fractional errors are greatest. The values of the square root of the microwave power at half-saturation for the delipidated protein samples (component b) are $(P^0_{1/2,b})^{1/2} = 6.2$ and 8.1 $mW^{1/2}$ at 4 and 30°C, respectively, and for the pure lipid samples (component f) are $(P^0_{1/2,f})^{1/2} = 16.1$ and 23.8 $mW^{1/2}$ at 4 and 30°C, respectively. It will be noted from Fig. 2 that some of the saturation curves do not extend up to the value of $P_{1/2}$, the extreme case being $P \approx P_{1/2}/3$. From Eq. 2, it can be estimated that the fractional error in $P_{1/2}$ is $(\frac{1}{3})P_{1/2}/P$ times the error in $(S^0_{CW}/S_{CW})^2$, i.e., is $\sim 3/\sqrt{2}$ times greater in the extreme

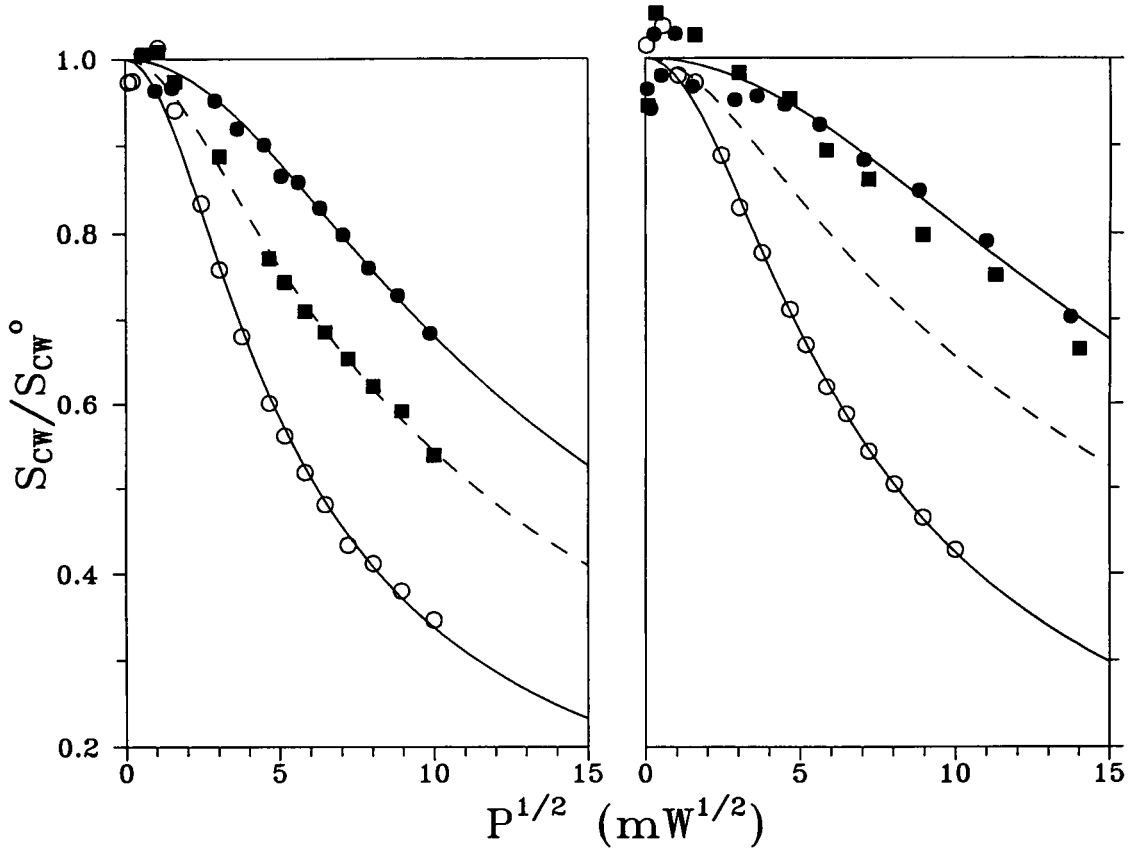

FIGURE 2 Continuous wave saturation curves of the integral ESR intensity of spin-labeled phosphatidylcholine (14-PCSL) in dimyristoyl phosphatidylcholine bilayers alone (●), myelin proteolipid protein/dimyristoyl phosphatidylcholine complexes of lipid/protein ratio 24:1 mol/mol (■), and myelin proteolipid protein alone (○). The microwave power (P) dependence of the degree of saturation, S_{CW}/S_{CW}^{0}, is recorded at 4°C (left-hand panel) and 30°C (right-hand panel). Full lines represent fits to the saturation of a single component according to Eq. 2 and dashed lines represent the predicted saturation behavior of the lipid/protein complexes, assuming no exchange between the two components and a fraction f = 0.40 of the protein-associated component. For the data recorded at 4°C, the latter corresponds to a nonlinear least-squares fit to the data points (see text).

case than would be a measurement at $P = P_{1/2}$. The rms error in the fit for the extreme case (ca. 0.10 in S_{CW}^{0}/S_{CW}, $N = 17$), corresponds to ≤28% error in $P_{1/2}$, which is an overestimate because the standard error will be considerably lower.

Clearly, the spin-labeled phosphatidylcholine in pure lipid bilayers saturates far less readily than when it is associated with the protein, indicating a pronounced difference in the lipid chain dynamics in these two environments. In the fluid lipid phase, this difference in mobility is clear from the conventional ESR spectra (cf. Fig. 1 and reference 12), i.e., on the timescale of the spin label T_2 relaxation. The CW saturation results demonstrate that this difference in mobility of the lipids interacting with the protein extends also to lipids in the gel phase, indicating differences in mobility on the timescale of the spin-label T_1 relaxation in the gel phase.

The saturation behavior of the spin-labeled lipid in the lipid/protein complexes shown in Fig. 2 is intermediate between that found in the pure lipid and protein-alone environments, but there is a very pronounced difference between the data recorded in the gel and fluid lipid phases. In the gel phase, the saturation curve for the lipid/protein complex lies approximately midway between that for the pure lipid sample and the sample containing the protein alone, whereas in the fluid phase the lipid/protein complex saturates less readily and its saturation curve lies much closer to that of the pure lipid. This behavior is that expected if the exchange rate between the two lipid environments is slow on the timescale of the spin-lattice relaxation in the gel phase but fast in the fluid phase. In the former case (of no exchange), the saturation of the integrated intensity of the two-component spectrum will simply be the weighted average (according to f) of that for the two constituent components. The dashed lines in Fig. 2 are the saturation curves predicted for the lipid/protein complexes in the absence of exchange with a value of $f = 0.40$, which represents a nonlinear least-squares fit to the data obtained at 4°C (left-hand panel of Fig. 2). This value for f is quite close to that obtained from subtractions of the conventional ESR spectra in the fluid phase (see above).

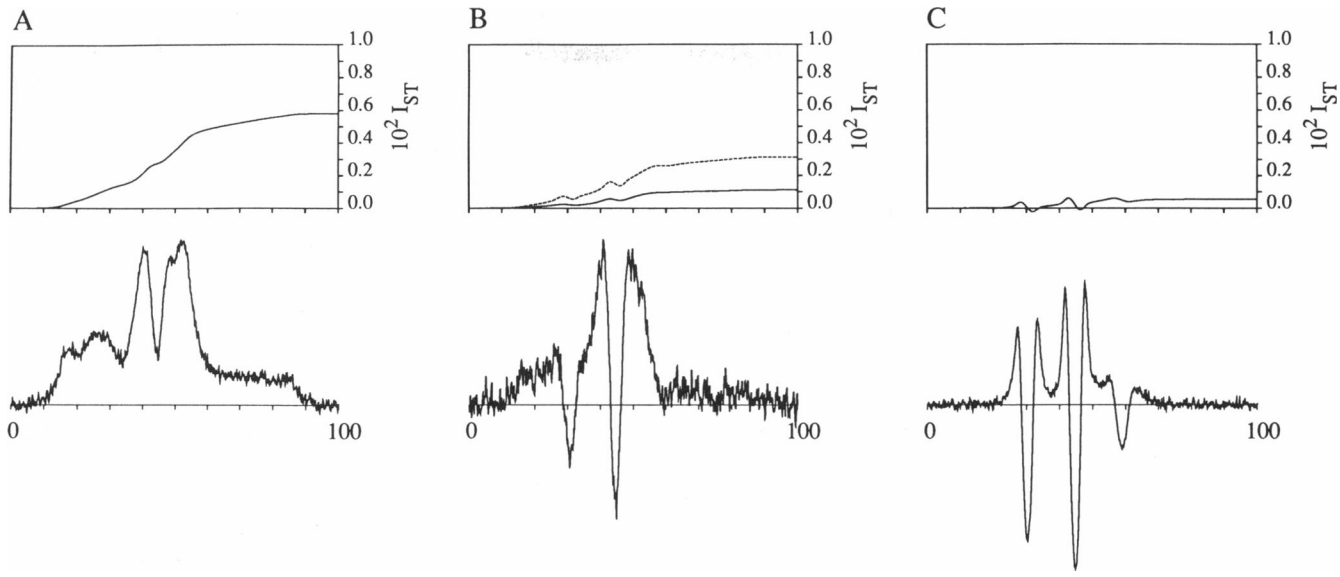

FIGURE 3 Second harmonic, 90° out-of-phase, absorption STESR spectra (V'_2-display) and their normalized first integrals of the 14-PCSL phosphatidylcholine spin label in (*A*) myelin proteolipid protein alone, (*B*) myelin proteolipid protein/dimyristoyl phosphatidylcholine complex of lipid/protein ratio 24:1 mol/mol, and (*C*) dimyristoyl phosphatidylcholine bilayers. The spectra are given in the lower row and the integrals (I_{ST}) in the upper row. The dashed line in *B* corresponds to the weighted average predicted from the values of I_{ST} for the isolated components that are given in *A* and *B*. T = 30°C, scan range = 100 G.

The quality of the fit to the experimental data points obtained at 4°C indicates that the exchange at the lipid/protein interface is slow on the timescale of T_1 in the gel phase and that the size of the lipid population interacting with the protein is similar to that in the fluid phase. However, the degree of saturation of the lipid/protein complex that is observed experimentally in the fluid phase at 30°C is far less than that predicted in the absence of exchange between the two lipid populations. Clearly, the close proximity of the saturation curve for the lipid/protein complex to that for the pure lipid at 30°C indicates that there is an enhancement of the spin-lattice relaxation due to exchange on the T_1 timescale between the two lipid environments of the lipid/protein complex in the fluid phase. This result is consistent with the results of exchange-coupled lineshape simulations for PLP/DMPC systems in the fluid phase (13, 14).

STESR spectra of lipid/protein complexes

A further approach to the saturation properties of the lipid/protein complexes is to determine the intensities of the STESR spectra recorded in phase quadrature. The second-harmonic 90° out-of-phase absorption STESR spectra (V'_2-display) of the 14-PCSL spin label and the corresponding normalized spectral integrals are given in Fig. 3 for samples of the delipidated PLP protein, bilayer dispersions of the pure DMPC lipid, and a PLP/DMPC complex. The spectra were recorded at 30°C corresponding to the fluid phase of DMPC bilayers. In addition to the expected differences in spectral lineshape, there are large differences in the integrated intensities of the spectra from the different samples. The normalized STESR integral, I_{ST}, for the delipidated PLP is much larger than that for the pure DMPC dispersion, corresponding to the much greater rotational mobility of the spin-labeled lipid in fluid lipid bilayers than when associated with the delipidated protein (cf. reference 27). The normalized STESR integral of the lipid spin label in the PLP/DMPC complex is intermediate between that for the pure lipid and the PLP alone. However, the value of I_{ST} is considerably less than the average value weighted by the relative populations (f = 0.49, cf. above) of the two individual components. This lack of additivity of the spectral intensities indicates the presence of an additional motion giving rise to further saturation transfer that it is not present in the isolated components. The most likely candidate for this extra motion is the exchange between the two lipid environments in the lipid/protein complex in the fluid phase.

The myelin proteolipid protein displays a selectivity in interaction with different spin-labeled lipids (12). This gives a convenient method of varying both the fraction of protein-associated lipid spin label and its exchange rate in a given lipid/protein complex (13, 14). The integrated intensity, I_{ST}, of the STESR spectra from various lipid spin labels in PLP/DMPC complexes of fixed lipid/protein ratio is given as a function of the fraction of motionally restricted spin labels, f, in Fig. 4. Values of I_{ST} are given for STESR spectra recorded at temperatures for which the bulk lipid in the complexes is either in the gel phase (4°C) or in the fluid phase (30°C), as

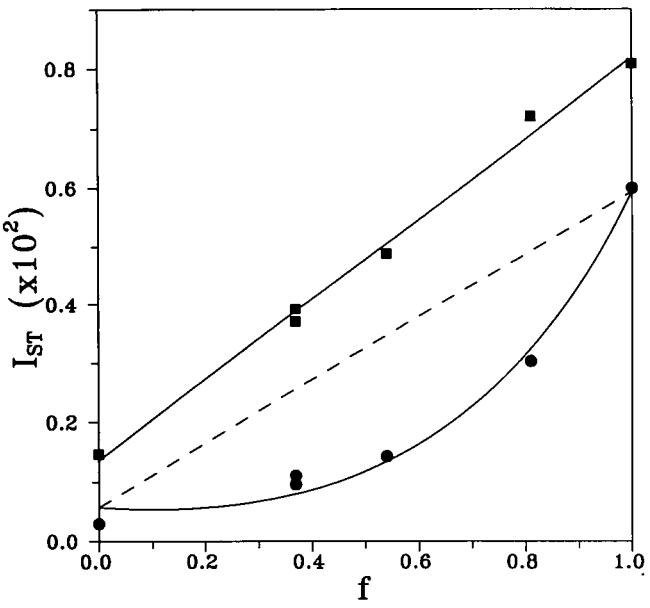

FIGURE 4 Dependence of the integrated intensity, I_{ST}, of the STESR spectra from spin-labeled lipids, 14-PLSL, in PLP/DMPC (1:37 mol/mol) complexes, on the fraction, f, of motionally restricted spin label determined from spectral subtractions of the conventional ESR spectra. Data correspond to spin labels with different selectivities for the protein (cf. Table 1) and were recorded at 30°C (●) and at 4°C (■). Solid lines correspond to the optimal fits to Eq. 9, obtained by a linear regression with $T^0_{1,b}\tau_f^{-1} = 0$ for the data at 4°C and a nonlinear least-squares fit for the data at 30°C, yielding $T^0_{1,b}\tau_f^{-1} = 2.88$ (rms = 2.1×10^{-4}, $N = 6$). The dashed line is the corresponding dependence that would be predicted in the absence of exchange ($T^0_{1,b}\tau_f^{-1} = 0$) at 30°C.

indicated by the conventional ESR spectral lineshapes. The values of f were determined from subtractions with the conventional spectra recorded in the fluid phase at 30°C (cf. Table 1).

In the gel phase, the values of I_{ST} increase linearly with increasing values of f. A similar linear dependence is also obtained when f is changed by varying the lipid/protein ratio for a given spin label (cf. reference 27). It will be noted that the value of I_{ST} for the protein-alone sample ($f = 1$) in Fig. 4 does not deviate from the linear dependence on f observed for the lipid/protein complexes. This suggests that, when delipidated, the protein is in a similar state to that in the reconstituted complexes in the gel phase, as is found also to be the case in the fluid phase from the similarity of the conventional ESR spectra (cf. above). The linear dependence in Fig. 4 is consistent with the existence of two lipid populations for which the protein-interacting component has a mobility that is restricted relative to that of the bulk gel phase lipids and there is no exchange between the two populations on the STESR timescale. In such a situation, the observed value of I_{ST} is expected to be the weighted average of the values corresponding to the protein-interacting and bulk lipid components in the complex (cf. reference 27). Since the values of f were deduced from measurements at 30°C, it therefore follows that the stoichiometry and specificity of the lipid-protein interaction with the various spin labels in the gel phase is similar to that in the fluid phase. Furthermore, the linear dependence of I_{ST} on f indicates that there is no exchange between the two lipid populations on the timescale of the spin-lattice relaxation of the spin labels in the gel phase.

In contrast to the situation in the gel phase, the values of I_{ST} in the fluid phase increase nonlinearly with increasing values of f, in a manner that is consistent with the selectivity for interaction with the protein of the different lipid spin labels. The lineshapes of the conventional ESR spectra in the fluid phase clearly indicate the presence of two lipid populations, and spectral subtraction allows a determination of the relative proportions of these two components that is directly applicable to the STESR spectra obtained in the fluid phase. The nonlinear dependence of the STESR integrals on these values of f therefore indicates that, in the fluid phase, exchange between the two lipid populations is taking place on the STESR timescale. It will be shown later in the Discussion how the exchange rates can be determined in terms of the intrinsic spin-lattice relaxation rates of the lipid spin labels from the STESR spectra recorded in the fluid phase.

Comparable data to those of Fig. 4 were also obtained for the microwave power at half-saturation, $P_{1/2}$, obtained from CW saturation experiments on the different spin labels in PLP/DMPC complexes of fixed lipid/protein ratio. These values were determined as indicated in Fig. 2 and are given as a function of f in Fig. 5. The data display trends similar to those for the STESR integrals, except that a decreasing degree of saturation corresponds to an increased value of $P_{1/2}$, as opposed to a decreased value of I_{ST}. However, a nonlinear dependence of $P_{1/2}$ on f is expected even in the absence of exchange, and therefore further analysis is deferred until after that of the STESR integrals in the Discussion.

TABLE 1 STESR integrals, fraction of motionally restricted lipid, and normalized exchange rates for spin-labeled lipids in PLP/DMPC complexes in the fluid phase

Spin label	f	$I_{ST} \times 10^2$	$T^0_{1,b}\tau_b^{-1}$	
14-SASL	0.81	0.303	0.73*	0.67‡
14-PASL§	0.77	0.195	1.9*	0.86‡
14-PSSL	0.54	0.143	1.6*	2.45‡
14-PGSL	0.37	0.111	1.5*	4.9‡
14-PCSL	0.37	0.096	2.0*	4.9‡

* Deduced from Eq. 8.
‡ Deduced from nonlinear least-squares fit to Eq. 9.
§ The value of I_{ST} for 14-PASL is atypically low, because of spin-spin broadening.
Normalized integrated intensity, I_{ST}, in the saturation transfer ESR spectra of spin-labeled lipids, 14-PLSL, in PLP/DMPC complexes (1:37 mol/mol) at 30°C, and the fraction, f, of motionally restricted spin labels deduced from subtractions of the conventional ESR spectra. The normalized off-rate constants, $T^0_{1,b}\tau_b^{-1}$, deduced from Eq. 8 and alternatively from the value of $T^0_{1,b}\tau_f^{-1} = 2.88$ obtained by a nonlinear least-squares fit to Eq. 9 (see Fig. 4), are given separately.

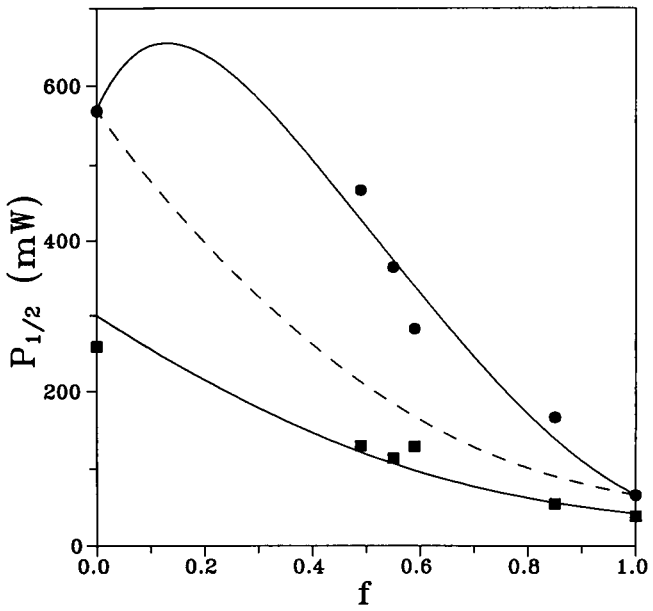

FIGURE 5 Dependence of the microwave power at half-saturation, $P_{1/2}$, for the double integrated intensity of the conventional ESR spectra from spin-labeled lipids, 14-PLSL, in PLP/DMPC (1:24 mol/mol) complexes, on the fraction, f, of motionally restricted spin label determined from spectral subtractions. Data correspond to spin labels with different selectivities for the protein and were recorded at 30°C (●) and at 4°C (■). Solid lines correspond to nonlinear least-squares fits to Eq. 11, obtained with $T^0_{1,b}\tau_f^{-1} = 0$ for the data at 4°C and $T^0_{1,b}\tau_f^{-1} = 4.15$, for the data at 30°C. The dashed line is the corresponding dependence that would be predicted in the absence of exchange ($T^0_{1,b}\tau_f^{-1} = 0$) at 30°C.

DISCUSSION

Previous studies of the stoichiometry and specificity of lipid interaction with the myelin proteolipid protein using conventional ESR are extended in this paper to investigation of the saturation properties of lipid spin labels in PLP/DMPC complexes. Both conventional CW saturation and saturation transfer ESR spectroscopy have been used for this purpose. The results demonstrate, in contrast to the conventional lineshape studies that were necessarily restricted to analysis of complexes in the fluid lipid phase, that a lipid population interacting with the intramembranous surface of the PLP protein can be distinguished also in gel phase lipid complexes by means of its different saturation properties. This protein-associated lipid component has a stoichiometry and specificity for interaction with the protein similar to that found previously in the fluid phase. The exchange between the two lipid components is slow on the timescale of the spin-lattice relaxation when the bulk lipid is in the gel phase but rapid when it is in the fluid phase. The ESR saturation properties are now analyzed below in terms of the lipid exchange rates at the protein interface in the fluid phase. The measurements in the gel phase where no exchange takes place constitute a valuable control for the method of analysis that is used. Derivation of the effective spin-lattice relaxation times governing the saturation properties in the presence of two-site exchange is given in the Appendix.

Analysis of the STESR spectra

The dependence of the normalized integrated intensity, I_{ST}, of the STESR spectrum on the selectivity of the different spin-labeled lipids for the protein can be related to the exchange rates of the lipid at the lipid-protein interface. For an STESR spectrum composed of two components, the observed STESR integral is given by a weighted average of the integrals from the components b and f(27):

$$I_{ST} = f \cdot I_{ST,b} + (1 - f) I_{ST,f}, \quad (3)$$

where f is the fractional population of component b and $I_{ST,i}$ is the normalized value of the saturation transfer integral for component i (with $i \equiv b$ or f). At exchange equilibrium, the condition for mass balance yields the following relation between the individual exchange rates for the two components (13):

$$f\tau_b^{-1} = (1 - f)\tau_f^{-1}, \quad (4)$$

where τ_i^{-1} is the rate of transfer per unit time from component i. It is assumed that the normalized integral intensity, $I_{ST,i}$, contributed by each component i in the STESR spectrum is proportional to the effective spin-lattice relaxation time, $T^{eff}_{1,i}$, of the spin labels giving rise to that component (8, 30, 31):

$$I_{ST,i} = (I^0_{ST,i}/T^0_{1,i})T^{eff}_{1,i}, \quad (5)$$

where $I^0_{ST,i}$ and $T^0_{1,i}$ are the STESR integral and effective T_1, respectively, in the absence of lipid exchange. In the presence of lipid exchange, the effective values of T_1 for each component, b and f, are given by (see Appendix and reference 4):

$$1/T^{eff}_{1,b} = 1/T^0_{1,b} + (1 - f)\tau_b^{-1}/(1 - f + fT^0_{1,f}\tau_b^{-1}), \quad (6)$$

$$1/T^{eff}_{1,f} = 1/T^0_{1,f} + f\tau_b^{-1}/[(1 - f)(1 + T^0_{1,b}\tau_b^{-1})]. \quad (7)$$

Substituting Eqs. 5–7 in Eq. 3 then yields the following expression for the dependence of the STESR integral on the fraction, f, of motionally restricted lipid and the intrinsic rate of exchange, τ_b^{-1}, off the surface of the protein:

$$I_{ST} = [I^0_{ST,b} f(1 - f + fT^0_{1,f}\tau_b^{-1}) + I^0_{ST,f}(1 - f)^2(1 + T^0_{1,b}\tau_b^{-1})]/\{1 - f + [fT^0_{1,f} + (1 - f)T^0_{1,b}]\tau_b^{-1}\}, \quad (8)$$

where the relation $T^0_{1,f} = (I^0_{ST,f}/I^0_{ST,b})T^0_{1,b}$ from Eq. 5 can be substituted to express I_{ST} in terms of the single scaled exchange rate: $T^0_{1,b}\tau_b^{-1}$.

The scaled values of the exchange rates, $T^0_{1,b}\tau_b^{-1}$, for the different lipid spin labels at the hydrophobic surface

of the PLP in fluid-phase DMPC recombinants, as deduced from Eq. 8, are given in Table 1. The values of I_{ST} obtained from the STESR spectra recorded at 30°C and the values of f deduced from spectral subtractions performed with the conventional ESR spectra were used in this calculation. The data all refer to recombinants with the same lipid/protein ratio. It is seen from Table 1 that the rates of exchange generally tend to decrease as the lipid selectivity for the protein (reflected by the values of f) increases, as is expected on thermodynamic grounds (cf. references 13 and 14).

The absolute values for the exchange rates, τ_b^{-1}, can be determined if the effective T_1 for component b in the absence of lipid exchange is known. These latter values must take account of the reduction in effective T_1 by the rotational motion of the lipid chains at the hydrophobic surface of the protein. Using calibrations for the dependence of I_{ST} on rotational correlation time given previously (32), it can be estimated that $T_{1,b}^0$ is reduced relative to the value in the absence of rotational diffusion by a factor of 0.56 at 30°C, corresponding to a rotational correlation time of $\tau_R \approx 42$ μs.

Assuming that the value of T_1^0 in the absence of rotational diffusion is ~1 μs (cf. reference 8), the values of τ_b^{-1} deduced from Table 1 range from 1.3 to 3.5 × 10^6 s^{-1} for 14-SASL and 14-PCSL, respectively, at 30°C. Corresponding values obtained previously by simulation of the conventional ESR spectra with the exchange-coupled Bloch equations range from 2 to 12 × 10^6 s^{-1} for 14-SASL and 14-PCSL, respectively, under similar conditions (14). These latter values are systematically higher than those obtained from the present STESR measurements. Both methods contain potential systematic uncertainties depending on the choice of $T_{1,b}^0$ in the case of STESR and on the choice of the single component lineshapes in the case of the conventional lineshape simulations. Nevertheless, both sets of determinations yield exchange rates of a similar order of magnitude. A somewhat better agreement is obtained by a combined analysis of the lipid selectivity data obtained from the STESR measurements that now follows.

It is possible to express Eq. 8 alternatively in terms of the on-rate constant, τ_f^{-1}, at the lipid-protein interface by using the relationship given in Eq. 4:

$$I_{ST} = \{I_{ST,b}^0 f^2(1 + T_{1,f}^0\tau_f^{-1}) + I_{ST,f}^0(1-f)[f + (1-f)T_{1,b}^0\tau_f^{-1})]\}/\{f + [fT_{1,f}^0 + (1-f)T_{1,b}^0]\tau_f^{-1}\}. \quad (9)$$

This formulation is particularly useful in analyzing the dependence of the STESR integrals on the lipid selectivity in complexes of a fixed lipid/protein ratio. In such cases, the on-rate, τ_f^{-1}, is expected to remain constant because it is diffusion controlled, as established from an analysis of the conventional ESR spectra (14). A nonlinear least-squares fit to Eq. 9 for the data recorded at 30°C with complexes of fixed lipid/protein ratio is given in Fig. 4. It is seen that the dependence of the STESR integral for the fluid phase can be described reasonably well by this equation, with a constant value for the scaled on-rate of $T_{1,b}^0\tau_f^{-1} = 2.88$ at 30°C. By contrast, the corresponding data obtained at 4°C display an approximately linear dependence on the fraction of motionally restricted spin labels, which implies that the exchange rates at the lipid-protein interface are extremely slow when the bulk lipid is in the gel phase. A linear regression to Eq. 9, assuming that $T_{1,b}^0\tau_f^{-1} = 0$, is given in Fig. 4, and it can be seen that this fits the data recorded at 4°C rather well.

The scaled values of the off-rate constants, $T_{1,b}^0\tau_b^{-1}$, for the different spin labels deduced from Eq. 4, with the constant on-rate of $T_{1,b}^0\tau_f^{-1} = 2.88$ at 30°C obtained by fitting the full data set to Eq. 9, are also given in Table 1. These combined values display a wider variation with the lipid selectivity than obtained from the individual determinations and, in particular, yield somewhat faster exchange rates for 14-PCSL and 14-PGSL. Using the same value for $T_{1,b}^0$ as that used above gives absolute values for the intrinsic exchange rate ranging from 1.2 to 8.7 × 10^6 s^{-1} for 14-SASL and 14-PCSL, respectively. These values lie quite close to those deduced from the conventional ESR lineshape simulations. The latter are likely to yield an upper bound for the exchange rates, since it was assumed that the lineshape of the fluid lipid component was unaffected by longer range effects of the lipid-protein interaction (13, 14).

Analysis of CW saturation

The saturation of the double-integrated intensity, S_{CW}, of the conventional two-component ESR spectrum in the CW experiment is given by:

$$S_{CW} = S_{CW}^0[f/(1 + \sigma_b^{eff})^{1/2} + (1-f)/(1 + \sigma_f^{eff})^{1/2}], \quad (10)$$

where S_{CW}^0 is the value that S_{CW} would have in the absence of saturation and $\sigma_i^{eff} = \gamma^2 H_1^2 T_{1,i}^{eff} T_{2,i}^{eff}$ is the effective saturation parameter of component i with $T_{2,i}^{eff}$ being the effective spin-spin relaxation time of component i (with $i \equiv b$ or f). This equation is valid independent of the degree of inhomogeneous broadening in the composite powder spectrum (29, 33). Assuming that changes in $T_{2,i}^{eff}$ due to exchange can be neglected, the value of $P_{1/2} \equiv H_{1,1/2}^2$ at half-saturation (i.e., for $S_{CW}/S_{CW}^0 = ½$) is given by the condition:

$$0.5 = f/[1 + \sigma_{1/2}^S(P_{1/2}/P_{1/2,b}^0)(T_{1,b}^{eff}/T_{1,b}^0)]^{1/2} + (1-f)/[1 + \sigma_{1/2}^S(P_{1/2}/P_{1/2,f}^0)(T_{1,f}^{eff}/T_{1,f}^0)]^{1/2}, \quad (11)$$

where $\sigma_{1/2}^S = 3$ is the saturation parameter for a single component at half-saturation and $P_{1/2,i}^0 = \sigma_{1/2}^S/\gamma^2 T_{1,i}^0 T_{2,i}^0$ is the value of $P_{1/2}$ at half-saturation for the isolated component i. Substitution from Eqs. 6 and 7 in Eq. 11 then allows determination of the values of the scaled exchange rate from the measured values of $P_{1/2}$ (and $P_{1/2,i}^0$). The version of Eq. 11 corresponding to no

exchange is obtained by putting $T^{eff}_{1,b}/T^0_{1,b} = 1 = T^{eff}_{1,f}/T^0_{1,f}$.

The powers at half-saturation of the double integral from the conventional ESR spectra of PLP/DMPC complexes with fixed lipid/protein ratio are given as a function of the fraction, f, of the motionally restricted component in Fig. 5. Measurements both in the gel and fluid phases are included in the figure. In the gel phase, the data can be fitted reasonably well by Eq. 11, on the assumption of no exchange between the two lipid components. The values of $P^0_{1/2,b}$ and $P^0_{1/2,f}$ obtained from the fit are close to those measured for samples of the protein-alone and the pure lipid, respectively, at the same temperature. The CW saturation data are therefore in agreement with those obtained from STESR in that, although the lipid spin labels interacting directly with the protein can be distinguished by their saturation behavior from those in the gel phase, they are not undergoing exchange on the timescale of the spin-lattice relaxation.

The dependence of the half-saturation power on f calculated from Eq. 11 on the assumption of no exchange by using the values of $P^0_{1/2,b}$ and $P^0_{1/2,f}$ for the protein-alone and pure lipid samples obtained in the fluid phase are given by the dashed line in Fig. 5. It is seen that all spin labels in the lipid/protein complexes saturate less easily than would be expected in the absence of exchange. Once again, this indicates that, in the fluid phase, the spin-labeled lipids undergo exchange on and off the surface of the protein on the timescale of T_1. The scatter in the data is greater than that in Fig. 4, at least in part because of the lower sensitivity of CW saturation than STESR to changes in T_1 (cf. reference 30). A nonlinear least-squares fit of the data in the fluid phase to Eq. 11 (analogous to that given for the STESR data to Eq. 8 in Fig. 4 and including the same substitution for $T^0_{1,f}$) is given by the full line in Fig. 5. The biphasic character of the predicted dependence on f arises because the relaxation of both components is enhanced by exchange, in a manner that is dependent differently on f for the two components (cf. Eqs. 6 and 7). The value for the scaled on-rate of $T^0_{1,b}\tau_f^{-1} = 4.15$ at 30°C that is obtained from the fit is larger than that obtained from the similar fit (to STESR data) which is given in Fig. 4, because the lipid/protein ratio is lower. Values deduced for the intrinsic scaled off-rate, $T^0_{1,b}\tau_b^{-1}$, from Eq. 4 range from 0.73 to 4.3 for 14-SASL and 14-PCSL, respectively, which are comparable to the values given in Table 1. Thus, in spite of the inherently lower sensitivity to T_1, it is clear that the CW saturation experiments fully support the conclusions reached from the STESR measurements with regard to the exchange at the lipid-protein interface in the fluid and gel phases.

In summary, studies of the saturation behavior of spin-labeled lipids provide a valuable additional ESR method for investigating the interactions of lipids with integral membrane proteins. Unlike conventional ESR lineshape analysis, such methods are applicable also to lipid/protein complexes in the gel phase, and their primary use is in studying lipid exchange at the protein surface over a wider range of rates than is available by conventional means, extending both to the gel and fluid lipid phases.

APPENDIX

Spin-lattice relaxation and two-site exchange

The effects of two-site exchange in CW saturation experiments can be analyzed by using the rate equations for the population differences, n_b and n_f, between the $M_S = \pm\frac{1}{2}$ levels of spins at sites b and f, respectively. If transitions of the spins at site b are induced by the H_1 field, then the steady-state condition for the population difference is:

$$dn_b/dt = -2Wn_b + (n^0_b - n_b)/T^0_{1,b} - n_b\tau_b^{-1} + n_f\tau_f^{-1} = 0, \quad (A.1)$$

where W is the rate at which transitions are induced by the H_1 field, n^0_b is the Boltzmann equilibrium value of n_b, and other symbols are as defined previously. The corresponding steady-state condition for spins at site f that is not irradiated by the H_1 field is:

$$dn_f/dt = (n^0_f - n_f)/T^0_{1,f} - n_f\tau_f^{-1} + n_b\tau_b^{-1} = 0, \quad (A.2)$$

where n^0_f is the Boltzmann equilibrium value of n_f. Solution of Eqs. A.1 and A.2, together with the condition for detailed balance (Eq. 4, given previously) yields the standard expression for the saturation of the spin system at site b (cf. reference 34):

$$n_b = n^0_b/(1 + 2WT^{eff}_{1,b}), \quad (A.3)$$

where the effective spin-lattice relaxation rate, $1/T^{eff}_{1,b}$, in the presence of two-site exchange is that given by Eq. 6 above. Similarly, it is found that when the spins at site f are irradiated by the H_1 field, their saturation is characterized by the effective spin-lattice relaxation rate, $1/T^{eff}_{1,f}$, that is given by Eq. 7 above. These expressions for the effective spin-lattice relaxation rates of the two species are valid as long as the corresponding resonances remain distinct (cf. Eqs. A.1 and A.2). Thus, the treatment essentially is equivalent to that of Luz and Meiboom (35) for the situation in which one species is in excess and to one of the cases considered by McConnell (4). The effective spin-lattice relaxation times derived in this way should be applicable also to the calculation of the normalized integrated intensity of the STESR spectrum by using Eq. 5 above.

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