Protein-Induced Vertical Lipid Dislocation in a Model Membrane System: Spin-Label Relaxation Studies on Avidin-Biotinylphosphatidylethanolamine Interactions

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ABSTRACT The change in vertical location of spin-labeled *N*-biotinyl phosphatidylethanolamine in fluid-phase dimyristoyl phosphatidylcholine bilayer membranes, on binding avidin to the biotinyl headgroup, has been investigated by progressive saturation electron spin resonance measurements. Spin-labeled phospholipids were present at a concentration of 1 mol%, relative to total membrane lipids. For avidin-bound *N*-biotinyl phosphatidylethanolamine spin-labeled on the 8 C atom of the *sn*-2 chain, the relaxation enhancement induced by 30 mM Ni^{2+} ions confined to the aqueous phase was 2.5 times that induced by saturating molecular oxygen, which is preferentially concentrated in the hydrophobic core of the membrane. For phosphatidylcholine also spin-labeled at the 8 position of the *sn*-2 chain, this ratio was reversed: the relaxation enhancement by Ni^{2+} ions was half that induced by molecular oxygen. In the absence of avidin, the enhancement by either relaxant was the same for both spin-labeled phospholipids. For a double-labeled system, in which both *N*-biotinyl phosphatidylethanolamine and phosphatidylcholine were spin-labeled on the 12 C atom of the *sn*-2 chain, the relaxation rate in the absence of avidin was greater than that predicted from linear additivity of the corresponding singly labeled systems, because of mutual spin-spin interactions between the two labeled lipid species. On binding of avidin to the *N*-biotinyl phosphatidylethanolamine, this relaxation enhancement by mutual spin-spin interaction was very much decreased. These results indicate that, on binding of avidin to the lipid headgroup, *N*-biotinyl phosphatidylethanolamine is lifted vertically within the membrane, relative to the phosphatidylcholine host lipids. The specific binding of avidin to *N*-biotinyl phosphatidylethanolamine parallels the liftase activity proposed for activator proteins associated with the action of certain gangliosidases.

INTRODUCTION

The binding of avidin to *N*-biotinyl phosphatidylethanolamines in lipid bilayer membranes provides a useful model for studying highly specific lipid-protein interactions (Swamy and Marsh, 1993). Recent electron spin resonance (ESR) investigations on the interaction of avidin with *N*-biotinyl phosphatidylethanolamines (biotin-PEs) bearing a spin-label reporter group at different positions throughout the length of the *sn*-2 chain have shown that the binding of avidin produced a strong and selective restriction of the biotin-PE lipid mobility at all positions of the chain labeling (Swamy and Marsh, 1997). Furthermore, comparison of the outer hyperfine splittings from the different positionally labeled, avidin-bound and free biotin-PEs in the fluid phase membranes of the indifferent host lipid dimyristoylphosphatidylcholine has suggested a possible vertical displacement, of ~7–8 Å, from the membrane, of biotin-PEs on complexation with avidin. This vertical displacement of biotin-PE on avidin binding approximates a similar lipid displacement that is thought to be required in other systems of biochemical interest. For example, the active site of phospholipase A_2 is located ~15 Å from the protein surface, in the x-ray crystal structure (Scott et al., 1990). A movement of the phospholipid substrate out of the membrane, through a hydrophobic channel that leads to the catalytic site, is therefore required for the enzymatic hydrolysis. A similar "lifting" of the lipid molecule out of the membrane is postulated in the activator protein-mediated hydrolysis of the ganglioside G_{M2} by β-hexosaminidase A, in the lysosomal compartment (Sandhoff and Kolter, 1996). It is proposed that the essential cofactor, G_{M2}-activator protein, acts by binding one molecule of G_{M2} and lifting it either fully or by a few angstroms out of the membrane, for recognition of the activator-lipid complex by the water-soluble β-hexosaminidase A. The complexation of avidin with the headgroups of biotinylated membrane lipids hence provides a model system for the lipid dislocation in the initial steps of these membrane enzyme mechanisms.

In the present study, for the model avidin biotin-PE system, we have used two approaches to characterize the vertical displacement of the biotinylated lipid molecule on avidin binding, by using progressive saturation ESR spectroscopy. For the first series of experiments, use is made of aqueous and lipid-dissolved paramagnetic relaxants to study the differential relaxation enhancement for avidin-bound spin-labeled *N*-biotinyl phosphatidylethanolamine and free spin-labeled phosphatidylcholine in fluid-phase phosphatidylcholine membranes. For the second series of experiments, relaxation enhancement was induced differentially by spin exchange interactions between spin labels on *N*-biotinyl phosphatidylethanolamine and on phosphatidylcholine, respectively, in the presence and absence of avidin.

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The results of these studies demonstrate that the specific effect of avidin binding to the biotinylated lipid is to lift the bound phospholipid vertically in the membrane, relative to the average position of the host membrane phospholipid. This vertical displacement is required for the biotin moiety to reach the binding site within the avidin molecule. Overall, this work presents a unique approach to the study of systems involving vertical lipid dislocation in the enzyme-substrate interactions of the type discussed above, and it shows promise in characterizing the thermodynamic and dynamic aspects of such interactions.

MATERIALS AND METHODS

Materials

Hen egg white avidin was procured from Fluka (Buchs, Switzerland). Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Phosphatidylcholine spin labels labeled at different positions, *n*, in the *sn*-2 chain of the lipid (1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphocholines, *n*-PCSLs) and the analogous spin-labeled phosphatidylethanolamines (1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoethanolamines, *n*-PESLs) were synthesized as described earlier (Marsh and Watts, 1982). The corresponding spin-labeled *N*-biotinylphosphatidylethanolamines (1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-(*N*-biotinyl)phosphoethanolamines, *n*-BPESLs) were synthesized from the spin-labeled *n*-PESLs as described earlier (Swamy and Marsh, 1997). All other chemicals were of analytical grade purity.

Sample preparation

A greater extent of avidin binding to the *n*-BPESLs in DMPC membrane matrix was achieved by a slight modification of the method used previously (Swamy and Marsh, 1997). Briefly, 2 mg of DMPC and 1 mol% spin-labeled *N*-biotinyl phosphatidylethanolamine (or corresponding *n*-PCSL) were codissolved in chloroform:methanol mixture (2:1, v/v), and the solvent was evaporated under a stream of dry N_2 gas so as to leave a thin film of the mixture on the walls of the glass tube. The residual solvent was removed by evacuation for 6 h or more. The lipid film was then hydrated with 0.2 ml of avidin-containing (5 mg/ml) HEPES buffer (2 mM, containing 1 mM EDTA, pH 7.4). The sample was incubated at 37°C for 10 min, gently vortexed, and subjected to three 30-s pulses of sonication in a Branson bath-type sonicator. The resulting suspension was freeze-thawed three times and then transferred to a 1-mm i.d. glass capillary. The avidin-bound lipid membranes were pelleted by centrifugation. The lipid film and avidin-containing buffer were thoroughly flushed with argon and, during the various steps of sample preparation, an atmosphere of argon was always carefully maintained. For experiments in the presence of oxygen, buffers were saturated with oxygen, and the sample tubes and capillaries were also flushed with oxygen.

ESR measurements

ESR spectra were recorded on a Varian Century Line 9-GHz spectrometer equipped with nitrogen gas flow temperature regulation. Samples in 1-mm i.d. glass capillaries were placed in a standard quartz holder (4 mm i.d.) containing light silicone oil for thermal stability. Spectral data were collected digitally on an IBM personal computer using software written by M. D. King of this institute. Spectral subtractions were performed as described earlier (Marsh, 1982). Conventional, in-phase absorption spectra (V_1 display) were recorded, with a scan range of 160 gauss and a modulation amplitude of 1.25 gauss p-p, at various microwave powers decreasing from 200 to 0.2 mW, which corresponds to a root mean square (r.m.s.) microwave magnetic field, $\langle H_1^2\rangle^{1/2}$, of 600–6 mgauss at the sample. A standardized sample configuration was used in all progressive saturation experiments (Fajer and Marsh, 1982), and all measurements were performed under critical coupling conditions. The microwave H_1-field at the sample was calibrated as described by Fajer and Marsh (1982). The saturation curves were obtained by plotting the spectral intensity (second integral of the first-derivative spin-label ESR spectrum) as a function of the microwave magnetic field, H_1, at the sample, and were analyzed as described by Snel and Marsh (1993) and Páli et al. (1993). The H_1 dependence of the double-integrated intensity, *S*, of the spin-label ESR spectrum is given by (Páli et al., 1993)

$$S = \frac{S_o' H_1}{\sqrt{1 + \gamma_e^2 \langle H_1^2\rangle (T_1 T_2)^{\text{eff}}}} \quad (1)$$

where γ_e is the electron gyromagnetic ratio, T_1 is the spin-lattice relaxation time, T_2 is the transverse spin relaxation time, $(T_1T_2)^{\text{eff}}$ is the effective T_1T_2 relaxation time product, and S_o' is a normalization constant. Spectral double integrals were evaluated over the full scan range of 160 gauss. Peak-to-peak linewidths of the central hyperfine line were determined by local fitting to the peaks.

RESULTS AND DISCUSSION

Two approaches have been used to detect the possible vertical displacement in the membrane of the biotinylated lipid on binding avidin to its polar headgroup. In both cases, the biotinyl phosphatidylethanolamine is spin-labeled in the *sn*-2 acyl chain and is present at a concentration of 1 mol% in fluid-phase bilayer membranes of the zwitterionic phospholipid dimyristoylphosphatidylcholine. In the first series of experiments, Ni^{2+} ions that are confined to the aqueous phase, or molecular oxygen that displays a profile of increasing concentration toward the hydrophobic core of the membrane, were used as paramagnetic relaxants (see Hubbell and Altenbach, 1994; Snel and Marsh, 1993). The relaxation enhancement by aqueous Ni^{2+} ions takes place via a dipole-dipole interaction and depends directly on the distance of the spin-labeled group from the membrane surface (Páli et al., 1992). The bilayer profile of oxygen concentration is steepest in the region of the 8 C atom of the lipid *sn*-2 chain (Snel and Marsh, 1993), and therefore this position (i.e., 8-BPESL) was chosen for spin labeling. In the second series of experiments, double labeling was used with two different spin-labeled lipids. Relaxation enhancements were induced by spin-exchange interactions between biotinyl phosphatidylethanolamine spin-labeled at a fixed position in the *sn*-2 chain and phosphatidylcholine spin-labeled at the same or a different position in its *sn*-2 chain. In this case, the maximum relaxation enhancement is expected when the spin-labeled groups on the two lipids are located at the same vertical height in the membrane (Snel and Marsh, 1994).

Paramagnetic relaxation enhancement by Ni^{2+} ions and molecular oxygen

The ESR spectra of the biotinyl spin-label 8-BPESL and the corresponding phosphatidylcholine spin-label 8-PCSL in fluid-phase DMPC membranes, in the presence of avidin,

are given in Fig. 1 as a function of increasing microwave power. The ESR spectra of 8-BPESL in the absence of avidin (not shown) are closely similar to those given for 8-PCSL in the presence of avidin (see also Swamy and Marsh (1997) for low-power spectra of *n*-BPESL in the presence and absence of avidin). The anisotropy of the 8-BPESL spin-label spectra is increased by binding avidin, as found previously (Swamy and Marsh, 1997). The spectrum of 8-BPESL in the presence of avidin (*solid line*) contains only a small contribution from unbound spin label (cf. *dashed line*). From spectral subtraction using spectra recorded at subsaturating microwave power, it is found that this free biotin-PE spin label corresponds to only 10% or less of the total. The spectra in Fig. 1 are all normalized to the maximum line height in the respective spectra. Therefore, they show only the saturation broadening with increasing microwave power and do not reflect the increases in spectral intensity. The power dependences of the double-integrated intensities of the first-derivative ESR spectra of 8-BPESL and 8-PCSL are given in Fig. 2, for samples in the presence or absence of the relaxation agents aqueous Ni^{2+} ions and molecular oxygen. For 8-BPESL in the presence of avidin, aqueous Ni^{2+} ions alleviate saturation more efficiently than does molecular oxygen (Fig. 2, *top*), but for 8 PCSL (and 8-BPESL in the absence of avidin) the reverse is true (Fig. 2, *bottom*).

The saturation curves in Fig. 2 have been fitted to the H_1 dependence of the double-integrated ESR intensity that is given by Eq. 1. The resulting values of the reciprocal effective T_1T_2 relaxation time product for 8-BPESL complexed with avidin, in the absence and in the presence of aqueous Ni^{2+} ions or oxygen, are given in Table 1. Corresponding control values for 8-PCSL in DMPC with 2 mol% avidin-bound 1,2-dimyristoyl-*sn*-glycero-3-(*N*-biotinyl)-phosphoethanolamine (DMBPE) are also given in the same table. For the control, complexation of avidin with the 2 mol% DMBPE in the membrane does not change the relaxation behavior of 8-PCSL. The effective relaxation rate for the 8-PCSL control in the absence of avidin is $1/(T_1T_2)^{\text{eff}} = 12.5 \times 10^{13}\ \text{s}^{-2}$, i.e., close to that in the presence of avidin.

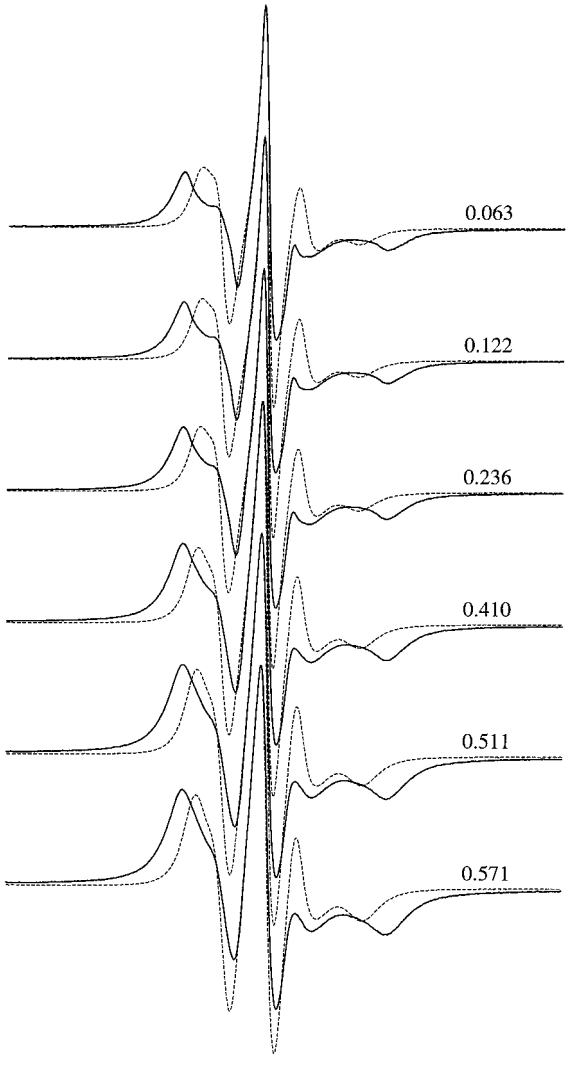

FIGURE 1 ESR spectra of 8-BPESL (——) and 8-PCSL (– – –) in DMPC membranes at 30°C, in the presence of avidin, recorded at the microwave H_1-field intensities, $\langle H_1^2 \rangle^{1/2}$ (gauss), indicated. Spectra are normalized to the maximum positive line height. Total scan range = 160 gauss.

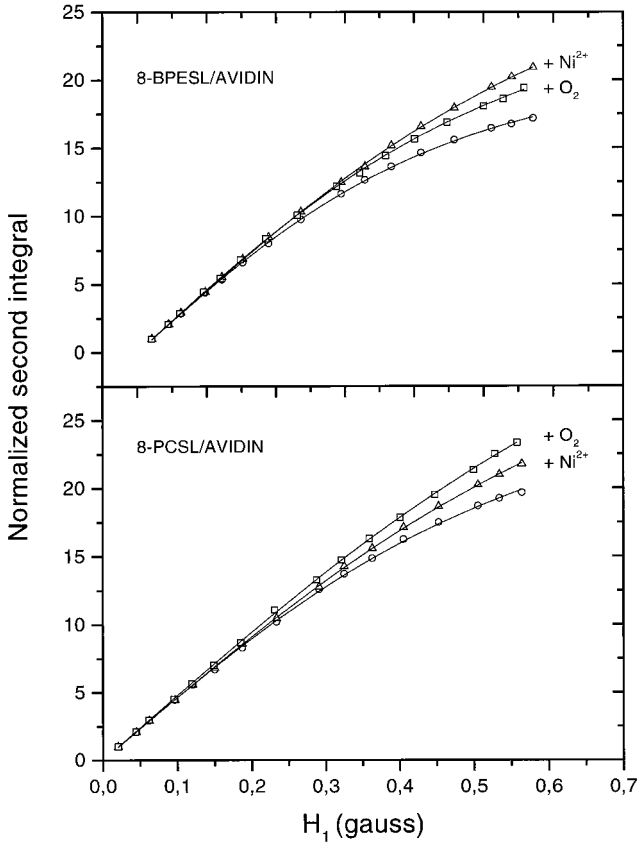

FIGURE 2 Saturation curves, as a function of H_1-field intensity, for the double-integrated intensity of the ESR spectra of 8-BPESL (*top*) and 8-PCSL (*bottom*) in DMPC membranes at 30°C in the presence of avidin. ○, Argonated samples; □, oxygenated samples; △, argonated samples in 30 mM $NiCl_2$. Curves represent nonlinear least-squares fitting to Eq. 1.

TABLE 1 Effect of paramagnetic relaxation agents on the saturation behavior of 8-BPESL complexed with avidin, in DMPC bilayers at 30°C

	$1/(T_1T_2)^{eff}$ (10^{13} s^{-2})	$\Delta[1/(T_1T_2)^{eff}]$ (10^{13} s^{-2})
8-BPESL + avidin*	7.7	—
+ 30 mM Ni^{2+}	18.2	10.5
+ sat. O_2	11.7	4.0
8-PCSL + 2% DMBPE + avidin#	12.6	—
+ 30 mM Ni^{2+}	21.1	8.5
+ sat. O_2	29.1	16.5

Values of the effective reciprocal relaxation time product, $1/(T_1T_2)^{eff}$, are given for argonated samples in the presence and absence of 30 mM $NiCl_2$, and for samples saturated with oxygen. Corresponding values are also given for 8-PCSL in bilayers of DMPC containing 2 mol% DMBPE, in the presence of excess avidin.

*In the absence of avidin $1/(T_1T_2)^{eff} = 11.7 \times 10^{13}$ s^{-2}.

#In the absence of avidin $1/(T_1T_2)^{eff} = 12.5 \times 10^{13}$ s^{-2}.

For 8-BPESL, however, complexation with avidin gives rise to a decrease in the effective relaxation rate. In the absence of avidin $1/(T_1T_2)^{eff} = 11.7 \times 10^{13}$ s^{-2} for 8-BPESL, which is close to the corresponding value for 8-PCSL, but considerably higher than that in the presence of avidin (see Table 1). This confirms that, in the absence of avidin, 8-BPESL is in an environment with dynamics similar to that for 8-PCSL. Values of the effective relaxation rate, $1/(T_1T_2)^{eff}$, have been shown both experimentally and theoretically to be sensitively dependent on the rotational correlation time, i.e., on dynamics (Livshits et al., 1998; Squier and Thomas, 1989). On binding avidin, however, the chain dynamics of 8-BPESL as monitored by the saturation behavior are changed considerably, consistent with the change in the spectral lineshape (see Fig. 1 and Swamy and Marsh, 1997).

The paramagnetic relaxation agents, Ni^{2+} ions and molecular oxygen, increase the effective relaxation rate to different extents and in a different manner for the two membrane-bound spin labels, 8-BPESL and 8-PCSL, in the presence of avidin. The values for the enhancement in effective relaxation rate, $\Delta(1/T_1T_2) = 1/(T_1T_2)^{eff} - 1/(T_1T_2)°$, where $(T_1T_2)°$ is the relaxation time product in the absence of paramagnetic agents, are given in Table 1. It is clearly seen that the relaxation enhancement of 8-BPESL by Ni^{2+} ions is greater than that by oxygen in the presence of avidin, whereas for 8-PCSL the reverse is true. The relaxation enhancements of the two spin labels cannot be compared directly because, as mentioned above, the saturation behavior of 8-BPESL is changed by binding avidin, whereas that of 8-PCSL is not. However, because interaction with 30 mM $NiCl_2$ in the aqueous phase or with molecular oxygen in the membrane phase is unlikely to change the effective T_2 relaxation time appreciably, the ratios of the relaxation enhancements by the two relaxation agents may be compared directly. The effective T_2 relaxation times cancel in the Ni^{2+}/O_2 relaxation enhancement ratio for a given spin label in a fixed system (i.e., with or without avidin). The ratios of $(T_1T_2)^{eff}$ then correspond to the ratios of the effective spin-lattice relaxation rates in the presence of Ni^{2+} and of oxygen; these are $\Delta(1/T_1)_{Ni}/\Delta(1/T_1)_{O_2} = 2.6$ for 8-BPESL and $\Delta(1/T_1)_{Ni}/\Delta(1/T_1)_{O_2} = 0.5$ for 8-PCSL, in the presence of avidin. These relaxation enhancement ratios clearly demonstrate that the spin-labeled group on 8-BPESL complexed to avidin is situated in a region closer to the polar-apolar interface of the membrane than is that of the control spin-label 8-PCSL. The spin label on 8-BPESL bound to avidin is preferentially relaxed by Ni^{2+} ions in the aqueous phase, whereas that on 8-PCSL is preferentially relaxed by molecular oxygen that is concentrated in the hydrophobic interior of the membrane (cf. Hubbell and Altenbach, 1994; Snel and Marsh, 1993).

It is of interest to compare the relaxation enhancements of 8-BPESL by Ni^{2+} ions and oxygen with those obtained recently with spin-labeled M13 bacteriophage coat protein and spin-labeled lipids in lipid bilayers (Stopar et al., 1997). To do this, the data in Table 1 were converted to effective spin-lattice relaxation rates by using measurements of the (Lorentzian) spectral linewidths. The relaxation enhancement of avidin-bound 8-BPESL by Ni^{2+} is found to be approximately two times smaller than that for a spin label next to the C-terminal of the M13 coat protein, which was located in the aqueous phase. Correspondingly, the enhancement by oxygen is two times larger than that of the aqueous-exposed spin label on the M13 coat protein. Relative to the calibrations with spin-labeled lipids in bilayer membranes, the relaxation enhancements of avidin-bound 8-BPESL by both Ni^{2+} and oxygen are intermediate between those for a spin label in the lipid headgroup region and a spin label on the 5 C position of the *sn*-2 lipid chain.

Because, in the absence of avidin, the effective relaxation rates (Table 1) and spectral lineshapes (Swamy and Marsh, 1997) of 8-BPESL and 8-PCSL are very similar, their spin-label groups must be situated at the same vertical location in the membrane, as expected for identical spin-labeled diacyl phospholipid positional isomers. Therefore, the effect of binding avidin to the biotinylated lipid 8-BPESL is to lift the bound phospholipid vertically in the membrane, relative to the average position of the host membrane phospholipids. The cause of this is the complexation of the biotin lipid headgroup in the binding pocket of avidin, which is situated in the interior of the protein molecule (see Fig. 5).

Relaxation enhancement by spin-spin interactions between spin labels

The microwave power dependences of the ESR spectra of a double-labeled DMPC sample (12-BPESL + 5-PCSL, 1:1 mol/mol) in the fluid phase, in the presence and absence of avidin, are given in Fig. 3. The fraction of total spin label that is bound by avidin, determined by spectral subtraction, is $f_B = 0.35$ for the 12-BPESL/12-PCSL pair and $f_B = 0.45$ for the 12-BPESL/8-PCSL and 12-BPESL/5-PCSL pairs. The saturation curves and fits to Eq. 1 for the 12-BPESL/12-PCSL pair (and corresponding single-labeled systems)

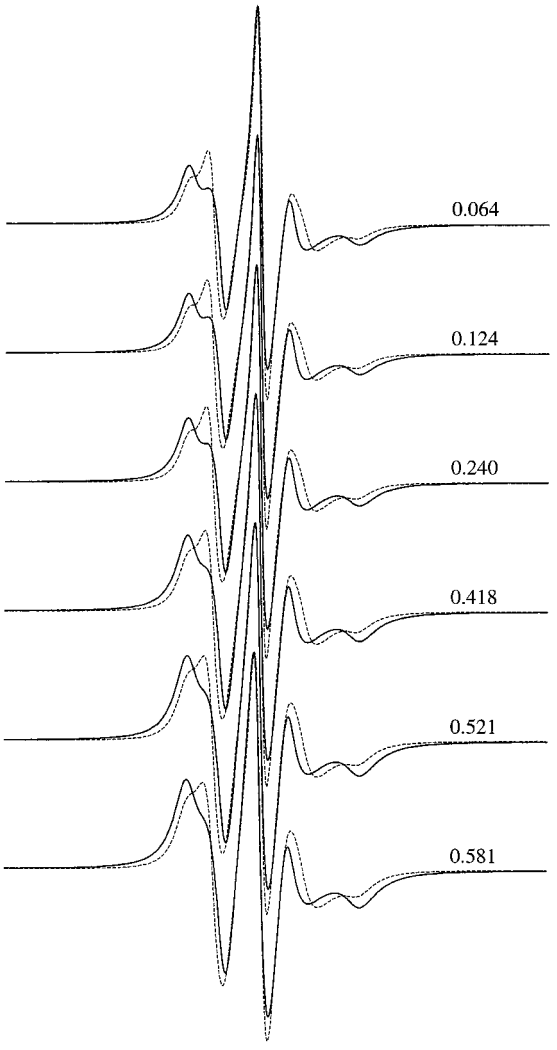

FIGURE 3 ESR spectra of 12-BPESL plus 5-PCSL (1:1 mol/mol) in DMPC membranes at 30°C, in the presence (——) and absence (– – –) of avidin, recorded at the microwave H_1-field intensities, $\langle H_1^2 \rangle^{1/2}$ (gauss), indicated. Spectra are normalized to the maximum positive line height. Total scan range = 160 gauss.

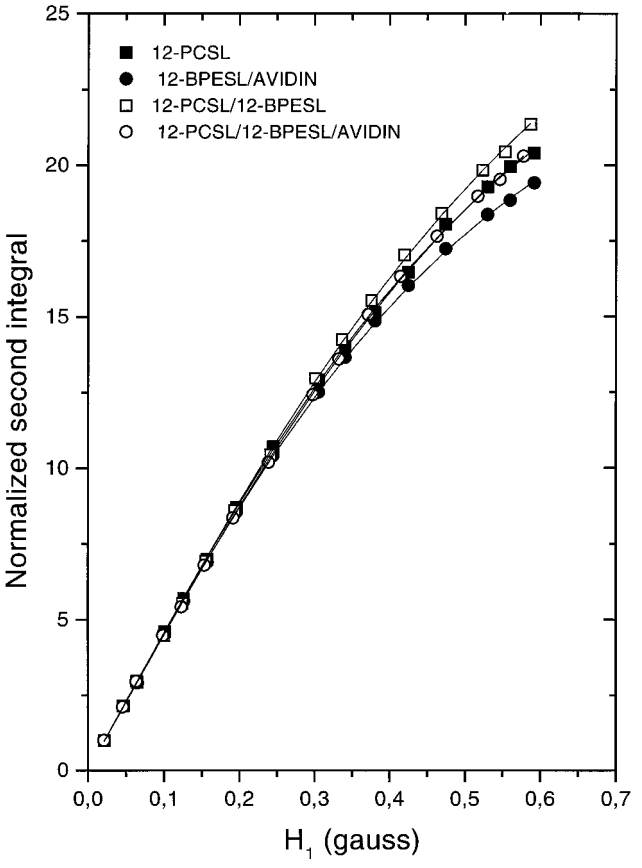

FIGURE 4 Saturation curves, as a function of H_1-field intensity, for the double-integrated ESR intensity of double-labeled samples: 12-BPESL + 12-PCSL (1:1 mol/mol) in DMPC membranes at 30°C in the presence (○) and absence (□) of avidin, and of single-labeled samples: 12-BPESL in the presence of avidin (●) and 12-PCSL alone (■). Curves represent nonlinear least-squares fits according to Eq. 1.

are given in Fig. 4. The single-component formalism given by Eq. 1 can account with reasonable precision for the H_1 dependence of the two-component spectra. This has previously been shown to be the case whenever the effective T_1T_2 products of the two components differ by less than an order of magnitude (Páli et al., 1993). It is seen from Fig. 4 that, in the absence of avidin, saturation of the 12-BPESL/12-PCSL pair is alleviated, relative to that of the single-labeled systems. Binding of avidin then increases the degree of saturation of the 12-BPESL/12-PCSL pair to the level of the single-labeled system in the absence of avidin. The situation is complicated, however, by the fact that binding avidin to 12-BPESL also increases the degree of saturation in the single-labeled system. This therefore requires deeper analysis of the effects of spin-spin interactions on the saturation behavior in the presence and absence of avidin.

The values of the effective reciprocal T_1T_2 relaxation time product resulting from fitting the saturation curves of the double-labeled systems, along with those for the corresponding single spin-labeled systems, are given in Table 2. For the 12-BPESL/12-PCSL couple in the absence of avidin, the effective value of $1/(T_1T_2)$ is higher than for the single spin-labeled systems alone. On the other hand, for the 12-BPESL/5-PCSL couple in the absence of avidin, the effective value of $1/(T_1T_2)$ is intermediate between those for the single-labeled systems. This difference corresponds to an enhancement in relaxation rate by spin-spin interactions between the lipids that are labeled in register on the 12 C position, relative to the 12 and 5 position label pair. On the addition of avidin, the effective value of $1/(T_1T_2)$ for the 12-BPESL spin label alone is reduced and becomes closer to that of the 5-PCSL label alone than to that of the 12-PCSL label alone. Simultaneously, the value of $1/(T_1T_2)_{BL}$ for the 12-BPESL/12-PCSL couple is reduced. The value of $1/(T_1T_2)_{BL}$ for the 12-BPESL/5-PCSL pair, however, is less affected by binding of avidin but comes closer to that for 12-BPESL alone. The results for the 12-BPESL/8-PCSL couple are intermediate between those of the 12-BPESL/12-PCSL and 12-BPESL/5-PCSL couples, but qualitatively resemble those of the former.

TABLE 2 Effective reciprocal relaxation time products for 12-BPESL, $1/(T_1T_2)^o_B$, for *n*-PCSLs, $1/(T_1T_2)^o_L$, and for both 12-BPESL and *n*-PCSL simultaneously, $1/(T_1T_2)_{BL}$, in DMPC bilayers at 30°C, in the presence and absence of avidin

L	$1/(T_1T_2)_{BL}$ ($10^{13} \times s^{-2}$)	$1/(T_1T_2)^o_L$ ($10^{13} \times s^{-2}$)	$1/(T_1T_2)^o_B$ ($10^{13} \times s^{-2}$)
12-BPESL			
12-PCSL	18.2	14.2	(14.2)*
8-PCSL	14.7	12.5	(14.2)*
5-PCSL	12.3	10.5	(14.2)*
12-BPESL + avidin			
12-PCSL	15.6§	14.2	11.6#
8-PCSL	12.8¶	12.6	11.6#
5-PCSL	11.1¶	10.5	11.6#

Samples were saturated with argon.

*Assumed equal to 12-PCSL alone, as found for the 8-position spin-labeled isomers (see Table 1). With samples in air, $1/(T_1T_2)^o_B = 15.0 \times 10^{13}\ s^{-2}$ for 12-BPESL, in the absence of avidin.

#>90% of 12-BPESL is bound by avidin.

§35% of total spin label is bound by avidin.

¶45% of total spin label is bound by avidin.

To assess the effects of mutual spin-spin interactions between the 12-BPESL/*n*-PCSL couples, the effective values of $1/(T_1T_2)^o_{BL}$ that are predicted from the measurements on the single-labeled systems are given in Table 3. Assuming no relaxation enhancement by mutual spin exchange, the integrated intensity of the double-spin-labeled system is given by (cf. Eq. 1)

$$S = S_o\left(\frac{f_B}{\sqrt{1 + \gamma_e^2\langle H_1^2\rangle (T_1T_2)^o_B}} + \frac{1 - f_B}{\sqrt{1 + \gamma_e^2\langle H_1^2\rangle (T_1T_2)^o_L}}\right) \tag{2}$$

where S_o is the value that S would have in the absence of saturation. This equation may be evaluated at the H_1-field corresponding to half-saturation, i.e., for $S/S_o = 1/2$ (Snel and Marsh, 1994). From Eq. 1, the latter can be defined by $\gamma_e^2\langle H_1^2\rangle = 3/(T_1T_2)^o_{BL}$. From Table 3 it can be seen that there is a large discrepancy between the measured value of $1/(T_1T_2)_{BL}$ for the 12-BPESL/12-PCSL couple in the absence of avidin and that of $1/(T_1T_2)^o_{BL}$ predicted for this system in the absence of mutual spin exchange. With the addition of avidin, the value of $1/(T_1T_2)_{BL}$ is reduced and becomes much closer to that predicted for the absence of exchange. Qualitatively similar trends are found for the 12-BPESL/8PCSL couple, although the overall differences are smaller. For the 12-BPESL/5-PCSL couple, on the other hand, the measured values of $1/(T_1T_2)_{BL}$ are close to those predicted for this system in the absence of exchange, in both the presence and absence of avidin.

TABLE 3 Effective relaxation time products $1/(T_1T_2)_{BL}$ for DMPC membranes double-labeled with 12-BPESL and *n*-PCSL spin labels, at 30°C, in the presence and absence of avidin

L	$1/(T_1T_2)_{BL}$ ($10^{13} \times s^{-2}$)	f_B	$1/(T_1T_2)^o_{BL}$ ($10^{13} \times s^{-2}$)	$T^o_{1,L}\tau_{ex}^{-1}$
12-BPESL				
12-PCSL	18.2	0.5	14.2	0.8
8-PCSL	14.7	0.45	13.3	0.2
5-PCSL	12.3	0.45	12.0	0.03
12-BPESL + avidin				
12-PCSL	15.6	0.35	14.0*	0.2
8-PCSL	12.8	0.45	12.2	0.08
5-PCSL	11.1	0.45	11.0	0.01

The values calculated by assuming no mutual exchange, $1/(T_1T_2)^o_{BL}$, are obtained from measurements on the single-labeled systems according to Eq. 2. Effective normalized mutual exchange frequencies, $T^o_{1,L}\tau_{ex}^{-1}$, are obtained from Eq. 3 for 12-BPESL/12-PCSL in the absence of avidin and from Eqs. 4–6 for the remainder. In each case, $T^o_{1,L}$ is the effective spin-lattice relaxation time of 12-PCSL in the absence of exchange. f_B is the fraction of 12-BPESL (bound by avidin).

*Corrected for spin-spin interaction between 12-PCSL and free 12-BPESL.

To make a suitably normalized comparison between the different double-labeled systems, in the presence and absence of avidin, it is necessary to extract the spin exchange frequencies, τ_{ex}^{-1}, from the effective relaxation time products that are determined in the saturation measurements (Snel and Marsh, 1994). To do this, it is assumed that the spin exchange is sufficiently weak that it affects only the T_1 relaxation time and does not change T_2 appreciably. For like spin labels, i.e., 12-BPESL and 12-PCSL in the absence of avidin, the normalized exchange frequency may be obtained directly from (Marsh, 1993)

$$\frac{1}{(T_1T_2)^{eff}_{BL}} = \frac{1}{(T_1T_2)^o_L}\cdot\frac{1 + T^o_{1,L}\tau_{ex}^{-1}}{1 + ZT^o_{1,L}\tau_{ex}^{-1}} \tag{3}$$

where $T^o_{1,L}$ is the effective spin-lattice relaxation time of 12-PCSL in the absence of exchange, and the degeneracy is $Z = 1/2$ for a simple two-site model. The corresponding value of $T^o_{1,L}\tau_{ex}^{-1}$ for the 12-BPESL/12-PCSL couple in the absence of avidin is given in Table 3. For unlike spin labels, the two-site exchange model gives rise to the following effective spin-lattice relaxation rates (Snel and Marsh, 1994):

$$\frac{1}{T^{eff}_{1,B}} = \frac{1}{T^o_{1,B}}\left[1 + \frac{(1 - f_B)T^o_{1,B}\tau_{ex}^{-1}}{1 + f_BT^o_{1,L}\tau_{ex}^{-1}}\right] \tag{4}$$

$$\frac{1}{T^{eff}_{1,L}} = \frac{1}{T^o_{1,L}}\left[1 + \frac{f_BT^o_{1,L}\tau_{ex}^{-1}}{1 + (1 - f_B)T^o_{1,B}\tau_{ex}^{-1}}\right] \tag{5}$$

where the right side may be expressed in terms of the single normalized exchange frequency $T^o_{1,L}\tau_{ex}^{-1}$, together with the ratio $T^o_{1,L}/T^o_{1,B}$, which can be obtained from the single-component saturation behavior together with linewidth measurements. The expression for the spectral intensity corresponding to Eq. 2, but in the presence of spin exchange, is then (Snel and Marsh, 1994)

$$S = S_o\left\{\frac{f_B}{[1 + \sigma^{eff}_{BL}(T_1T_2)^o_B/(T_1T_2)^{eff}_{BL}(T^{eff}_{1,B}/T^o_{1,B})]^{1/2}} + \frac{1 - f_B}{[1 + \sigma^{eff}_{BL}(T_1T_2)^o_L/(T_1T_2)^{eff}_{BL}(T^{eff}_{1,L}/T^o_{1,L})]^{1/2}}\right\} \tag{6}$$

where $\sigma_{BL}^{eff} = 3$ at half-saturation, i.e., for $S/S_o = 1/2$. Hence the exchange frequency $T_{1,L}^{o}\tau_{eff}^{-1}$ can be obtained by substituting into Eq. 6 from Eqs. 4 and 5. For all unlike spin-labeled lipid couples, the values of $T_{1,L}^{o}\tau_{ex}^{-1}$ obtained in this way are given in Table 3. (Equations 4 and 5 reduce to Eq. 3 for like spin labels, i.e., for $T_{1,B}^{o} = T_{1,L}^{o}$ and $f_B = 1/2$.)

All exchange frequencies given in Table 3 are normalized to the same value of $T_{1,L}^{o}$, that of 12-PCSL (which is unchanged on binding avidin—see Table 2). Therefore they may be compared directly. This common normalization of the different exchange frequencies was made by using ratios of the respective linewidths (cf. above).

It is seen that the spin exchange frequency between 12-PCSL and 12-BPESL is quite high ($\sim 1/T_{1,L}^{o}$) in the absence of avidin but is reduced by a factor of 4 on binding of avidin to 12-BPESL. This reduction in exchange frequency is consistent with 12-BPESL moving vertically toward the membrane surface on binding of avidin, as found already from the relaxation enhancement of 8-BPESL by aqueous Ni^{2+} ions and molecular oxygen. Measurable spin exchange occurs between 8-PCSL and 12-BPESL in the absence of avidin, but to a much lesser extent than for 12-PCSL. This limited exchange in the 12-BPESL/8-PCSL couple is reduced further by binding avidin, again suggesting that 12-BPESL moves vertically in the membrane to a region where the collision frequency with the label on 8-PCSL is less. On the other hand, however, the frequency of spin exchange between 5-PCSL and 12-BPESL is low, in both the presence and absence of avidin. In the absence of avidin this is to be expected from the difference in vertical location of the two spin labels. However, it might have been expected that the exchange frequency between 5-PCSL and avidin-bound 12-BPESL would have been higher. This is because the profile of lipid chain segmental flexibility of the *n*-BPESLs appears to be shifted by 7–8 C atoms toward the membrane surface on binding of avidin (Swamy and Marsh, 1997). Possibly this degree of motional restriction in the chain of 12-BPESL bound to avidin does not allow easy accessibility to lipid chains labeled closer to the membrane surface. Alternatively, perhaps the vertical movement of 12-BPESL is not as great, on binding of avidin, as suggested by the change in the chain flexibility profile. In the latter case, more of the restriction observed in chain motion must be attributed to a direct interaction of avidin with the biotin lipid.

CONCLUSIONS

The experiments with aqueous and apolar paramagnetic relaxants clearly demonstrate the vertical movement of *N*-biotinylphosphatidylethanolamine, relative to the host membrane phosphatidylcholine lipids, on the specific binding of avidin to the biotinyl headgroup. This conclusion is also supported by double-labeling experiments involving spin-labeled *N*-biotinylphosphatidylethanolamine and spin-labeled phosphatidylcholine in the same membrane. The vertical displacement of *N*-biotinylphosphatidylethano-

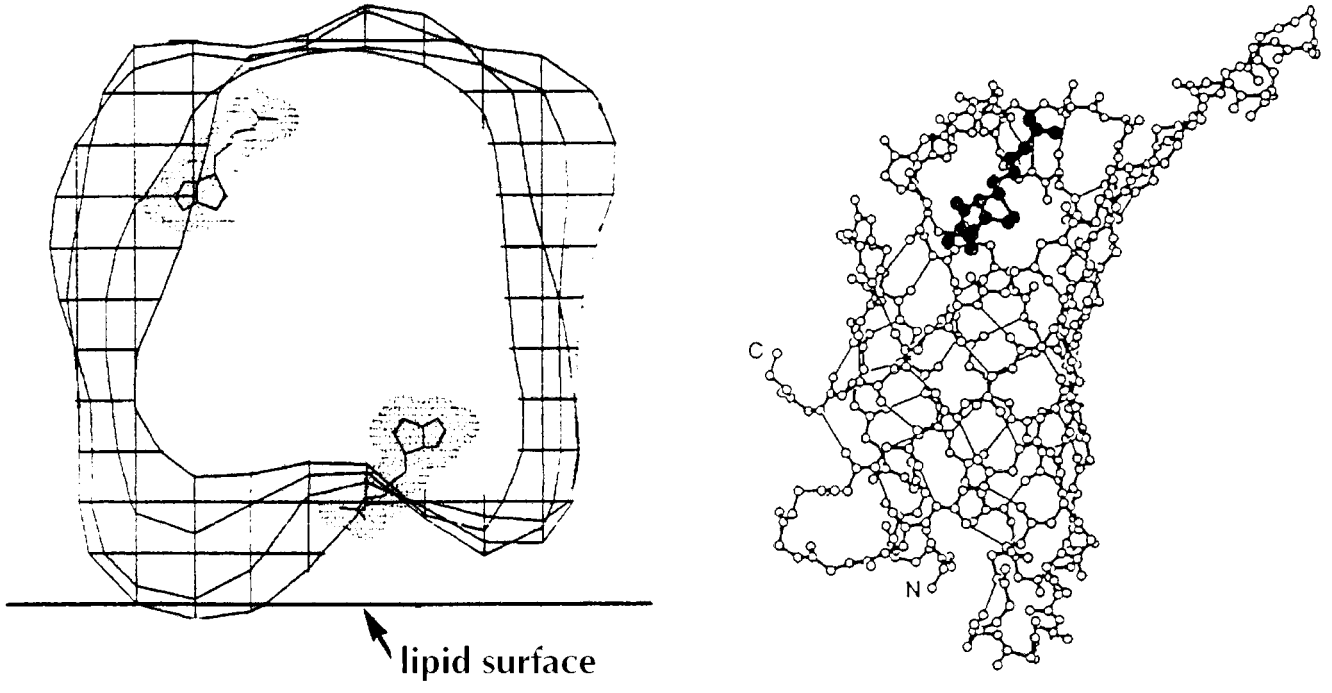

FIGURE 5 (*Left*) Approximate location of the streptavidin tetramer, relative to the lipid surface, when bound in a two-dimensional array to a layer of biotinylated lipids at the air-water interface (Darst et al., 1991). (*Right*) Backbone crystal structure of streptavidin, with bound biotin (*shaded*) (Weber et al., 1989). Reprinted with permission from Weber, P. C., D. H. Ohlendorf, J. J. Wendoloski, and F. R. Salemme. 1989. Structural origins of high-affinity biotin binding to streptavidin. Science, 243:85–88. Copyright 1989. American Association for the Advancement of Science.

lamine is required for the biotin moiety to reach the binding site within the avidin molecule (see Fig. 5). The extent of movement required is ~8 Å (Darst et al., 1991). This would locate the spin-labeled group on 12-BPESL at approximately the same level, or higher, in the membrane as that on 5-PCSL. The lack of appreciable mutual spin-spin interactions for this spin-label couple on binding avidin to 12-BPESL therefore suggests that a motional restriction, which is evidenced by the 12-BPESL ESR lineshape, reduces collision frequencies in this region of the membrane complexed with avidin. The effective relaxation rates $1/(T_1T_2)^{eff}$ show a systematic dependence on label position, n, for the n-PCSL phosphatidylcholines (Table 2). This is consistent with the expected dependence on rotational correlation time in the motional narrowing regime that is appropriate here (Livshits et al., 1998; Squier and Thomas, 1989). Comparison with the values for the biotin-PE spin labels suggests that avidin-bound 12-BPESL may be positioned between 8-PCSL and 5-PCSL and that avidin-bound 8-BPESL is located closer to the membrane surface than is 5-PCSL. Conversely, the direct demonstration of vertical lipid movement given here indicates that a large part of this restriction in chain motion is caused by the change in environment of the avidin-bound lipid, rather than by binding per se. The avidin/*N*-biotinylphosphatidylethanolamine interaction provides a model system that illustrates a vertical lipid dislocation of the type proposed for the activator proteins involved as essential cofactors in ganglioside catabolism. The spin-label relaxation experiments described in this paper provide a strategy for studying such mechanisms in other systems, including lipid interactions with phospholipase A_2.

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