Interactions between Lipid-Anchored and Transmembrane Proteins. Spin-Label ESR Studies on Avidin−Biotinyl Phosphatidylethanolamine in Membrane Recombinants with Myelin Proteolipid Proteins

Musti J. Swamy,[‡,§] László I. Horváth,[||] Peter J. Brophy,[⊥] and Derek Marsh*[,‡]

Max-Planck-Institut für biophysikalische Chemie, Abt. Spektroskopie, D-37070 Göttingen, Germany, Institute of Biophysics, Biological Research Centre, H-6701 Szeged, Hungary, and Department of Preclinical Veterinary Science, Royal School of Veterinary Studies, Edinburgh EH9 1QU, U.K.

Received July 12, 1999; Revised Manuscript Received September 2, 1999

ABSTRACT: Interactions between lipid-anchored and transmembrane proteins are relevant to the intracellular membrane sorting of glycosyl phosphatidylinositol-linked proteins. We have studied the interaction of a spin-labeled biotinyl diacyl phospholipid, with and without specifically bound avidin, with the myelin proteolipid protein (or the DM-20 isoform) reconstituted in dimyristoylphosphatidylcholine. Tetrameric avidin bound to the *N*-biotinyl lipid headgroup is a surface-anchored protein, and the myelin proteolipid is an integral protein containing four transmembrane helices. The electron spin resonance (ESR) spectrum of *N*-biotinyl phosphatidylethanolamine spin-labeled at the C-14 position of the *sn*-2 chain consists of two components in fluid-phase membranes of dimyristoylphosphatidylcholine containing the proteolipid. In the absence of avidin, this is characteristic of lipid−protein interactions with integral transmembrane proteins. The more motionally restricted component represents the lipid population in direct contact with the intramembranous surface of the integral protein, and the more mobile component corresponds to the bulk fluid lipid environment of the bilayer. In the presence of avidin, the biotin-lipid chains have reduced mobility because of the binding to avidin, even in the absence of the proteolipid [Swamy, M. J., and Marsh, D. (1997) *Biochemistry 36*, 7403−7407]. In the presence of the proteolipid, the major fraction of the avidin-anchored chains is further restricted in its mobility by interaction with the transmembrane protein. At a biotin-lipid concentration of 1 mol %, approximately 80% of the avidin-linked chains are restricted in membranes with a phosphatidylcholine:proteolipid molar ratio of 37:1. This relatively high stoichiometry of interaction can be explained when allowance is made for the closest interaction distance between the lipid-anchored avidin tetramer and the transmembrane proteolipid hexamer, without any specific interaction between the two types of membrane-associated proteins. The interaction is essentially one of steric exclusion, but the lipid chains are rendered more sensitive to interaction with the integral protein by being linked to avidin, even though they are removed from the immediate intramembrane protein−lipid interface. This could have implications for the tendency of lipid-anchored chains to associate with membrane domains with reduced lipid mobility.

In polarized epithelial cells, proteins that are attached to the membrane by glycosyl phosphatidylinositol (GPI)[1] lipid anchors are almost exclusively targeted to the apical membrane (*1*). Integral proteins, on the other hand, are largely committed to a basolateral membrane location. Sorting of these two critically different types of membrane proteins takes place within the cell at the level of the trans Golgi network. Current models place emphasis on lipid composition as a controlling factor in membrane sorting (*2*). Microdomains, or "rafts" that are enriched specifically with sphingolipids and cholesterol, are proposed as the vehicles for apical membrane traffic (*3*). Nevertheless, the possible influence on the sorting process of mutual interactions between lipid-anchored and transmembrane proteins, especially at the stage of the trans Golgi, also deserves consideration.

In the work presented here, we have investigated the interactions between a lipid-anchored protein and an integral membrane protein reconstituted in membranes of an indifferent host phospholipid, phosphatidylcholine. The lipid-anchored protein is tetrameric avidin bound to the *N*-biotinyl headgroup of a long-chain diacylphosphatidylethanolamine (PE). The biotin-PE additionally bears a spin-label group on

* To whom correspondence should be addressed: Max-Planck-Institut für biophysikalische Chemie, Abt. Spektroskopie, D-37070 Göttingen, Germany. Telephone: +49-551-201 1285. Fax: +49-551-201 1501. E-mail: dmarsh@gwdg.de.

‡ Max-Planck-Institut für biophysikalische Chemie.

§ Permanent address: School of Chemistry, University of Hyderabad, General University P. O., Hyderabad 500046, India.

|| Biological Research Centre.

⊥ Royal School of Veterinary Studies.

[1] Abbreviations: GPI, glycosyl phosphatidylinositol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; PE, phosphatidylethanolamine; 14-BPESL, 1-acyl-2-[14-(4,4-dimethyloxazolidine-*N*-oxyl)-stearoyl]-*sn*-glycero-3-(*N*-biotinyl)phosphoethanolamine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PLP, myelin proteolipid protein; DM-20, isoform of PLP in which residues 116−150 have been deleted.

10.1021/bi991601+ CCC: $18.00 © 1999 American Chemical Society
Published on Web 11/10/1999

the *sn*-2 chain to allow study of the interactions with the integral protein via ESR spectroscopy. Interactions of the avidin−biotin-PE conjugate with lipid membranes alone were characterized previously by ESR methods (*4*, *5*). The extremely high affinity of avidin for biotin ensures that the protein is very firmly anchored to the PE lipid (*6*). This particular model system lacks the features that are specific to the glycosyl and inositol moieties of GPI-linked proteins. Nevertheless, it possesses the features that must be general to all protein-linked lipid chains, including, for instance, the doubly acylated tyrosine kinases of the Src family that also are thought to be associated with sphingolipid rafts (*3*). The aim here is to study the aspects of the interaction with integral proteins that are common to all types of protein-conjugated chains.

The integral proteins chosen for this study are the myelin proteolipid protein (PLP), which traverses the membrane as a four-helix bundle (*7*), and its DM-20 isoform. In the DM-20 isoform, the major polar loop of the parent proteolipid (comprising residues 116−150) is deleted, but the transmembrane portion of PLP is retained intact. Lipid−protein interactions with PLP and the DM-20 isoform have already been studied extensively in reconstituted membranes by ESR spectroscopy (*8*−*10*). Choosing the avidin−biotin-PE conjugate as the lipid-anchored partner protein is particularly advantageous because comparison can readily be made with the lipid−protein interactions of the unconjugated biotin-PE with PLP and DM-20. It is found that there is little difference between the two proteolipids in their interactions with the biotin-PE lipid. However, these lipid−protein interactions differ greatly from the interactions of either proteolipid with the lipid-anchored avidin conjugates.

MATERIALS AND METHODS

DMPC was obtained from Avanti Polar Lipids (Alabaster, AL) and avidin from Molecular Probes (Eugene, OR) or from Fluka (Buchs, Switzerland). *N*-Biotinyl phosphatidylethanolamine spin-labeled at the C-14 position of the *sn*-2 chain was synthesized as described previously (*4*, *11*). Myelin proteolipid proteins, PLP and DM-20, were purified from bovine spinal cord as described in refs *12* and *13*, respectively.

Reconstitution of the proteolipids in DMPC membranes was performed by dialysis of lipid and protein codissolved in freshly distilled 2-chloroethanol, against a buffer containing 2 mM Hepes, 0.1 M NaCl, and 1 mM EDTA (pH 7.4) (*12*). This procedure produces proteolipid-containing vesicles that are unilamellar (*12*). Reconstituted membranes were demonstrated to have a homogeneous lipid:protein ratio by centrifugation on continuous sucrose density gradients (*10*). Protein:lipid molar ratios of the PLP/DMPC and DM-20/DMPC recombinants were determined by protein (*14*) and lipid phosphate (*15*) assays. Spin-labeled biotin lipid was incorporated in reconstituted membrane dispersions, at a level of ca. 1 mol %, from a small volume of a concentrated stock solution in ethanol, followed by centrifugation and washing. Quantitatively identical results are obtained when the spin-labeled lipid is added before dialysis, indicating that spin-labeled lipid introduced by the present method is distributed throughout the entire sample (*8*). Avidin was bound to the 14-BPESL biotin lipid by adding a stoichiometric excess (5:1 w/w protein:lipid) of a protein stock solution in the same buffer.

Reconstituted, spin-labeled membranes were resuspended in 2 mM Hepes, 0.1 M NaCl, and 1 mM EDTA (pH 7.4) and packed to a height of ca. 5 mm in 1 mm inside diameter glass capillaries by using a benchtop centrifuge. Excess supernatant was removed from the membrane pellets, and the capillaries were flame sealed.

ESR spectra were recorded on a Varian Century Line 9 GHz spectrometer that was equipped with nitrogen gas-flow-temperature regulation. ESR sample capillaries were accommodated within standard 4 mm diameter quartz ESR tubes that contained light silicone oil for thermal stability. The temperature was measured with a fine-wire thermocouple placed in the silicone oil, close to the sample capillary. ESR spectra were signal-averaged on an IBM personal computer equipped with a Labmaster analogue to digital converter interface. Spectral subtraction and other spectral data analysis were performed as described in ref *16*.

RESULTS

The spin-labeled biotin-PE, 14-BPESL, was incorporated at a level of ca. 1 mol % relative to total lipid in membranes containing the myelin proteolipid proteins PLP or DM-20 which were reconstituted in DMPC host lipid at a fixed protein:lipid ratio. The PLP proteolipid preparation consists of a mixture of the parent proteolipid and the DM-20 isoform in a 2.5:1 ratio (*10*). For convenience, this mixture is simply termed PLP. ESR spectra of the reconstituted membranes in the absence of avidin reflect lipid−protein interactions between biotin-PE and the respective proteolipids (see, for example, refs *17* and *18*). In the presence of a saturating amount of avidin bound to the biotin-PE headgroup, the ESR spectra are used to investigate the interactions between the lipid-anchored avidin protein and the transmembrane proteolipid proteins.

DM-20/DMPC Membranes in the Presence and Absence of Avidin. The ESR spectra of 14-BPESL in reconstituted membranes that contain the integral protein DM-20 are given in Figure 1. Dotted lines represent the spectra from membranes in the absence of avidin, and solid lines represent the spectra from membranes in the presence of excess avidin. Spectra are shown for the samples at various temperatures above the chain-melting transition (23 °C) of the host DMPC bilayer membrane. The sharp, three-line spectral components seen in Figure 1 indicate that the membranes are in the fluid phase, at least for temperatures of $\geq$30 °C.

In the absence of avidin, the spectra in Figure 1 (dotted lines) all consist of two components. The sharp three-line spectral component corresponds to the fluid lipid bilayer regions of the reconstituted membranes. The broader spectral component that is resolved in the outer wings of the spectrum corresponds to lipids with reduced rotational mobility, which interact directly with the intramembranous surface of the integral DM-20 protein (*10*). These spectra indicate that the spin-label is incorporated normally and homogeneously within the membrane. They correspond to those characteristic of many lipid−protein systems (including natural membranes), at low labeling levels, and the sharp spectral component is that well known for fluid lipid bilayers (see, for example, refs *17* and *18*).

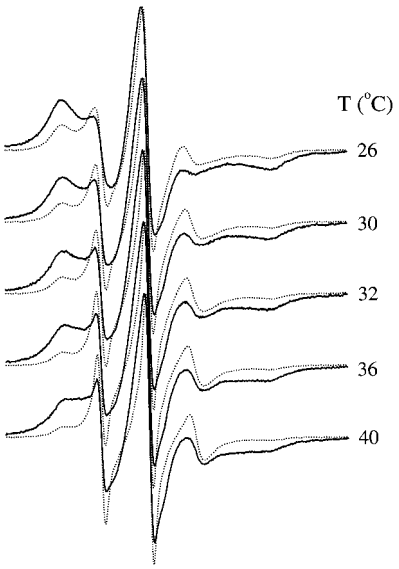

FIGURE 1: ESR spectra of the 14-BPESL biotin-PE spin-label in DM-20 recombinant membranes with DMPC at a lipid:protein molar ratio of 37:1. Solid lines are for samples in the presence of a 5:1 w/w ratio of avidin:DMPC, and dotted lines are for samples without avidin. Spectra are recorded at the indicated temperatures. The total scan width is 100 G.

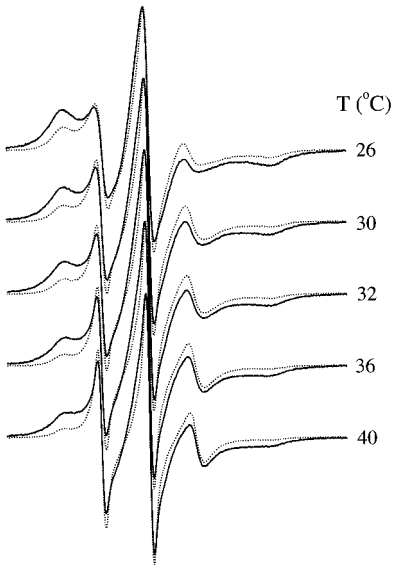

FIGURE 2: ESR spectra of the 14-BPESL biotin-PE spin-label in PLP recombinant membranes with DMPC at a lipid:protein molar ratio of 37:1. Solid lines are for samples in the presence of a 5:1 w/w ratio of avidin/DMPC, and dotted lines are for samples without avidin. Spectra are recorded at the indicated temperatures. The total scan width is 100 G.

In the presence of excess avidin, the sharp three-line spectral component is largely suppressed by the binding of avidin to the 14-BPESL headgroup (Figure 1, solid lines). This is because, even in the absence of transmembrane proteins, the mobility of the spin-labeled 14-BPESL chain is reduced considerably by binding avidin to the biotin headgroup (*4*, *6*). In addition to the minor residual sharp, three-line component, the spectra of Figure 1 (solid lines) contain two major components at higher temperatures. One of these has a large spectral splitting, which is comparable to that of the component associated with DM-20 that is observed in the absence of avidin (Figure 1, dotted lines). This therefore corresponds to avidin anchored to the biotin lipid that is interacting directly with DM-20. The other component has an intermediate spectral splitting that is poorly resolved, but is seen best in the spectra recorded at higher temperatures (Figure 1). This component corresponds to avidin-linked 14-BPESL in the fluid bilayer regions of the DM-20/DMPC membranes. It will be investigated further below by using difference spectroscopy.

PLP/DMPC Membranes in the Presence and Absence of Avidin. Corresponding ESR spectra of 14-BPESL in reconstituted membranes that contain the PLP protein are given in Figure 2. Again, dotted lines represent spectra from membranes without avidin, and solid lines represent spectra from membranes in the presence of excess avidin. For membranes without avidin, the spectra of 14-BPESL are very similar to those from DMPC membranes containing the DM-20 protein at the same lipid:protein ratio that are represented by the dotted lines in Figure 1. For PLP/DMPC membranes in the presence of excess avidin, however, the spectra differ from the corresponding ones from the DM-20/DMPC membranes in that a smaller proportion of the 14-BPESL spin-label is bound by avidin. This is indicated by a larger relative intensity of the sharp three-line spectra at higher temperatures in Figure 2 (solid lines). Multiple-difference spectroscopy is therefore required to remove the contribution from 14-BPESL not bound by avidin, prior to analysis of the interactions of the lipid-anchored avidin−14-BPESL conjugate with the transmembrane protein.

Difference Spectroscopy with the Avidin−14-BPESL Conjugate. The dotted-line spectra in Figures 1 and 2 were subtracted from the corresponding solid-line spectra recorded at the same temperature to remove the spectral contribution from 14-BPESL to which no avidin is bound. The resulting spectra of the avidin−14-BPESL conjugate in DM-20/DMPC and PLP/DMPC membranes at 36 °C are represented by the solid and dotted lines, respectively, in Figure 3A. It is seen that, after allowing for the different proportions of 14-BPESL that is not bound by avidin in the PLP and DM-20 membranes, the spectra of the avidin−14-BPESL conjugate are rather similar for membranes containing either proteolipid at the same lipid:protein ratio. The spectrum of the avidin−14-BPESL conjugate in membranes of DMPC alone at 32 °C is given in Figure 3B. This again is a difference spectrum from which the contribution of 14-BPESL not bound by avidin is removed (*6*). (A slightly lower temperature is chosen for the spectrum of Figure 3B to give best matching with the more mobile component in the spectrum of Figure 3A. This allows for a small perturbation of the latter by the presence of PLP/DM-20.) Subtracting the spectrum of Figure 3B from the spectrum of Figure 3A yields the spectra given in Figure 3C. These difference spectra correspond to the avidin-linked lipids that are interacting with the transmembrane protein, PLP or DM-20, respectively.

Data derived from difference spectroscopy for the avidin−14-BPESL conjugate, and correspondingly for unconjugated 14-BPESL, both in DM-20/DMPC membranes and in PLP/DMPC membranes are given in Table 1. Control values for spin-labeled phosphatidylcholine, 14-PCSL, are included in the same table. The values reported are for the fraction, *f*, of the total spin-label population that is interacting directly with the transmembrane protein, and the outer hyperfine splitting constant, A_{max}, of this motionally restricted population. The

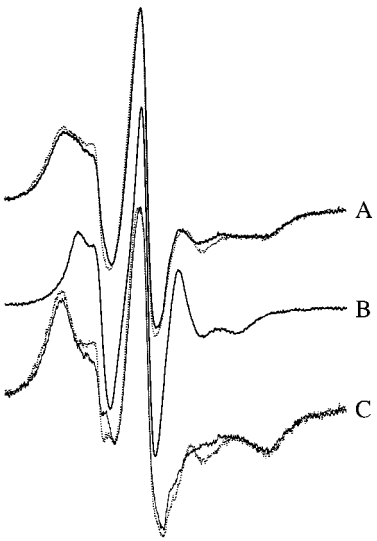

FIGURE 3: ESR difference spectra for the lipid-anchored avidin−14-BPESL conjugate in reconstituted DMPC membranes containing PLP or DM-20 (lipid:protein molar ratio of 37:1) at 36 °C. (A) Difference spectra obtained by subtracting the spectrum of 14-BPESL in the absence of avidin from that in the presence of avidin, for PLP/DMPC (dotted line) and DM-20/DMPC (solid line). (B) Spectrum of the avidin−14-BPESL conjugate in membranes of DMPC alone (the spectral component from 14-BPESL with no avidin bound is already subtracted). (C) Difference spectra obtained by subtracting spectrum B from spectrum A. See the text for details. The total scan width is 100 G.

Table 1: Fraction, *f*, of Spin-Labeled Lipid Chains (14-BPESL or 14-PCSL) That Is Motionally Restricted by Interacting with PLP or DM-20 in DMPC Membranes, in the Presence and Absence of Excess Avidin, and the Outer Hyperfine Splitting Constants, A_{max}, of This Motionally Restricted Lipid Component[a]

		f		A_{max} (G)	
protein	spin-label	without avidin	with avidin	without avidin	with avidin
PLP	14-BPESL	0.51	0.80	30.7	30.2
	14-PCSL	0.29	—[b]	30.6	—[b]
DM-20	14-BPESL	0.57	0.79	30.5	30.1
	14-PCSL	0.30	—[b]	30.3	—[b]

[a] PLP/DMPC or DM-20/DMPC molar ratio of 1:37; $T = 36$ °C. [b] Avidin has no effect on 14-PCSL in fluid DMPC membranes (*6*).

temperature dependences of A_{max} for the avidin−14-BPESL conjugate interacting with the DM-20 or PLP protein are given in Figure 4. They are compared with corresponding data for the 14-BPESL lipid alone in DM-20/DMPC or PLP/DMPC recombinants. It is seen that, although the outer hyperfine splittings of the avidin−14-BPESL conjugate have sizes comparable to those of 14-BPESL without avidin, the values of A_{max} for avidin−14-BPESL display a much steeper temperature dependence than for 14-BPESL alone with PLP or DM-20. This demonstrates that the lipid chains of the avidin−14-BPESL conjugate have a considerably higher mobility (in the slow-motion regime of conventional spin-label ESR spectroscopy) when interacting with the integral proteolipids than do those of the unconjugated 14-BPESL phospholipid.

DISCUSSION

The experiments described here are designed to characterize the interactions between the lipid-anchored protein and

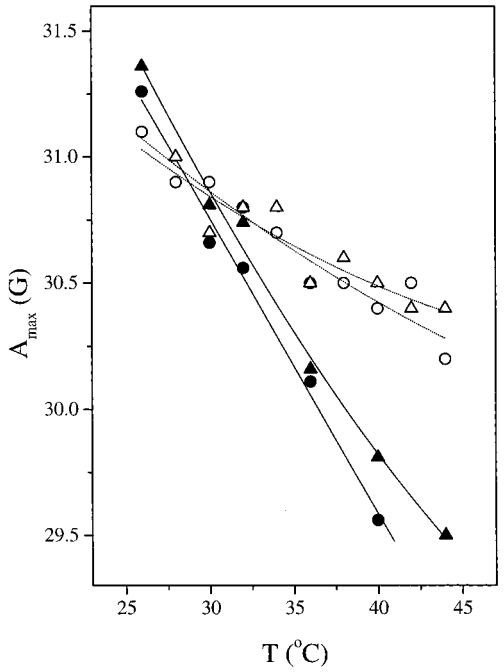

FIGURE 4: Temperature dependence of the outer hyperfine splitting constant, A_{max}, for the motionally restricted 14-BPESL lipid population in PLP/DMPC (circles) or DM-20/DMPC (triangles) recombinants, in the presence (black symbols) and absence (white symbols) of avidin.

two related transmembrane proteins by using ESR methods developed previously for studying lipid interactions with integral membrane proteins (see, for example, refs *17* and *18*). It is appropriate, therefore, first to discuss the lipid−protein interactions with unconjugated biotin-PE, before proceeding to consideration of lipid-anchored proteins.

Lipid−Protein Interactions. The fraction, *f*, of spin-labeled lipid that is motionally restricted at one of the N_b lipid sites at the intramembranous surface of the protein is given by the equation for equilibrium exchange association (*17*, *19*):

$$f = N_b K_r/[n_t - N_b(1 - K_r)] \quad (1)$$

where K_r is the association constant of the spin-labeled lipid relative to the host lipid (DMPC) and n_t is the total lipid:protein molar ratio. For 14-PCSL in proteolipid/DMPC membranes, $K_r \approx 1$, which is also true for other systems reconstituted in DMPC. The data for 14-PCSL in Table 1 therefore yield a value for N_b of ≈11 perimeter lipid sites per proteolipid monomer, in agreement with previous studies (*8*). This relatively low lipid:protein stoichiometry was shown previously to be consistent with hexamer formation of the myelin proteolipid, for which there is independent experimental evidence. The relatively low values of *f* for 14-PCSL further indicate that the protein:lipid ratio of the reconstituted membranes is not particularly high with regard to nonspecific lipid−protein interactions.

For 14-BPESL, the values of *f* given in Table 1 are greater than those for 14-PCSL. This reflects a selectivity of biotin-PE for interaction with the myelin proteolipids, and corresponds to association constants (K_r) relative to phosphatidylcholine of ≈3.1 for DM-20 and ≈2.5 for PLP. Biotin-PEs are negatively charged phospholipids because the coupling of biotin is by amide formation with the ethanolamine moiety of the PE headgroup. The selectivity of interaction with

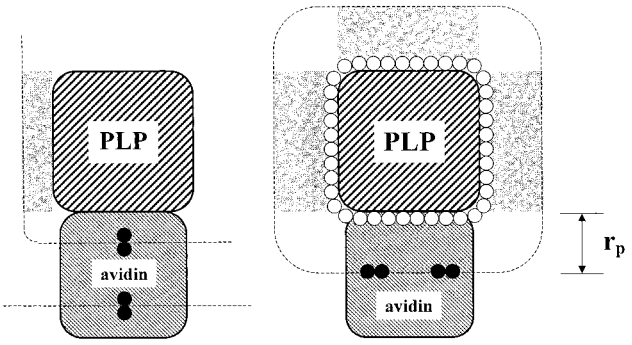

FIGURE 5: Schematic indication of the steric exclusion between the myelin proteolipid protein hexamer (PLP) and lipid-linked avidin tetramer. (Right) The avidin-linked chains (●) can approach only to within a distance r_p of the PLP perimeter. The number of lipids, per PLP monomer, that can be accommodated at this distance is estimated from eq 2 to be 15 (N_b), as compared with 10 sites for freely diffusible lipids at the PLP perimeter (○). The shaded areas correspond schematically to regions of lipid that are inaccessible to the avidin-linked chains. Minimally, these correspond to 17 (4 × 25/6) per PLP monomer. (Left) Results quantitatively identical to those deduced for the right configuration are obtained on taking the mean of the values for the two separate avidin-linked chain locations.

biotin-PEs is therefore in line with the selectivities of interaction with other negatively charged lipids that have been characterized previously (*8*). However, the comparable strength of selectivity for the DM-20 isoform relative to that for PLP is unlike the situation for phospholipids previously studied (*10*). In the latter case, the selectivity for DM-20 was strongly reduced relative to that for PLP. This suggests that the *N*-biotinyl moiety itself may contribute partly to the selectivity of interaction of biotin-PEs with the myelin proteolipids.

Lipid Anchor−Transmembrane Protein Interactions. Although the values of *f* for the 14-BPESL lipid are larger than those for 14-PCSL, they are exceeded by far by those for the avidin−14-BPESL conjugate. Because the cross-sectional dimensions of the avidin tetramer approach those of the myelin proteolipid hexamer, the interaction perimeter is considerably enlarged relative to that for simple lipid chains. A generalization of previous geometric models relating the number of perimeter lipids per monomer, N_b, to the number, n_α, of transmembrane α-helices and degree of oligomerization, n_{agg}, of the integral protein is for a helical sandwich structure (*20*, *21*):

$$N_b = \frac{\pi}{n_{agg}} \frac{(D_\alpha + 2r_p)}{d_{ch}} + \frac{n_\alpha D_\alpha}{d_{ch}} \qquad (2)$$

where D_α and d_{ch} are the diameters of an α-helix (1.0 nm) and a lipid chain (0.48 nm), respectively, and r_p is the distance from the intramembranous surface of the integral protein at which the perimeter is measured (cf. Figure 5). For conventional lipid−protein interactions, $r_p = d_{ch}/2$, and a value for N_b of 10 lipids per monomer is obtained for the myelin proteolipid hexamer ($n_{agg} = 6$), as opposed to an N_b of 18 for the monomeric four-helix bundle ($n_{agg} = 1$). The model is therefore in agreement with the experimental measurements using 14-PCSL that were discussed above. For the avidin−14-BPESL biotin−lipid conjugate, the interaction distance $r_p = 2.5$ nm (*22*), and hence, the perimeter length (N_b) is increased to 15 lipids per myelin proteolipid monomer, for the hexameric configuration of the latter.

In itself, this increase in perimeter is insufficient to account for the whole of the increase in population of motionally restricted avidin−14-BPESL lipid chains on interaction with the transmembrane proteolipid. However, another effect comes into play as a result of the size of the tetrameric avidin complex. Lipids within the interaction perimeter are not accessible to the spin-labeled chains of the avidin−14-BPESL conjugate, and thus, the effective DMPC:proteolipid ratio is reduced for interaction with the avidin-bound biotin-lipid. These further geometric factors must therefore also be taken into account.

Geometric Considerations. The surface area occupied by an avidin tetramer is approximately 30 nm^2 (*22*) and that by a DMPC molecule in the fluid phase is ∼0.6 nm^2 (see, for example, ref *23*). This corresponds to a surface coverage of 50 DMPC molecules by an avidin tetramer. The relative concentration of specific biotin-PE lipid is one per 100 DMPC molecules. Because the avidin tetramer has two biotin binding sites on one surface, the lipid-anchored avidin molecules occupy overall approximately 25% of the total lipid membrane surface. This degree of coverage is sufficiently small that the lipid-anchored avidin is relatively free to interact, or otherwise, with the transmembrane proteolipid. There is no overwhelming steric constraint that forces all lipid-anchored avidin tetramers to be in contact with the proteolipid hexamers.

The number of lipid-anchored avidin tetramers that can be accommodated at the perimeter of a proteolipid hexamer is minimally four, if contact between the two proteins is to be optimized (cf. Figure 5). The proteolipid hexamer will have dimensions in the region of 4 nm × 6 nm (see ref *20*) that are comparable to the mean cross-sectional diameter of tetrameric avidin, which is 5.1 nm (*22*). Because approximately half of the area under an avidin tetramer at the proteolipid perimeter is inaccessible to the 14-BPESL lipid chains, the number of inaccessible DMPC lipids corresponds to ∼17 per proteolipid monomer (see Figure 5). The effective lipid:protein ratio for the interaction of the avidin−14-BPESL conjugate with the transmembrane proteolipid is therefore approximately 20 mol/mol of proteolipid monomer, and correspondingly, the fraction of lipids at the interaction perimeter relative to the bulk is 0.75 for an N_b of 15. This fraction is close to the values of *f* determined for the avidin−14-BPESL conjugate (see Table 1). It therefore can be concluded that, after the geometric extent of the lipid-anchored avidin is taken into account, it displays little selective interaction with the transmembrane proteolipid.

Nature of the Lipid Anchor−Transmembrane Protein Interaction. Considerations about the relative size of the avidin tetramer and the myelin proteolipid hexamer, which were given above, suggest that the interactions of a freely diffusing lipid with the transmembrane protein will differ from those of the avidin-anchored lipid chains. Nevertheless, the ESR spectra of the avidin−14-BPESL conjugate in proteolipid-containing membranes clearly contain a major component that corresponds to avidin-anchored chains whose mobility is markedly reduced relative to that in lipid membranes without transmembrane protein (see Figures 1 and 3). The outer hyperfine splitting constants, A_{max}, of this

spin-labeled lipid population are somewhat lower than those for the nonanchored 14-BPESL chains, as is seen from Table 1 and the temperature dependences that are given in Figure 4. The significance of this difference becomes apparent when it is considered that the 14-BPESL chains are already partially restricted in their mobility by anchoring to avidin, even in the absence of transmembrane proteins (refs *4* and *6*, and see also Figure 3). The increase in outer hyperfine splitting constants resulting from interaction with the proteolipid (ΔA_{max}) is ~6 G for the avidin−14-BPESL conjugate, as opposed to ~14 G for free 14-BPESL. It is therefore possible that the restricted mobility of the avidin-anchored chains in the 14-BPESL conjugate renders their spectra more sensitive to interactions with transmembrane proteins, even if they are somewhat removed from the innermost protein−lipid interface. This is a common feature of spin-label spectra that are approaching the slow-motional regime of conventional nitroxide ESR spectroscopy (see, for example, refs *18* and *24*).

It should be noted, however, that the spectra of the spin-labeled lipid chains that are not anchored to avidin are also in a spectral region in which they potentially are sensitive to more distant interactions with the transmembrane protein. Although the spectral changes might be qualitatively different, such interactions would be reflected by changes in the line shape of the fluid lipid component as a function of the lipid:protein ratio. Studies of this type have been performed previously with reconstituted proteolipid/DMPC membranes and spin-labeled phospholipids that are not protein-conjugated. It was concluded that the proteolipid-dependent perturbation of the fluid lipid line shapes could be ascribed completely to the slow exchange of lipids that are removed from the protein with those in the first shell immediately surrounding the proteolipid (*25*, *26*). This is not the case here for the avidin-linked chains, as even the line shape changes are qualitatively different from those expected for an exchange process. Therefore, it is concluded that not only do the spectra of the avidin-linked chains exhibit enhanced sensitivity to interaction with the integral protein but also the chains themselves are inherently more sensitive to the lipid−protein interaction by virtue of their conjugation to the avidin protein.

The relative temperature dependences of the outer hyperfine splittings, A_{max}, that are given in Figure 4 clearly indicate that the chain mobility is higher for biotin-lipids attached to avidin that is interacting with the proteolipid than is that for biotin-lipids located directly at the intramembranous perimeter of the integral protein. In the latter case, the temperature dependence of A_{max} is relatively small because the chain mobility lies well into the slow-motional regime of conventional spin-label ESR spectroscopy (see also ref *27*). For biotin-lipids conjugated to avidin in association with the proteolipid, the chain mobility is much more responsive to changes in temperature and therefore lies closer to the fast-motional regime of spin-label ESR. This is because, in this case, the biotin-lipid chains do not contact the integral protein directly.

Comparison with Other Work. It is of interest to contrast the results presented here on a lipid-anchored protein with previous studies of the interaction of myelin proteolipids with a classical peripheral membrane protein, in double-reconstituted systems (*28*). In the latter work, it was found that the interaction of the peripheral myelin basic protein with the integral myelin proteolipid protein is primarily one of steric exclusion. This is not unlike the model proposed here with respect to the interaction distance of the avidin−lipid complex with the proteolipid. However, binding of myelin basic protein to PLP recombinant membranes with negatively charged lipids reduced the fraction of freely diffusible lipids interacting with the integral protein. This is in contrast to the increased fraction of avidin-bound lipid chains that are found here to be motionally restricted on interaction with the proteolipids. The explanation for this given above is based on the different locations of the lipid chains, in the freely diffusible and protein-anchored situations.

CONCLUSIONS

The lipid-anchored protein studied here differs in detail from GPI-linked proteins, but nevertheless should exhibit all features that are general to lipid-linked proteins as a whole. In addition, there is evidence that not only GPI-linked proteins but also the fatty acid-acylated Src family kinases are sorted to an apical destination in polarized cells (*3*). This suggests that features common to the entire class of lipid-linked proteins are significant in the sorting process.

The model proposed for interpreting the results on lipid−protein interactions is essentially one of steric exclusion between the proteolipid and the avidin conjugate. This, combined with the enhanced sensitivity of the avidin-linked spin-labeled chains to lipid−protein interaction, is sufficient to explain the stoichiometry of lipid−protein interaction with the proteolipid. In this sense, the results are consistent with those obtained previously on interactions between the proteolipid and the peripheral myelin basic protein (*28*). The steric exclusion seen with the basic protein is supposed to arise from repulsive interactions with the polar extramembranous section of the proteolipid. If the interpretation of the results presented here can be generalized, lipid-linked proteins do not possess an intrinsic tendency per se to associate with integral proteins that results from their lipid anchor. This does not exclude, of course, the possibility of specific protein−protein recognition (e.g., G-proteins and their receptors), but this is not a general property of the lipid-anchored class of proteins. To this extent, the findings presented here on the interactions between lipid-linked and integral proteins are fully consistent with the paradigm for membrane protein sorting in polarized epithelial cells. Generally, most integral proteins are destined for the basolateral membrane and are proposed not to enter sphingolipid rafts.

Despite the similarities in interpretation of the interactions of the lipid-linked avidin and of the peripheral basic protein with the integral proteolipids, the effect on lipid−protein interactions differs considerably in the two cases. The features of the lipid−protein interaction that are peculiar to the protein-conjugated lipid chains shed further light on the nature of the interaction of lipid-linked proteins with membranes that possibly is also relevant to the sorting process. Previous studies have shown that conjugation to avidin leads to a considerable decrease in the mobility of the biotin-lipid chains (*4*, *6*). The results presented here indicate that this renders the protein-linked chains much more

susceptible to interactions and perturbations within the lipid matrix, as compared with free lipid chains situated a corresponding distance from the integral protein. In comparison with lipid chains that are not anchored, the presence of the integral protein is felt rather strongly, in terms of chain immobilization, by the avidin-linked chains even though they are removed from direct contact with the membrane sector of the proteolipid. Correspondingly, it might be envisaged that a similar potentiation of the interaction with membrane lipids, e.g., sphingolipids and cholesterol, may take place. This could be a mechanism for the association of lipid-anchored proteins with the putative sphingolipid rafts, the lipids of which are thought to be in a liquid-ordered state (*2*). The loss of chain entropy on entering a liquid-ordered phase would be smaller for the protein-linked chains than for more disordered free lipids, which would energetically favor the preferential association of the lipid-linked proteins. The analogue in this work is the increase in chain restriction on lipid−protein interaction that is characterized by a ΔA_{max} of ∼6 G for the avidin-linked chains, as opposed to a ΔA_{max} of ∼14 G for the free lipid chains.

ACKNOWLEDGMENT

We gratefully thank Frau B. Angerstein for spin-label synthesis and Mrs. S. Chatterjee for preparation of proteolipids.

REFERENCES

1. Lisanti, M. P., and Rodriguez-Boulan, E. (1990) *Trends Biochem. Sci. 15*, 113−118.
2. Brown, D. A., and London, E. (1998) *Annu. Rev. Cell Dev. Biol. 14*, 111−116.
3. Simons, K., and Ikonen, E. (1997) *Nature 387*, 569−572.
4. Swamy, M. J., and Marsh, D. (1997) *Biochemistry 36*, 7403−7407.
5. Arora, A., and Marsh, D. (1998) *Biophys. J. 75*, 2915−2922.
6. Swamy, M. J., and Marsh, D. (1993) *FEBS Lett. 324*, 56−58.
7. Weimbs, T., and Stoffel, W. (1992) *Biochemistry 31*, 12289−12296.
8. Brophy, P. J., Horváth, L. I., and Marsh, D. (1984) *Biochemistry 23*, 860−865.
9. Horváth, L. I., Brophy, P. J., and Marsh, D. (1988) *Biochemistry 27*, 5296−5304.
10. Horváth, L. I., Brophy, P. J., and Marsh, D. (1990) *Biochemistry 29*, 2635−2638.
11. Swamy, M. J., Angerstein, B., and Marsh, D. (1994) *Biophys. J. 66*, 31−39.
12. Brophy, P. J. (1977) *FEBS Lett. 84*, 92−95.
13. Trifilieff, E., Skalidis, G., Helynck, G., Lepage, P., Sorokine, O., van Dorsselaer, A., and Luu, B. (1985) *C. R. Acad. Sci., Ser. III 300*, 241−246.
14. Lowry, O. H., Rosebrough, N. J., Farr, L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
15. Eibl, H., and Lands, W. E. M. (1969) *Anal. Biochem. 30*, 51−57.
16. Marsh, D. (1982) in *Techniques in Lipid and Membrane Biochemistry* (Metcalfe, J. C., and Hesketh, T. R., Eds.) Vol. B4/II, pp B426-1−B426-44, Elsevier, Amsterdam.
17. Marsh, D. (1985) in *Progress in Protein−Lipid Interactions* (Watts, A., and de Pont, J. J. H. H. M., Eds.) Vol. 1, pp 143−172, Elsevier, Amsterdam.
18. Marsh, D., and Horváth, L. I. (1998) *Biochim. Biophys. Acta 1376*, 267−296.
19. Brotherus, J. R., Griffith, O. H., Brotherus, M. O., Jost, P. C., Silvius, J. R., and Hokin, L. E. (1981) *Biochemistry 20*, 5261−5267.
20. Marsh, D. (1993) in *New Comprehensive Biochemistry, Vol. 25. Protein−Lipid Interactions* (Watts, A., Ed.) pp 41−66, Elsevier, Amsterdam.
21. Marsh, D. (1997) *Eur. Biophys. J. 26*, 203−208.
22. Hendrickson, W. A., Pahler, A., Smith, J. L., Satow, Y., Merritt, E. A., and Phizackerley, R. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2190−2194.
23. Marsh, D. (1990) *Handbook of Lipid Bilayers*, pp 387, CRC Press, Boca Raton, FL.
24. Freed, J. H. (1976) in *Spin Labeling. Theory and Applications* (Berliner, L. J., Ed.) pp 53−132, Academic Press, New York.
25. Horváth, L. I., Brophy, P. J., and Marsh, D. (1988) *Biochemistry 27*, 46−52.
26. Horváth, L. I., Brophy, P. J., and Marsh, D. (1994) *J. Magn. Reson., Ser. B 105*, 120−128.
27. Pates, R. D., and Marsh, D. (1987) *Biochemistry 26*, 29−39.
28. Sankaram, M. B., Brophy, P. J., and Marsh, D. (1991) *Biochemistry 30*, 5866−5873.

BI991601+