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Spin-label electron paramagnetic resonance studies on the interaction of avidin with dimyristoyl-phosphatidylglycerol membranes

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Abstract

The interaction of avidin – a basic protein from hen egg-white – with dimyristoyl-phosphatidylglycerol membranes was investigated by spin-label electron paramagnetic resonance spectroscopy. Phosphatidylcholines, bearing the nitroxide spin label at different positions along the *sn*-2 acyl chain of the lipid were used to investigate the effect of protein binding on the lipid chain-melting phase transition and acyl chain dynamics. Binding of the protein at saturating levels results in abolition of the chain-melting phase transition of the lipid and accompanying perturbation of the lipid acyl chain mobility. In the fluid phase region, the outer hyperfine splitting increases for all phosphatidylcholine spin-label positional isomers, indicating that the chain mobility is decreased by binding avidin. However, there was no evidence for direct interaction of the protein with the lipid acyl chains, clearly indicating that the protein does not penetrate the hydrophobic interior of the membrane. Selectivity experiments with different spin-labelled lipid probes indicate that avidin exhibits a preference for negatively charged lipid species, although all spin-labelled lipid species indirectly sense the protein binding. The interaction with negatively charged lipids is relevant to the use of avidin in applications such as the ultrastructural localization of biotinylated lipids in histochemical studies.

Keywords: 1,2-Dimyristoyl-*sn*-glycero-3-phosphoglycerol; Lipid bilayer; Chain mobility; Spin label; Electron paramagnetic resonance; Peripheral protein; Avidin

1. Introduction

The egg-white protein, avidin, has attracted much attention in view of its ability to recognize the vitamin, biotin, with high affinity ($K_a \approx 10^{15}$ M^{-1}; see [1]). In view of this high affinity, the avidin–biotin

Abbreviations: EPR, electron paramagnetic resonance; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; *n*-SASL, *n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; *n*-PCSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphocholine; 5-PESL, -PGSL, -PSSL, -PASL, 1-acyl-2-[5-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphoethanolamine, -phosphoglycerol, -phosphoserine, -phosphatidic acid; 5-DGSL, 1-acyl-2-[*n*-(4,4′-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycerol; 5-NAPESL, 1,2-dipalmitoyl-*sn*-glycero-3-[*N*-5-(4,4-dimethyloxazolidine-*N*′-oxyl)stearoyl]-phosphoethanolamine; 5-CLSL, 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-(5-(4,4′-dimethyloxazolidine-*N*-oxyl)stearoyl)-*sn*-glycero(3)phospho]-*sn*-glycerol; HBS, 10 mM HEPES buffer containing 1 mM EDTA, pH 7.4; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid

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system is used extensively for various applications in biology and medicine (for reviews, see [2,3]). In several applications, biotinylated lipids are used in which the biotin moiety is attached to the headgroup of phosphatidylethanolamine or phosphatidylserine. These are then identified by their interaction with avidin that is suitably derivatized for visualization. Localization of liposomes in cell–liposome interactions, non-covalent attachment of antibodies to cells, and targeting of drug-loaded liposomes to tumor cells are some examples where the interaction of avidin with biotinylated phospholipids constitutes the key step [4–7].

The main principle underlying such applications is the extremely strong and highly specific, non-covalent interaction between avidin and the biotin moiety covalently attached to the lipid headgroup. However, avidin is a strongly basic protein with an isoelectric point of p$I \approx 10.5$. Therefore it is expected to interact non-specifically with negatively charged lipids [8], in addition to its specific interaction with any biotinylated species present. Consequently, it is of interest to study the interaction of avidin with negatively charged lipid membranes. This not only is relevant to the application of avidin–biotin conjugates in ultrastructural localization and related studies, but also serves as a model for the interaction of peripheral proteins with membranes. In the latter connection, comparative studies may be particularly of interest because the secondary structure of avidin differs from many peripheral proteins in belonging to the class of β-barrel proteins. Also avidin is a stable tetramer, whereas many classical peripheral proteins are monomeric.

In the present work, we have studied the binding of avidin to negatively charged dimyristoyl-phosphatidylglycerol (DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol) membranes by using spin-label electron paramagnetic resonance (EPR) spectroscopy. Lipids spin-labelled in their *sn*-2 acyl chain are used to characterize the effects on the cooperative chain-melting transition, and on the segmental chain mobility and its profile in the membrane. Spin-labelled lipids with different polar headgroups are also used to detect any selectivity of interaction with particular lipid species.

2. Materials and methods

2.1. Materials

Avidin was purchased from Molecular Probes (Eugene, OR, USA). DMPG was obtained from Avanti Polar Lipids (Alabaster, AL, USA). The spin-labelled fatty acids (*n*-SASL, *n*-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid) and phosphatidylcholine phospholipids (*n*-PCSL, 1-acyl-2-[*n*-(4,4-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphocholine) were synthesized according to procedures outlined in [9]. Spin-labelled phospholipids with different polar headgroups (5-PGSL, phosphatidylglycerol; 5-PESL, phosphatidylethanolamine; 5-PSSL, phosphatidylserine; 5-PASL, phosphatidic acid) were prepared from spin-labelled phosphatidylcholine (5-PCSL) by phospholipase D-catalyzed headgroup exchange, as described in the same reference. 1-Acyl-2-[*n*-(4,4′-dimethyloxazolidine-*N*-oxyl)]stearoyl-*sn*-glycerol (5-DGSL) was prepared from 5-PCSL by enzymatic cleavage with phospholipase C as described earlier [10]. Spin-labelled cardiolipin (5-CLSL, 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-(5-(4,4′-dimethyloxazolidine-*N*-oxyl)stearoyl)-*sn*-glycero(3) phospho]-*sn*-glycerol) was synthesized from monolyso cardiolipin (Avanti Polar Lipids) and 5-SASL by acylation as described for the $n = 14$ positional isomer [11]. Spin-labelled *N*-acyl phosphatidylethanolamine, 5-NAPESL, with the spin label in the *N*-acyl chain was synthesized as described in [12].

2.2. Sample preparation

Samples for EPR spectroscopy were prepared as follows. The lipid and 1 mol% of the spin label were co-dissolved in CH_2Cl_2 and a thin film of the lipid was formed by evaporating the solvent with dry nitrogen gas. The final traces of solvent were removed by subjecting the sample to vacuum desiccation for at least 3 h. The sample was then hydrated with approximately 100 μl of 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4 buffer (HBS) and vortexed. For measurements at pH 5.0 and 9.0 the samples were hydrated with 10 mM acetate/1 mM EDTA and 10 mM borate/1 mM EDTA, respectively. The lipid suspension obtained

was transferred into a 100-μl glass capillary and pelleted in a tabletop centrifuge. The excess supernatant was removed and the capillary was flame-sealed. Samples containing avidin were prepared in a similar manner, except that avidin was added to the lipid suspension and vortexed repeatedly and subjected to several freeze–thaw cycles in order to produce a homogeneous sample. This was then transferred into a 100-μl glass capillary and processed as above.

2.3. EPR spectroscopy

EPR spectra were recorded on a Varian E-12 Century line 9 GHz EPR spectrometer. Samples in 100-μl glass capillaries were placed in a standard 4-mm quartz sample tube containing light silicone oil for thermal stability. The temperature of the sample was maintained constant by blowing thermostatted nitrogen gas through a quartz dewar, and was measured with a fine-wire thermocouple positioned in the silicone oil adjacent to the sample capillary. Spectra were quantitated in terms of the maximum hyperfine splitting, $2A_{max}$, between the outer peaks of the anisotropic powder patterns.

3. Results

3.1. Binding of avidin to DMPG membranes

Binding of avidin to DMPG membranes was monitored by following changes in the outer hyperfine splittings ($2A_{max}$) in the EPR spectra of spin-labelled lipids, bearing the nitroxide moiety on the C-5 atom of the *sn*-2 acyl chain. The EPR spectra of the C-5 atom spin labels in pure DMPG dispersions consist of an axially symmetric, partially motionally averaged anisotropic powder pattern. Binding of avidin leads to a considerable decrease in the motional averaging of the anisotropy in the spectra of the different spin-labelled phospholipids. A plot of the increase in the $2A_{max}$ values ($\Delta 2A_{max}$) of 5-PCSL in DMPG membranes as a function of the ratio of avidin added to DMPG is given in Fig. 1. From this figure it can be seen that while the $2A_{max}$ values change relatively steeply at low protein/lipid ratios, they are more gradual at higher ratios of added protein, thus displaying a progressive saturation behavior. All further experiments were performed using a 5:1 (w/w) avidin/DMPG ratio of protein added, which corresponds to a molar ratio of about 20 DMPG molecules per avidin tetramer.

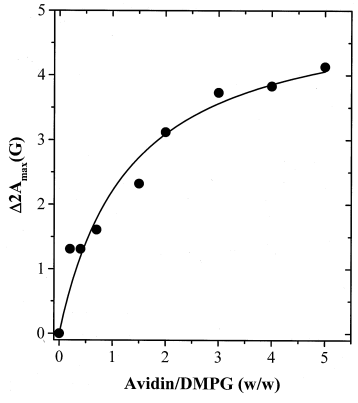

Fig. 1. Dependence of spin-label outer hyperfine splitting, $2A_{max}$, of 5-PCSL in DMPG membranes on the weight ratio of added avidin. Plotted is the increase, $\Delta 2A_{max}$, on adding avidin. Temperature: 40°C.

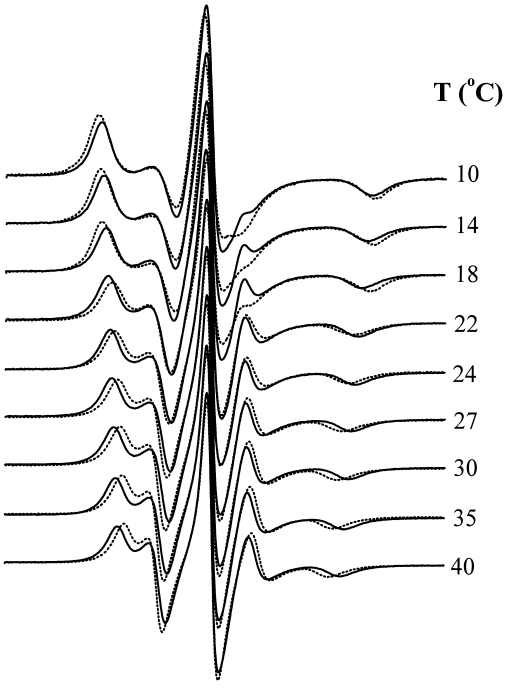

Fig. 2. EPR spectra of the phosphatidylcholine spin label, 5-PCSL, bearing the nitroxide moiety at the C-5 position of the *sn*-2 acyl chain, in DMPG membranes (dotted lines) and DMPG/avidin (1:5, w/w) complexes (solid lines). The temperature at which the spectra were recorded is indicated in the figure. The spectral width is 100 G.

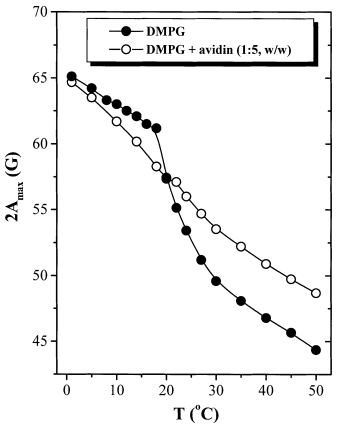

Fig. 3. Temperature dependence of the outer hyperfine splitting, $2A_{max}$, for the 5-PCSL spin label in DMPG membranes in the absence (○) and in the presence (●) of a 5:1 weight ratio of added avidin.

3.2. Effect of avidin binding on the chain-melting phase transition of DMPG

DMPG undergoes a cooperative chain-melting phase transition around 23°C when it is heated from a lower temperature. The EPR spectra of 5-PCSL, which was present at probe concentrations in DMPG membranes, recorded at different temperatures in the gel phase and the liquid-crystalline phase, are shown in Fig. 2 (dotted lines). From these spectra it can be seen that the outer hyperfine splitting, $2A_{max}$, decreases quite abruptly at the phase transition, in the region of 22°C. For samples in the presence of a 5:1 (w/w) ratio of added avidin, such abrupt changes are not seen in the temperature dependence of the 5-PCSL spectra (see Fig. 2, solid lines). On the contrary, the spectra appear to change more gradually throughout the temperature range studied.

The temperature dependences of the values of $2A_{max}$ for 5-PCSL in DMPG dispersions alone and in the presence of a 5:1 (w/w) ratio of added avidin are shown in Fig. 3. It is seen that binding of avidin to DMPG causes a decrease in the values of $2A_{max}$ in the gel phase, whereas these values are increased by avidin in the fluid phase. This suggests that binding of avidin makes the gel phase more fluid-like and the fluid phase less mobile.

The effect of avidin binding on the chain-melting phase transition of DMPG was investigated at different protein/lipid ratios. As monitored by the values of $2A_{max}$ for 5-PCSL, increasing binding of avidin results in a progressive broadening of the chain-melting phase transition (not shown). At a 5:1 (w/w) avidin/DMPG ratio the chain-melting phase transition was almost completely abolished (Fig. 3).

3.3. Lipid chain flexibility gradient

The effect of binding avidin on the chain flexibility gradient of DMPG membranes has been investigated by employing phosphatidylcholine spin labels, bearing the nitroxide moiety at different positions in the *sn*-2 acyl chain. EPR spectra recorded at 30°C, corresponding to the fluid phase of the hydrated lipid, clearly indicate a substantial decrease of chain mobility in the bilayer interior, upon binding of avidin (see Fig. 4). This applies to all positions of chain labelling and is especially evident in the outer wings of the EPR spectra of the different positional isomers of *n*-PCSL that are shown in Fig. 4. The outer hyperfine splitting, $2A_{max}$, for each of the spin labels is

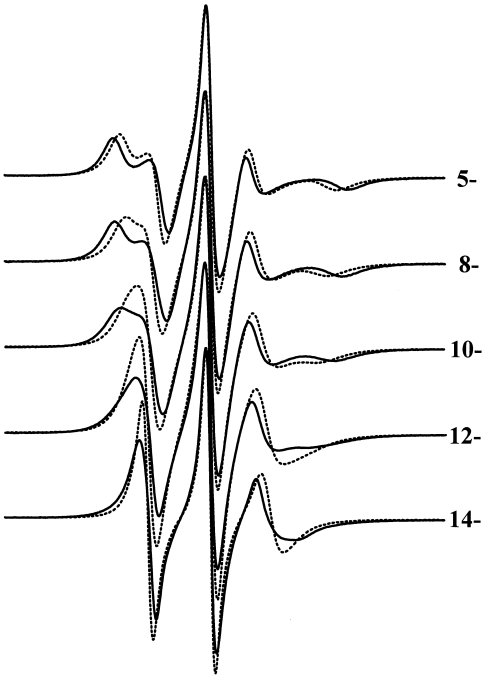

Fig. 4. EPR spectra of phosphatidylcholine spin-label positional isomers, *n*-PCSL, at 30°C in the fluid phase of DMPG membranes (dotted lines) and DMPG/avidin (1:5, w/w) complexes (solid lines). The spin-label position, *n*, in the *sn*-2 chain is indicated on the figure. The spectral width is 100 G.

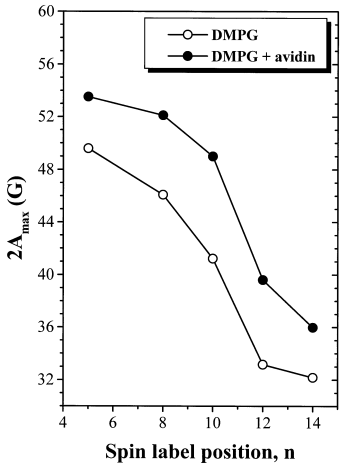

Fig. 5. Positional dependence of the outer hyperfine splitting, $2A_{max}$, for phosphatidylcholine spin labels, *n*-PCSL, in DMPG membranes (○) and in DMPG/avidin (1:5, w/w) complexes (●), at 30°C, corresponding to the fluid-phase region of DMPG alone.

higher in the presence of avidin than for the fluid-phase DMPG dispersions alone. The flexibility gradient that is characterized by the values of $2A_{max}$ for the different positions, *n*, of chain labelling is given in Fig. 5 .

3.4. Selectivity of interaction with different lipids

The selectivity of interaction of avidin with different lipid species was investigated using phospholipids with different polar headgroups, all spin-labelled at the C-5 atom of the *sn*-2 acyl chain. The corresponding spin-labelled stearic acid (5-SASL) and diacylglycerol (5-DGSL), as well as *N*-acyl phosphatidylethanolamine spin-labelled on the C-5 atom of the *N*-stearoyl chain, were also included in the study. Spin labels bearing headgroups titratable in the accessible range (5-PASL and 5-SASL) were examined in their different protonation (i.e., charge) states.

Spectral measurements were made at 30°C, corresponding to the fluid phase of the lipid. The EPR spectra of the different spin labels in DMPG dispersions alone and in the presence of a 5:1 (w/w) ratio of added avidin are shown in Fig. 6. For all the spin labels, protein binding results in an increase of the outer hyperfine splitting, $2A_{max}$, but to differing extents for the different lipids. The increases ($\Delta 2A_{max}$) in the value of $2A_{max}$ that are induced by binding avidin are given in Table 1. The selectivity series for the different spin-labelled lipids for interacting with avidin, as defined by the extent of perturbation in chain mobility that is measured by $\Delta 2A_{max}$, is in the following order: $SASLH \geq CLSL^{2-} \gg PASL^{-} > DGSL > NAPESL^{-} \approx PSSL^{-} > PCSL \geq PGSL \gg PASL^{2-} \approx PESL \gg SASL^{-}$. It should be remembered that this applies to spin-labelled lipid species at probe concentrations in DMPG membranes to which avidin is bound. In this sense, it refers to selectivity for the avidin–DMPG complex as a whole. The way in which this is interpreted depends on the model employed and is considered further in Section 4.

Table 1
Outer hyperfine splitting, $2A_{max}$, in the EPR spectra from lipids spin-labelled on the C-5 atom of the acyl chain in DMPG host membranes at 30°C

Spin label	Charge	pH	$2A_{max}$ (G)		$\Delta 2A_{max}$ (G)
			DMPG	DMPG+avidin (1:5, w/w)	
SASLH	0	9.0	43.7	49.8	6.1
CLSL	−2	7.4	44.1	50.1	6.0
PASL	−1	5.0	49.0	54.1	5.1
DGSL	0	7.4	47.8	52.6	4.8
NAPESL	−1	7.4	50.7	55.1	4.4
PSSL	−1	7.4	54.3	49.9	4.4
PCSL	±	7.4	49.6	53.6	4.0
PGSL	−1	7.4	50.3	54.2	3.9
PASL	−2	9.0	48.5	53.5	2.3
PESL	±	7.4	51.6	53.9	2.3
SASL	−1	5.0	49.8	51.1	1.3

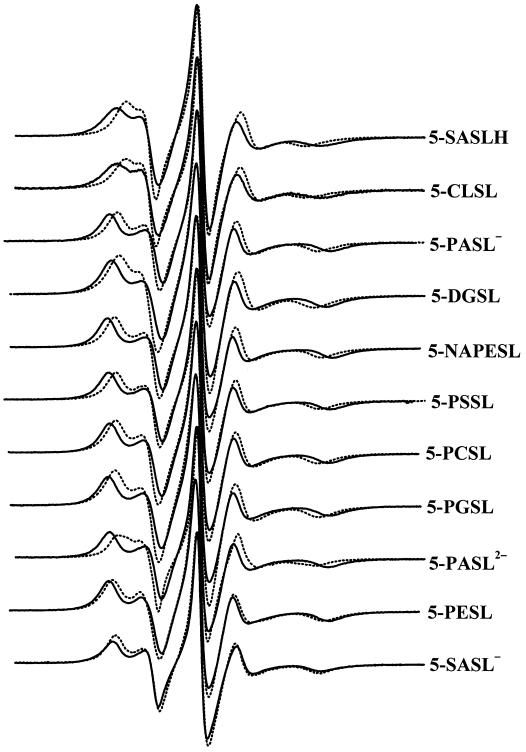

Fig. 6. EPR spectra of different lipid species, spin-labelled at the C-5 atom of the *sn*-2 acyl chain in the fluid phase of DMPG membranes (dotted lines) and of DMPG/avidin (1:5, w/w) complexes (solid lines), recorded at 30°C. The spin-label species used in the sample for each pair of spectra is indicated in the figure. The spectral width is 100 G.

4. Discussion

The finding that avidin binds strongly to membranes composed of negatively charged lipids has implications for the design of experiments that exploit the specific recognition of biotin labels by avidin. High ionic strength and low concentrations of avidin will reduce non-specific background and favor specific binding, respectively. At low avidin concentrations the specific interaction will dominate by competition. Negatively charged lipid surfaces may then even facilitate specific binding by channelling avidin (via two-dimensional diffusion) to dilute biotin-conjugated sites at the membrane surface. It is of interest that biotinylated phosphatidylethanolamines and phosphatidylserines are themselves negatively charged lipids. This could, on the one hand, potentiate specific binding but, on the other hand, may engender non-specific binding if access to the biotinylated site is in some way sterically hindered.

The remainder of Section 4 is devoted to those aspects of membrane binding that relate to avidin as a model peripheral membrane protein.

4.1. Avidin binding

As judged from Fig. 1, initial binding is relatively tight. Weaker binding is to be expected at higher avidin concentrations, at least in part, because the membrane surface potential is progressively neutralized by the bound protein [13]. As saturation binding is approached, $\Delta 2A_{max}$ tends to a limiting value of $\sim$4 G. Extrapolation to this value from the initial part of the binding curve yields a stoichiometry $\sim 1.4 \pm 0.4$ w/w avidin/DMPG. This corresponds to approximately 60–100 DMPG molecules per avidin tetramer. From X-ray diffraction studies the cross-section of the avidin tetramer is $\sim 5 \times 5$ nm [14,15] which corresponds to the surface area of approximately 40 DMPG molecules. Therefore the saturation electrostatic binding of avidin to DMPG membranes approaches, although possibly does not reach, complete surface coverage.

4.2. Perturbation of lipid chain packing

Saturation binding of avidin almost entirely abolishes the cooperative chain-melting transition of DMPG membranes (see Fig. 3). Similar effects were observed on binding the basic peripheral proteins apocytochrome *c* [16] and myelin basic protein [17] to DMPG membranes, but not with holocytochrome *c* [18]. In the cases of apocytochrome *c* [19] and myelin basic protein [20], however, abolition of the chain melting is accompanied by a partial penetration of the protein into the membrane which would disrupt the cooperative chain packing in the gel phase. This was evidenced by the appearance of a second more motionally restricted component in the EPR spectra of lipids labelled close to the terminal methyl end of the chain. For avidin binding to DMPG, however, no such second component that can be attributed to a direct interaction of the protein with the lipid chains as in the case of integral membrane proteins [21] is seen in the spectra given in Fig. 4.

This different behavior is emphasized by detailed comparison with experiments analogous to those of Fig. 3 that were performed with the myelin basic protein [17]. In the latter case, the lipid chain mobility is decreased in both the gel and fluid phases of DMPG. This is in contrast to the situation with avidin binding for which the lipid chain mobility is increased in the gel phase. The interaction of avidin with the DMPG headgroups decreases the lipid close packing in the gel phase, thus removing the cooperativity of chain melting. In the more expanded fluid phase, the opposite takes place. Interaction with the DMPG headgroup increases the lateral lipid packing density and decreases the chain mobility. This is the situation that normally holds in the electrostatic interaction of peripheral proteins with negatively charged lipid membranes [21].

One major difference between avidin and apocytochrome *c* or myelin basic protein is that the latter proteins are predominantly unfolded in solution and only take on secondary structure when binding to negatively charged lipid membranes. Holocytochrome *c*, although structured in solution, experiences loosening of its tertiary structure on binding to negatively charged lipid membranes [18,22]. The backbone of avidin, on the other hand, is a rigid β-barrel structure. It is possibly this feature that determines the optimum interaction with negatively charged lipid headgroups. Tightly packed lipids in the gel phase are expanded and loosely packed lipids in the fluid phase are condensed to move towards this optimum. The looser tertiary structure of lipid-bound holocytochrome *c*, by contrast, does not exert such a pronounced effect. The cooperativity of packing in the lipid gel phase is then not reduced to such an extent that cooperative chain melting is abolished (see [23]).

4.3. Lipid chain flexibility profile

The progressive decrease in outer hyperfine splitting with spin label position down the *sn*-2 chain that is shown for fluid DMPG bilayers in Fig. 5 defines the gradient in chain segmental flexibility. This profile is a characteristic hallmark for this type of spin label in fluid liquid-crystalline lipid phases. From Fig. 5 it is seen that this characteristic profile is preserved when avidin is bound. The values of $2A_{max}$ are increased more or less uniformly at all positions of chain labelling. This effect is characteristic of the surface binding of peripheral membrane proteins, e.g., holocytochrome *c* (H. Görrissen and D. Marsh, unpublished). It also extends to the basic (i.e., surface-binding) regions of apocytochrome *c* [19] and myelin basic protein [17] that do not come in direct contact with the lipid chains, and additionally to the sublytic binding of melittin to phosphatidylcholine membranes [24]. In contrast to the situation for surface binding, the transmembrane insertion of α-lactalbumin at low pH drastically changes the shape of the chain flexibility profile [25].

4.4. Lipid selectivity

Differences in outer hyperfine splitting between various lipid species spin-labelled on the C-5 atom of the *sn*-2 chain is a common feature of peripheral protein–lipid complexes [26]. In terms of the magnitudes of ΔA_{max}, those given in Table 1 for avidin lie in an intermediate range, relative to the surface-binding peptides and proteins studied so far (cf. for example [20,27]). They are significantly higher than those for cytochrome *c* and apocytochrome *c* [27]. They do not, however, approach the value of $\Delta A_{max} \approx 7$ G for 5-PSSL in complexes of myelin basic protein with DMPG at 30°C [20]. The latter is by far the largest value of ΔA_{max} reported in such systems until now. To achieve such a specific interaction may require a conformational adaptability of the protein that is not accessible to the rigid β-barrel core of avidin. In general, all values of ΔA_{max} for myelin basic protein exceed those for avidin. This general result, but not the remarkably high selectivity for 5-PSSL, may partly be attributed to the penetration of the membrane by myelin basic protein (see above).

Because the spin-labelled lipids are intercalated at probe concentrations in the protein-bound lipid membranes, selectivities between the values of ΔA_{max} can have at least two possible origins. One arises from the change in the bulk lipid environment on binding the protein. The other is a direct selectivity for interaction of the lipid headgroups with the protein, possibly at 'hot spots' on the protein surface. The first is illustrated by the fact that the detailed sequence in values of ΔA_{max} depends to some extent

on membrane lipid composition [20,24,28]. This is the predominant and possibly only mechanism for zwitterionic and neutral lipids. In the latter case, the effects can be anomalously large because it is known that both protonated fatty acids (i.e., SASLH) and diacylglycerols (i.e., DGSL) are situated deeper in the membrane than are phospholipids and also charged lipids in general [10,29]. Increases in outer hyperfine splitting can then occur from upward relocation of the spin label in response to protein binding (cf. Fig. 6). In consequence, it is not uncommon that protonated 5-SASL exhibits the largest value of ΔA_{max} [24,28,30], as found here for avidin. Correspondingly, 5-DGSL does not invariably have the lowest value of ΔA_{max}. In several instances [20] it assumes an intermediate value as seen for avidin in Table 1.

Leaving aside the neutral lipids, the greatest selectivity for avidin is displayed by certain, but not all, of the negatively charged lipids (see Table 1). Lipids with the same formal charge do not necessarily display similar selectivities. These are unifying features for all peripheral proteins and peptides studied to date (see e.g., [26]). Differences between the zwitterionic lipids phosphatidylcholine and phosphatidylethanolamine are also not uncommon and may possibly arise from differences in headgroup hydration and hydrogen bonding capability. Overall the pattern of selectivity for different lipid species depends in detail on the particular peripheral protein, and avidin is no exception. As might be expected, this latter aspect does not differ from the situation that holds for the headgroup selectivity of lipid interactions with different integral membrane proteins [21].

Acknowledgements

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