Spin-Label Studies on the Anchoring and Lipid−Protein Interactions of Avidin with *N*-Biotinylphosphatidylethanolamines in Lipid Bilayer Membranes

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ABSTRACT: The specific binding of hen egg white avidin to phosphatidylcholine lipid membranes containing spin-labeled *N*-biotinylphosphatidylethanolamines (biotin-PESLs) was investigated by using ESR spectroscopy. Spin-labeled biotin-PEs were prepared with the nitroxide group at position C-5, C-8, C-10, C-12, or C-14 of the *sn*-2 chain and were incorporated at 1 mol % in lipid bilayer membranes of dimyristoylphosphatidylcholine. Binding of avidin produced a strong and selective restriction of the biotin-PE lipid mobility at all positions of chain labeling, as shown by the ESR spectra recorded in the fluid lipid phase. The spectral components of the fraction of the biotin-PESLs that were not complexed by avidin indicated that the mobility of the bulk membrane lipids was unperturbed by binding avidin, as demonstrated by difference spectroscopy. Comparison of the positional profiles and temperature dependences of the outer hyperfine splittings from the biotin-PESLs suggests that the C-12 and C-14 positions of the avidin-bound biotin-PEs are in register with the C-5 and C-7/C-6 positions, respectively, of the chains of the bulk membrane lipids. This indicates that the biotin-PEs are partially withdrawn from the membrane, with a vertical displacement of ca. 7−8 Å, on complexation with avidin. In addition, the specific lipid−protein interaction with avidin results in a selective reduction in the rates of lipid chain motion, as shown by the increased ESR line widths. These data define the way in which avidin is anchored to lipid membranes containing biotin-PEs.

Integral and peripheral proteins are bound to membranes largely by nonspecific interactions, hydrophobic in the one case and electrostatic in the other. In addition, there is a third type of membrane lipid−protein interaction that is highly specific. This involves either the selective tight binding of the protein to particular lipid head groups or the direct covalent attachment of the lipid to the protein. The first class of lipid−protein interactions of this type includes the binding of cholera toxin to membranes containing the ganglioside G_{M1} (Van Heyningen, 1983) and the interaction of lectins with glycolipids in membranes (Surolia et al., 1975; Curatolo, 1987). The second class of this type is exemplified by proteins that are anchored to the membrane by a covalent link to glycosylphosphatidylinositol, i.e. GPI anchors (Ferguson, 1991), or by an N-terminal or cysteine thioester acylated fatty acid (Towler et al., 1988). Whereas the lipid interactions with integral and peripheral proteins have been studied extensively [e.g. Marsh (1985) and Sankaram and Marsh (1993)], far less is known in detail about lipid−protein interactions of the third, highly specific type.

The binding of avidin to membranes containing phosphatidylethanolamine, the polar head group of which has been derivatized by the vitamin biotin (biotin-PE),[1] provides an extremely useful and versatile system for studying lipid−protein interactions of the highly specific type (Swamy & Marsh, 1993). Not only does avidin associate exclusively with the biotinylated lipid head group, but also the extremely high affinity of binding (Green, 1975) means that this tight association approximates rather well that of a covalently attached lipid. In addition to studies of surface recognition (Helm et al., 1991; Noppl-Simson & Needham, 1996), the avidin−biotin-PE system has become increasingly important for membrane sensor technologies (Ahlers et al., 1989; Herron et al., 1992; Zhao & Reichert, 1992) and also for cytological localization and targeting (Bayer et al., 1979) and protein structural determinations involving two-dimensional surface arrays (Kornberg & Darst, 1991; Darst et al., 1991). For all of the latter studies, the nature of the anchoring of the protein to the membrane via the specific lipid−protein interaction may be of crucial importance.

In initial studies, we have shown that spin-label electron spin resonance (ESR) spectroscopy combined with the avidin−biotin-PE system can provide a useful approach to studying lipid−protein interactions of the highly specific type. For the present work, we have synthesised biotin-PEs bearing a spin-label reporter group at five different positions throughout the length of the *sn*-2 chain in order to investigate their interaction with avidin when incorporated in membranes of the indifferent host lipid dimyristoylphosphatidylcholine. This system approximates the situation for specific lipid head group−protein interactions in natural membranes and corresponds to the relatively low levels of biotin-PE that are used in membrane applications of avidin−biotin technology. It is found that binding avidin induces a selective restriction in the mobility of the biotin-PE throughout the entire acyl chain. Comparison of the positional profile of chain mobility

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[1] Abbreviations: ESR, electron spin resonance; biotin-PE, *N*-biotinylphosphatidylethanolamine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; *n*-PESL, 1-acyl-2-[*n*-(4,4-dimethyl-*N*-oxyoxazolidinyl)stearoyl]-*sn*-glycero-3-phosphoethanolamine; *n*-PCSL, 1-acyl-2-[*n*-(4,4-dimethyl-*N*-oxyoxazolidinyl)stearoyl]-*sn*-glycero-3-phosphocholine; *n*-BPESL, 1-acyl-2-[*n*-(4,4-dimethyl-*N*-oxyoxazolidinyl)-stearoyl]-*sn*-glycero-3-(*N*-biotinyl)phosphoethanolamine; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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relative to the host lipid indicates that the specific biotin-PE lipids are partially withdrawn from the membrane by association with avidin. This unique type of lipid−protein interaction has many implications for the way in which the protein is anchored to the membrane.

MATERIALS AND METHODS

Materials. Hen egg white avidin and biotin−*N*-hydroxysuccinimide were obtained from Molecular Probes (Eugene, OR). Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Phosphatidylethanolamine spin-labels labeled at different positions, *n*, in the *sn*-2 chain of the lipid (*n*-PESLs) and the analogous spin-labeled phosphatidylcholine (*n*-PCSLs) were synthesised as described earlier (Marsh & Watts, 1982). The corresponding spin-labeled *N*-biotinylphosphatidylethanolamines (*n*-BPESLs) were synthesised from the spin-labeled PEs as described by Bayer et al. (1979).

Sample Preparation. Samples for ESR spectroscopy were prepared in the following manner. Approximately 1 mg of DMPC and 1 mol % spin-labeled *N*-biotinylphosphatidylethanolamine were codissolved in ca. 0.5 mL of dichloromethane, and the solvent was evaporated under a stream of dry nitrogen gas over a period of ca. 30 min. The residual solvent was removed by vacuum drying for at least 3 h. The lipid film was then hydrated by adding about 5 mg of avidin in 0.1 mL of 2 mM Hepes buffer (pH 7.4) containing 1 mM EDTA. After mild vortexing, the sample was incubated for approximately 1 h at 35−40 °C and then pelleted into a 1 mm inside diameter (i.d.) glass capillary. The excess supernatant was removed, and the capillary was flame-sealed and the capillary stored at 4 °C. Usually, samples were prepared on the day of the measurement and were never stored for more than 24 h before measurement.

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 ESR tube containing light silicone oil for thermal stability. Spectral data were collected digitally on a IBM personal computer using the software written by M. D. King of this institute. Spectral subtractions were performed as described previously (Marsh, 1982). The criterion for the subtraction end point is that a smoothly varying line shape is achieved for the difference spectrum, in the region of the outer peaks of the pure lipid spectrum being used to subtract. The quantitative data obtained from the difference spectrum, namely the outer hyperfine splitting ($2A_{max}$) of the avidin-bound biotinyl-PE spin-label, are insensitive to the fine details of the subtraction end point. The sensitivity of the subtraction end point is sufficient to ensure the close similarity between the spectrum being used to subtract and the spectral component being subtracted and to reveal the qualitative features of the line shape of the spectral component remaining.

RESULTS AND DISCUSSION

Spin-labeled biotin-PE was incorporated at a level of 1 mol % in bilayer membranes of dimyristoylphosphatidylcholine, a host phospholipid with which avidin does not interact. ESR spectra of the *n*-BPESL spin-labels bearing the nitroxide group at different positions in the *sn*-2 chain are given in Figure 1. The samples were at 30 °C, a temperature in the fluid phase of the DMPC membranes. For each spin-label position, *n*, the spectrum of membranes to which avidin was added (solid lines) is superimposed on that of membranes without added protein (dotted line). In the presence of avidin, the spectrum from each spin-label is a composite because it contains an additional component that corresponds to the spectrum in the absence of avidin and therefore is attributed to biotin-PE spin-labels to which avidin is not bound. This identity was confirmed by difference spectroscopy and demonstrates that the mobility of the bulk membrane lipids is unaffected by the specific binding of avidin. The difference spectra obtained by subtracting the spectra of the lipid membranes alone from the composite spectra of the membranes in the presence of avidin are given beneath the overlaid spectra for each of the *n*-BPESL spin-labels in Figure 1. These difference spectra represent biotin-PE spin-labels to which avidin is specifically bound and constitute 60−83% of the total spectral intensity. It is seen that complexation with avidin causes a large decrease in mobility of the biotin-PE for all positions of chain labeling. None of the spectra of the avidin-bound *n*-BPESL spin-labels, however, approach the rigid limit of conventional nitroxide ESR spectroscopy, and they also differ considerably between the different positions of chain labeling. Clearly, the origin of the lipid chain immobilization is quite different from that observed for the interaction of lipid chains with integral membrane proteins [see e.g. Marsh (1985)].

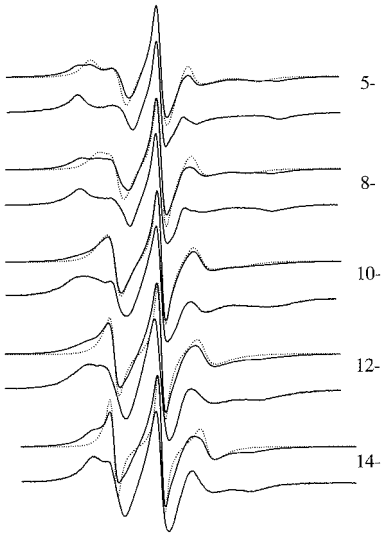

FIGURE 1: ESR spectra of biotin-PE spin-labels (1 mol %) in DMPC bilayers with and without added avidin. The position, *n*, of the spin-label in the *sn*-2 chain of the biotin-PE lipid (*n*-BPESL) is indicated on the right. In each set of three spectra, the composite spectrum recorded for the samples containing avidin (solid line) and the spectrum recorded without any added protein (dotted line) are shown overlaid on each other. The difference spectrum (rigid component) obtained by subtracting the spectrum of samples without avidin from that of samples containing avidin is shown below the overlaid spectra. The buffer is 2 mM Hepes/1 mM EDTA at pH 7.4. $T = 30$ °C. The spectral width is 100 G.

The positional profiles of the hyperfine splitting between the outer peaks ($2A_{max}$) in the ESR spectra of the different *n*-BPESL spin-labels are given for the free and avidin-bound components in Figure 2. Additional data for phospholipid spin-labels with other positions of chain labeling, for samples without added protein, are also included in the figure. The outer hyperfine splittings of the biotin-PE spin-labels with

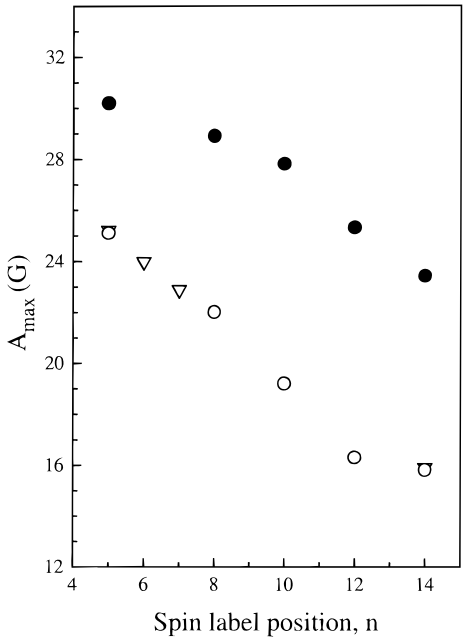

FIGURE 2: Dependence of the spectral outer hyperfine splitting ($2A_{max}$) on the spin-label position, *n*, in the *sn*-2 chain of the lipid. The values of A_{max} obtained for the rigid and fluid components of the different *n*-BPESL spin-labels in DMPC lipid bilayers in the presence of avidin are plotted: (●) rigid component in the presence of avidin, (○) fluid component (without any protein added), and (▽) *n*-PCSL labels in DMPC bilayers. $T = 30$ °C.

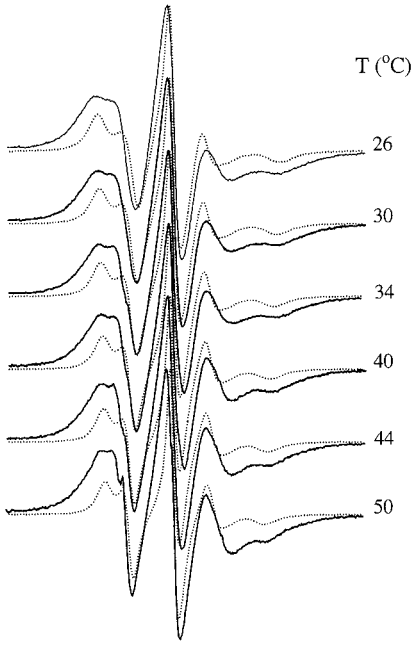

FIGURE 3: Temperature dependence of the ESR spectra of avidin-bound 12-BPESL and free 5-BPESL. ESR spectra of the rigid component obtained for the 12-BPESL spin-label in the presence of avidin (solid line) are compared with those of 5-BPESL in the absence of any added protein (dotted line). Samples were prepared as described in Materials and Methods. The temperature at which each spectrum was recorded is indicated in the figure. The buffer is 2 mM Hepes/1 mM EDTA at pH 7.4. The spectral width is 100 G.

avidin bound are consistently higher at each position of chain labeling than are those of the free biotin-PE lipids. Nevertheless, the avidin-bound *n*-BPESLs display a gradient of increasing mobility with the position, *n*, of labeling down the acyl chain that is characteristic of chain-labeled lipids in fluid phospholipid bilayers. Comparison of the chain mobility profiles in Figure 2 suggests that the profile for the avidin-bound biotin-PEs is shifted upward relative to that of the host lipid chains by approximately seven or eight C atoms. The values of A_{max} for the C-12 and C-14 chain positions of the avidin-bound lipids are approximately in register with those for the C-5 and C-6/C-7 chain positions, respectively, of the bulk membrane lipids. For the C-5, C-8, and C-10 chain positions of the avidin-bound lipids, the values of A_{max} are greater than any of those recorded for the uncomplexed bilayer lipids. This suggests that the biotin-PE lipids are partially drawn out of the lipid membrane on complexation of the biotin head group at the binding site of the avidin protein.

The data given in Figures 1 and 2 correspond to a single temperature in the fluid phase of the DMPC bilayer membranes. Similar effects are seen also at other temperatures in the fluid phase. The temperature dependence of the difference spectra corresponding to the avidin-bound 12-BPESL spin-label in DMPC bilayers is compared with that of the uncomplexed 5-PESL spin-label in Figure 3. It can be seen that the outer hyperfine splittings for these two spin-labels are comparable at the different temperatures throughout the fluid phase, in spite of the difference in spin-label position for the systems with and without avidin. The effect of binding avidin on selectively increasing the value of A_{max} for the biotin-PE spin-label is therefore preserved at higher temperatures in the fluid membrane phase. However, it will be noted that the widths of the spectral lines are increased for the 12-BPESL spin-label with bound avidin, relative to those for the uncomplexed 5-BPESL spin-label (which has comparable values of A_{max}). This indicates that the mobility of the biotin-PE is reduced below that of the uncomplexed lipids, for similar chain locations, by binding of avidin to the lipid head group. The latter represents a further, dynamic feature of the specific avidin−biotin-lipid interaction that is evident also for other label positions (see Figure 1). In addition to the differential line broadening, there is evidence for spin−spin broadening in the ESR spectra of 12-BPESL bound to avidin. This is possible, even at a relative spin-label concentration of 1 mol %, because the avidin tetramer may bind two biotin-PE molecules at one face, in which case there is a possibility of spin−spin interactions in the doubly liganded state.

The temperature dependence of the values of A_{max} in DMPC bilayers for the avidin-bound 12-BPESL spin-label is compared with those for the uncomplexed 5-BPESL and 5-PCSL spin-labels in Figure 4. A similar comparison is given for avidin-bound 14-BPESL and the uncomplexed 6-PCSL and 7-PCSL spin-labels in the same figure. It is seen that, throughout the range of 26−50 °C, not only the absolute values of A_{max} but also their temperature dependences are very similar when a biotin-PE spin-label with bound avidin is compared with an uncomplexed phospholipid spin-label that has the label group attached at a position seven or eight C atoms higher in the chain. The large differences between the outer hyperfine splittings of the same avidin-bound and uncomplexed biotin-PE spin-label are also preserved at higher temperatures, further illustrating the shift in the vertical location of the biotin-lipid in the membrane on binding avidin. The results given in Figure 4 therefore confirm that the spin-labels on positions C-12 and C-14 of the avidin-bound biotin-PE are located in a region of the

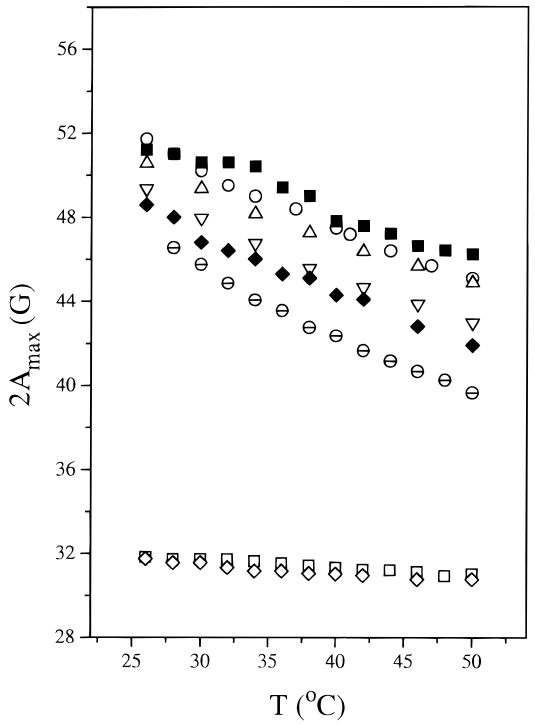

FIGURE 4: Temperature dependence of the outer hyperfine splitting ($2A_{max}$) for avidin-bound and free biotin-PE spin-labels. The values of $2A_{max}$ obtained from the ESR spectra of the avidin-bound 12-BPESL and 14-BPESL spin-labels (filled symbols) in DMPC bilayers are compared with those of the free 5-BPESL and 5-PCSL and 6-PCSL and 7-PCSL spin-labels: (■) 12-BPESL (rigid component), (□) 12-BPESL (fluid component), (◆) 14-BPESL (rigid component), (◇) 14-BPESL (fluid component), (○) 5-BPESL, (△) 5-PCSL, (▽) 6-PCSL, and (⊖) 7-PCSL. The buffer is 2 mM Hepes/1 mM EDTA at pH 7.4.

membrane similar to those on the C-5 and C-6/C-7 positions, respectively, of the host membrane lipids.

That the increase in spectral anisotropy of the *n*-BPESL spin-labels corresponds to an outward displacement of the biotin-PE from the membrane on binding avidin has been checked and confirmed by experiments on the comparative enhancements in the spin-label relaxation rate in response to aqueous Ni^{2+} ions and lipid-dissolved oxygen [cf. Altenbach and Hubbell (1988) and Páli et al. (1992)]. The $(T_1T_2)^{eff}$ effective relaxation time products of the spin-labeled lipid were determined by progressive saturation experiments as described by Páli et al. (1993). The relaxation enhancement induced by 30 mM Ni^{2+} was greater, and that induced by saturation with oxygen was smaller, for avidin-bound 8-BPESL than for control measurements on 8-PCSL in the same system. The ratio of the $1/(T_1T_2)^{eff}$ effective relaxation rate in the presence of oxygen to that in the presence of Ni^{2+} was 0.25 ± 0.02 for avidin-bound 8-BPESL compared with 1.2 ± 0.1 for 8-PCSL. This preferential relaxation enhancement by Ni^{2+} relative to that by oxygen directly documents the outward vertical displacement of the biotin-PE by binding to avidin.

The change in thickness with chain length of fluid phosphatidylcholine bilayers corresponds to an increment of 0.9−1 Å per CH_2 group per chain (Tardieu, 1972; Lewis & Engelman, 1983). The upward vertical movement in the membrane of the biotin-PE molecule that is proposed to be caused by binding avidin is therefore in the region of 7−8 Å. The binding site for biotin in avidin and streptavidin is buried in the interior of the protein; only the valeryl carboxyl group is partially accessible to solvent (Weber et al., 1989; Livnah et al., 1993). It therefore might be expected that the biotin-PE molecule must be partially withdrawn from the bilayer in order for the biotin moiety to be accommodated in the binding site of an avidin molecule situated at the membrane surface. Electron crystallographic studies on two-dimensional arrays of lipid-bound streptavidin have revealed that the carbonyl group of the bound biotin moiety is situated approximately 8 Å from the surface of the supporting lipid layer (Darst et al., 1991), consistent with the spin-label ESR results on the biotin lipid. A partial removal of the lipid acyl chains from the hydrophobic region of the membrane bilayer will be energetically unfavorable and weaken the avidin−biotin interaction relative to the binding of free biotin. The surface association constant for biotin lipids has been found to be 4−6 orders of magnitude lower than the association constant for binding with free biotin in solution (Zhao & Reichert, 1992). The hydrophobic free energy of a seven-C-atom segment of a diacyl lipid is in the region of $12RT$ (Cevc & Marsh, 1987) which corresponds to a (1.5×10^5)-fold difference in the association constant. This is, however, an upper estimate for the bound biotin-PE because the exposed chain section is unlikely to come fully into contact with water. The affinity for biotin-PE analogues with an extended linkage between the head group amine and the biotin moiety is found to be greater than that for the less accessible biotin-PE itself (Zhao & Reichert, 1992). This also is consistent with the finding that the biotin-PE must be partially withdrawn from the membrane to bind avidin, although the association constant for the biotin-lipid with the extended head group remains much smaller than that for free biotin.

CONCLUSION

The present results define the nature of the specific membrane interaction of avidin with biotinylated lipid head groups and the mode of protein anchoring in the lipid bilayer. The principal features are a partial withdrawal of the selected lipid from the membrane and an additional reduction in the lipid chain mobility resulting from binding to the lipid head group. The thermodynamic implications for other lipid−protein interactions of this type, e.g. the binding of lectins or toxins to glycolipids, will depend on the particular geometric relation between the protein binding site and the lipid head group structure which defines the extent of displacement of the bound lipid from its unperturbed membrane location. In the particular case of phospholipase A_2, the constitution of the lipid binding channel appears to have been organized to compensate energetically for the change in the hydrophobic chain environment on binding (White et al., 1990). For avidin−biotin-lipid membrane technologies, the implications for optimized design are clear and have to some extent been explored (Darst et al., 1991; Zhao & Reichert, 1992). In the more general context of the lipid anchoring of membrane proteins, the thermodynamic efficiency will again depend on the position of the attachment site and length of the linking group. The reduction in lipid mobility may also affect the lipid clustering and domain formation proposed to be involved in the sorting and targeting of certain GPI-anchored proteins, as has been discussed previously (Swamy & Marsh, 1993).

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