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Selectivity of interaction of spin-labelled lipids with peripheral proteins bound to dimyristoylphosphatidylglycerol bilayers, as determined by ESR spectroscopy

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The selectivity of interaction between spin-labelled lipids and the peripheral proteins, apocytochrome *c*, cytochrome *c*, lysozyme and polylysine has been studied using ESR spectroscopy. Derivatives of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol (PI), diphosphatidylglycerol (CL) and diacylglycerol (DG) spin-labelled at the 5-C atom position of the *sn*-2 chain were used to study the association of these proteins with bilayers of dimyristoylphosphatidylglycerol. Binding of the proteins increased the outer hyperfine splitting in the ESR spectra of the lipid spin labels to an extent which depended both on the spin-labelled lipid species involved and on the particular protein. The order of selectivity for apocytochrome *c* follows the sequence: $PI^- > CL^- \approx DG > PS^- > PC^\pm > PG^- > PE^\pm$. The selectivity pattern for cytochrome *c* is: $PI^- > PG^- > CL^- > DG > PS^- \approx PC^\pm > PE^\pm$; for lysozyme is: $CL^- > PG^- > DG > PE^\pm > PC^\pm > PS^- > PI^-$; and that for polylysine is: $CL^- > PS^- \geq PG^- > PI^- > PC^\pm > DG > PE^\pm$. The overall strength of interaction is in the order lysozyme > cytochrome *c* > apocytochrome *c*, for equivalent binding, and the spread of the selectivity for the different proteins is in the reverse order. Assuming fast exchange for the ESR spectra of the 5-C atom labelled lipids, the relative association constants of the different labels with the different proteins have been estimated

Introduction

Peripheral proteins can be displaced from membrane surfaces either by manipulations of the pH or by increasing the ionic strength, indicating the obligatory nature of electrostatic interactions for the membrane-protein association. The membranes of biological cells contain a variety of different negatively charged lipids and, in addition, a high proportion of zwitterionic lipids.

The selectivity of interaction with the different anionic lipid species and the interaction with zwitterionic lipids in the presence of negatively charged lipids are thus both important determinants of the peripheral protein binding.

In the present work, we have investigated the selectivity of interaction of seven spin-labelled glycerolipid species with different peripheral proteins in association with dimyristoylphosphatidylglycerol membranes, using ESR spectroscopy. The spin-labelled lipids employed for this study include both negatively charged and zwitterionic phospholipids and also the neutral diacylglycerol molecule. As peripheral proteins, we have taken cytochrome *c* and the precursor protein apocytochrome *c*, as well as polylysine and lysozyme. Although the latter are not true peripheral proteins, they also associate strongly with negatively charged lipid bilayers. Comparison of cytochrome *c* and the haem-less precursor, apocytochrome *c*, allows investigation of the differences in interaction between the native, folded protein and a structureless, essentially random coil, form of the protein with identical amino acid sequence.

Abbreviations: ESR, electron spin resonance; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; EDTA, ethylenediamine tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; 5-PGSL, -PSSL, -PISL, -PESL, -PCSL, 1-acyl-2-[5-(4,4-dimethyl-oxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero-3-phosphoglycerol, -phosphoric acid, -phosphoserine, -phosphoinositol, -phosphoethanolamine, -phosphocholine; 5-CLSL, 1-(3-*sn*-phosphatidyl)-3-[1-acyl-2-*O*-(4,4-dimethyl-oxazolidine-*N*-oxyl)]stearoyl-*sn*-glycero(3)phospho-*sn*-glycerol; 5-DGSL, 1-acyl-2-[5-(4,4-dimethyl-oxazolidine-*N*-oxyl)]stearoyl-*sn*-glycerol; MBP, bovine spinal cord myelin basic protein.

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These differences may be important for the mechanism of import of the precursor protein into mitochondria.

It is shown that the hyperfine splittings in the ESR spectra of the different spin-labelled lipids can be used to determine the strength and selectivity of interaction with peripheral proteins bound at the membrane surface. Previous, more limited studies with apocytochrome *c* and mixed lipid bilayers [1] have indicated that this might be the case, whereas studies with cytochrome *c* and mixed bilayers containing a lower proportion of negatively charged lipid have shown only little specific effects of the protein-lipid interaction [2]. The present results with dimyristoyl phosphatidylglycerol bilayers indicate that a well-defined pattern of lipid selectivity can be observed, which differs between the various proteins. The results with diacylglycerol may be relevant as model studies for the interaction of protein kinase C with cell plasma membranes, especially since this second messenger lipid is found to have a rather pronounced selectivity of interaction with two of the proteins investigated.

Materials and Methods

Materials. DMPG was synthesized from DMPC (Fluka, Buchs, Switzerland) by a headgroup exchange reaction catalyzed by phospholipase D [3]. Hen egg white lysozyme and poly(L-lysine)·HBr (15–30 kDa) were from Serva (Heidelberg, F.R.G.). 5-DGSL was synthesized from 5-PCSL by a phospholipase C (Boehringer-Mannheim) catalyzed hydrolysis reaction [4]. 5-PISL was synthesized from yeast phosphatidylinositol (Lipid Products, South Nutfield, U.K.) using a modification for spin-labelled lipids of the method described in Ref. 5. 5-CLSL was synthesized by condensation reaction between 5-PGSL and phosphatidic acid derived from egg-yolk phosphatidylcholine (Lipid Products, South Nutfield, U.K.), essentially according to the method given in Ref. 6. All other spin-labels were synthesized using the procedures detailed in Ref. 7.

Purification of cytochrome *c*. Horse-heart cytochrome *c* (Sigma, St. Louis, MO, type VI) was purified by chromatography on a carboxymethyl cellulose column as described in Ref. 8. The various deamidated forms present in the commercially available protein were separated on the CM-Cellulose column by eluting with 85 mM sodium phosphate buffer (pH 7.0). The pooled fractions of the major band were dialysed extensively against 10 mM NaCl, 10 mM Tris, 0.1 mM EDTA (pH 8.0) buffer.

Preparation of apocytochrome *c*. Apocytochrome *c* was prepared from cytochrome *c* (type VI, Sigma), by removing the haem moiety according to the method of Ref. 9 and subjecting the haem-free protein to a re-naturation procedure [10]. The protein was stored at -20°C in 0.5-ml aliquots at a concentration of approx.

1.5 mg/ml in 100 mM NaCl, 10 mM Pipes (pH 7.0), 0.01% (v/v) β -mercaptoethanol. The protein was always used immediately after thawing.

Sample preparation. The lipid-protein complexes were formed in each case by adding a solution of the protein to hydrated DMPG bilayers containing approximately 1 mol% spin label in the required buffer. Stock solutions of the proteins were at concentrations of 1.5 mg apocytochrome *c*/ml, 1 mg cytochrome *c*/ml, 1 mg lysozyme/ml and 2.5 mg polylysine/ml, and buffers were 10 mM Pipes, 0.1 M NaCl, 0.01% (v/v) β -mercaptoethanol (pH 7.0) for apocytochrome *c* and 10 mM Tris, 10 mM NaCl, 0.1 mM EDTA (pH 8.0) for lysozyme, cytochrome *c* and polylysine. Saturation levels of binding were achieved by adding 3 mg apocytochrome *c*, 3 mg cytochrome *c*, 4 mg lysozyme or 10 mg polylysine per mg DMPG. Upon addition of the protein solution, the samples were incubated at 35°C for approximately 1 h. The resulting lipid-protein complexes were isolated as pellets upon centrifugation in a bench top centrifuge. The pellets were transferred to the 100 μl capillaries used for ESR measurements. The samples were further concentrated by centrifugation. After the ESR measurements, the pellets were dissolved in a few drops of 1 M NaOH for determining the lipid and protein contents. Phospholipid concentration was determined by the method of Ref. 11. Protein assays were performed according to the method of Ref. 12. Polylysine concentrations were also determined by measuring the absorbance at 280 nm.

ESR spectroscopy. ESR spectra were recorded on a Varian Associates E-line 9 GHz spectrometer. Spectra were digitized and stored on an IBM personal computer interfaced to the ESR spectrometer using a Labmaster A/D converter and software written by Dr. M.D. King. Temperature was regulated using a nitrogen gas-flow system and measured with a fine-wire thermocouple placed adjacent to the sample capillary at the top of the cavity.

Results

The ESR spectra of selected diacyl lipids spin-labelled at the 5th position in the *sn*-2 chain and incorporated into complexes of DMPG with apocytochrome *c* are given in Fig. 1. The spectra included are those of spin-labelled phosphatidylinositol, 5-PISL; phosphatidylserine, 5-PSSL; phosphatidylethanolamine, 5-PESL; and phosphatidylcholine, 5-PCSL, at 30°C , both in the presence and absence of saturating amounts of apocytochrome *c*. The spectra of all labels exhibit a larger outer hyperfine splitting ($2A_{\text{max}}$) in the presence of protein than in the absence of protein, demonstrating a reduction in mobility of the acyl chains of all the different lipids on binding of the protein. The extent of this motional perturbation displays a clear dependence

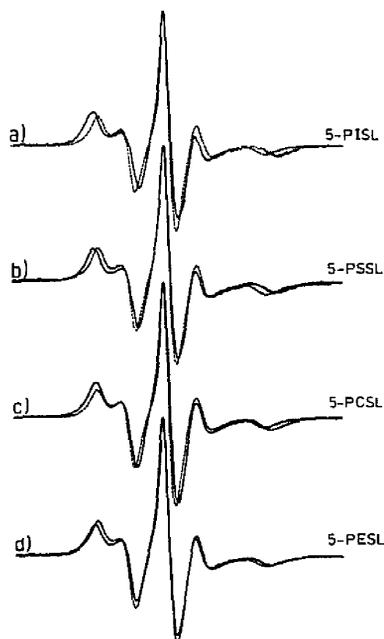


Fig. 1. ESR spectra at 30°C of phospholipid spin labels labelled at the 5th position of the *sn*-2 chain in DMPG bilayers and in DMPG-apocytochrome *c* complexes at saturation binding. Buffer: 10 mM Pipes, 0.1 M NaCl, 0.01% (v/v) β -mercaptoethanol (pH 7.0). The solid line of each pair is the spectrum from the lipid-protein complexes and the dotted line is the spectrum from DMPG bilayers. (a) phosphatidylinositol spin label, 5-PISL; (b) phosphatidylserine spin label, 5-PSSL; (c) phosphatidylcholine spin label, 5-PCSL; (d) phosphatidylethanolamine spin label, 5-PESL. Spectral width = 100 G.

on the lipid species, being greatest for phosphatidylinositol and smallest for phosphatidylethanolamine.

The ESR spectra of the 5-CLSL diphosphatidylglycerol and 5-PGSL phosphatidylglycerol spin labels in complexes of DMPG with saturating amounts of different extrinsic proteins (apocytochrome *c*, cytochrome *c* and lysozyme) are given in Fig. 2. The ionic strength of the buffer has been chosen such that comparable amounts of protein are bound for all three systems. The measured values of the lipid/protein ratio of the samples are: 10, 8 and 9 mol/mol for apocytochrome *c*, cytochrome *c* and lysozyme, respectively. (For polylysine the corresponding binding stoichiometry was calculated to be 25 mol/mol, based on a degree of polymerization of 80). For all the cases in Fig. 2, the outer hyperfine splitting in the presence of protein is greater than in the absence of protein, as was seen for different lipid spin labels in apocytochrome *c*-DMPG complexes in Fig. 1. The degree of motional perturbation, at comparable protein binding, is found to increase in the order apocytochrome *c* < cytochrome *c* < lysozyme, for both spin labels.

From Figs. 1 and 2, it can be seen that the spectra of all the various diacyl phospholipid spin-labels in pure DMPG bilayers are very similar (only that of diacylglycerol has an appreciably smaller spectral anisotropy). The values for the outer hyperfine splittings in spectra from the pure lipid at 30°C are nearly constant ($A_{\max} = 25.2 \pm 0.2$ G), indicating very similar motional characteristics for the different phospholipid spin-labels. Thus the difference in the values of A_{\max} between the lipid-protein complexes and the pure lipid can be taken as a measure of the degree of perturbation of the lipid chain motion and hence of the selectivity of interaction of the lipid with the protein. This is especially useful in the comparison with 5-DGSL (and to a lesser extent 5-CLSL) which has a smaller value of A_{\max} in the pure lipid. These values of ΔA_{\max} for the different spin labelled lipids with the different proteins and polylysine are given in Table I. A differential selectivity in the interaction of the various lipids with the proteins is seen clearly from the values of ΔA_{\max} . Relative to the 5-PGSL

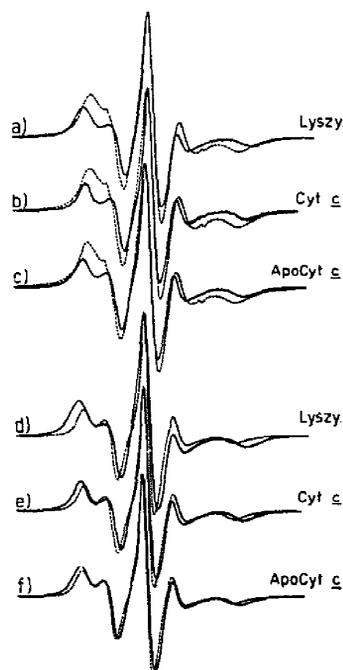


Fig. 2. ESR spectra at 30°C of the 5-CLSL and 5-PGSL spin labels in DMPG bilayers and in DMPG complexes with lysozyme, cytochrome *c* and apocytochrome *c*. Buffer: 10 mM Tris, 10 mM NaCl, 0.1 mM EDTA (pH 8.0) for lysozyme and cytochrome *c*, and as in the legend to Fig. 1 for apocytochrome *c*. The solid line of each pair is the spectrum from the lipid-protein complexes and the dotted line is the spectrum from DMPG bilayers. (a) 5-CLSL in lysozyme-DMPG, (b) 5-CLSL in cytochrome *c*-DMPG, (c) 5-CLSL in apocytochrome *c*-DMPG, (d) 5-PGSL in lysozyme-DMPG, (e) 5-PGSL in cytochrome *c*-DMPG, (f) 5-PGSL in apocytochrome *c*-DMPG. Spectral width = 100 G.

TABLE I

Increase in outer hyperfine splitting (ΔA_{\max}) of 5-C atom position spin-labelled lipids in DMPG bilayers, on binding saturating amounts of different peripheral proteins. $T = 30^\circ\text{C}$.

Spin label	ΔA_{\max} (G)			
	apocyt. <i>c</i>	cyt. <i>c</i>	lysozyme	polylysine
5-PSSL	2.01	1.82	2.26	3.21
5-CLSL	2.07	2.45	3.14	3.90
5-PGSL	1.44	2.57	3.01	3.08
5-PISL	2.70	2.83	2.14	2.85
5-PESL	1.13	1.51	2.45	0.82
5-PCSL	1.57	1.82	2.32	1.70
5-DGSL	2.07	2.20	2.95	1.30

spin-label in complexes of DMPG with apocytochrome *c*, the negatively charged lipids 5-PISL, 5-CLSL and 5-PSSL and the zwitterionic 5-PCSL, exhibit greater increases in A_{\max} on binding the protein, whereas the protein-induced motional restriction is less for the neutral 5-DGSL and zwitterionic 5-PESL lipid spin-labels than that for 5-PGSL. For the other two proteins and the polypeptide polylysine, the lipid selectivity patterns are different. For DMPG complexes with cytochrome *c*, only the 5-PISL spin label exhibits a greater protein-induced increase in A_{\max} than does the 5-PGSL spin label, and that for the 5-CLSL spin label is comparable to the value for 5-PGSL. For complexes with lysozyme, only the 5-CLSL spin label experiences a greater perturbation of the chain mobility by the protein than that for 5-PGSL spin label, although the value of ΔA_{\max} for the neutral 5-DGSL label is comparable to that for 5-PGSL. For complexes with polylysine, both the 5-CLSL and 5-PSSL spin labels exhibit a greater motional perturbation than does the 5-PGSL spin label, and only the zwitterionic labels 5-PCSL, 5-PESL and the neutral 5-DGSL are appreciably less perturbed by the protein than is the 5-PGSL spin label.

Discussion

The protein-induced changes in the outer hyperfine splittings can be used to establish the patterns in strength or selectivity of interaction of the various spin-labelled lipids with the different peripheral proteins. From the results of Table I, these are found to be: $\text{PI}^- > \text{CL}^- \approx \text{DG} > \text{PS}^- > \text{PC}^\pm > \text{PG}^- > \text{PE}^\pm$ for apocytochrome *c*; $\text{PI}^- > \text{PG}^- > \text{CL}^- > \text{DG} > \text{PS}^- \approx \text{PC}^\pm > \text{PE}^\pm$ for cytochrome *c*; $\text{CL}^- > \text{PG}^- > \text{DG} > \text{PE}^\pm > \text{PC}^\pm > \text{PS}^- > \text{PI}^-$ for lysozyme; and $\text{CL}^- > \text{PS}^- \geq \text{PG}^- > \text{PI}^- > \text{PC}^\pm > \text{DG} > \text{PE}^\pm$ for polylysine. Rather surprisingly, the neutral 5-DGSL and the zwitterionic 5-PCSL spin labels exhibit stronger interactions with apocytochrome *c* than does the negatively charged 5-PGSL spin label, which can be taken as representative of the background DMPG against which the spin labeled lipids are in competition.

In addition, the 5-DGSL spin label exhibits a stronger interaction with cytochrome *c* than does the 5-PGSL spin label, and the strengths of interaction of these two labels with polylysine are comparable. Although the 5-DGSL, 5-PCSL and 5-PESL spin labels have a weaker interaction with lysozyme than does 5-PGSL, these labels nonetheless exhibit a stronger interaction with this protein than do the negatively charged 5-PSSL and 5-PISL spin labels. It must be remembered, however, that the labels are present only at low concentration in the host DMPG bilayer and it is the latter which controls the binding of the various proteins. Therefore, other interactions may be involved than the direct electrostatic interaction which is the primary source of the binding energy for peripheral proteins. Amongst these are contributions from steric interactions, hydration and possibly also hydrophobic components of the interaction.

It is interesting to compare the selectivity patterns of the proteins studied here with that found for the myelin basic protein in complexes with DMPG. For the MBP, the selectivity pattern established from the protein-induced increases in A_{\max} is: $\text{PS}^- > \text{CL}^- > \text{PG}^- > \text{PI}^- > \text{PE}^\pm > \text{PC}^\pm > \text{DG}$ [13]. In this case the neutral lipid, 5-DGSL, exhibits the weakest interaction with the protein, and the zwitterionic lipids, 5-PCSL and 5-PESL, also exhibit weaker interactions than do any of the negatively charged lipids. This pattern conforms more closely with the conventional expectation that the negatively charged lipids have the stronger interaction with the basic peripheral protein. The diversity of the selectivity patterns for the different proteins emphasizes the complexity of the interactions which may occur with peripheral proteins in biological membranes and may give some rationale for the heterogeneity of natural membrane lipid compositions.

The relative sizes of the protein-induced increases in A_{\max} also give some idea of the different strengths of these selectivities. For apocytochrome *c*, the difference between the maximum and minimum values of ΔA_{\max} is 1.57 G; for cytochrome *c*, this difference is 1.32 G; and for lysozyme it is 1.00 G. By contrast, the mean values of ΔA_{\max} , which give an indication of the overall strength of the interaction, are: 1.86 G for apocytochrome *c*, 2.17 G for cytochrome *c*, and 2.61 G for lysozyme. Thus, although apocytochrome *c* exhibits the greatest range of lipid selectivity, the overall strength of lipid interaction with this protein is less than with lysozyme. This pattern is changed somewhat, if comparison is made with apocytochrome *c* at comparable ionic strength, rather than comparable protein binding. It has been found previously that at low ionic strength the value of ΔA_{\max} for 5-PGSL with apocytochrome *c* is considerably larger (≈ 4.1 G) than with cytochrome *c*, and that the protein binding is correspondingly higher [14]. The extent of binding is also different for poly-

lysine; in this case the spread in ΔA_{\max} is 3.1 G and the mean value is 2.4 G. For further comparison, the difference between the maximum and minimum values of ΔA_{\max} found for the MBP is 4.14 G, and the mean value of ΔA_{\max} for the different labels is 3.85 G [13]. The range of selectivity and the overall strength of the interaction is therefore greater for the MBP than for the proteins studied here. This may be in line with the structural role of the MBP in the myelin sheath, as opposed to the enzymatic role of cytochrome *c*, for instance, which requires an interaction only sufficiently strong to ensure a specific membrane localization.

The selectivity of the various spin-labelled lipids may be analyzed quantitatively, if it is assumed that the ESR spectra of the 5-C atom position labels correspond to fast exchange on the spin label ESR timescale. A model is used in which there are a limited number, n_p , of association sites on the protein at which the spin-labelled lipids have a maximum hyperfine splitting constant, A^p , and the remainder of the lipids have a lower hyperfine constant, A^f , which is taken as that in the pure lipid. The criterion for fast exchange is that the rate of exchange between the 'free' and protein-associated sites shall be greater than the difference between the hyperfine splittings of the corresponding spectral components. The largest value of $2\pi A_{\max}$ for the data in Table I (that for 5-CLSL with lysozyme) is approx. $5.5 \cdot 10^7$ s⁻¹. Typical lipid translational diffusion rates in fluid dimyristoyl phosphatidylcholine bilayers are: $\nu_{\text{diff}} = 4D_T / \langle x^2 \rangle \approx (7-9) \cdot 10^7$ s⁻¹ [15], and our unpublished values for DMPG are of a similar order of magnitude. Thus the fast exchange approximation may hold, at least for the spin-labelled lipids with smaller values of ΔA_{\max} .

With the above model, the maximum hyperfine splitting constant is then given by the standard expression for fast exchange:

$$A_{\max} = (A^p - A^f) \cdot f + A^f \quad (1)$$

where f is the fraction of the spin-labelled lipids that is associated with the specific sites (assumed to be totally occupied by either labelled or unlabelled lipids). The fraction of spin-labelled lipids occupying the specific sites is given by the equation for equilibrium lipid-protein association [16,17]:

$$f = [(n_t/n_p - 1)/K_r + 1]^{-1} \quad (2)$$

where K_r is the association constant of the labelled lipid relative to the unlabelled lipid (DMPG) and n_t is the total DMPG/protein ratio. Combining Eqns. 1 and 2, the relative association constant for the spin-labelled lipid is given by:

$$K_r = (n_t/n_p - 1) \cdot \Delta A_{\max} / (\Delta A_{\max}^0 - \Delta A_{\max}) \quad (3)$$

where $\Delta A_{\max}^0 = (A^p - A^f)$. The condition that $K_r = 1$ for the 5-PGSL label relative to the background DMPG, leads to the following relation for the limiting value of ΔA_{\max} :

$$\Delta A_{\max}^0 = (n_t/n_p) \Delta A_{\max}(\text{PG}) \quad (4)$$

where $\Delta A_{\max}(\text{PG})$ is the value of ΔA_{\max} for 5-PGSL. Substituting in Eqn. 3 yields:

$$K_r = (n_t/n_p - 1) \cdot \Delta A_{\max} / [(n_t/n_p) \Delta A_{\max}(\text{PG}) - \Delta A_{\max}] \quad (5)$$

Although the value for ΔA_{\max}^0 has been established by Eqn. 4, a value is also required for n_p before K_r can be determined. As will be seen below, strict limits are set on the values of n_p from the boundary values of ΔA_{\max}^0 , via Eqn. 4. The effect of the choice of n_p on the values derived for K_r is discussed later*.

An estimate for the relative number of protein-associated sites: $n_p/n_t \approx 0.5$, can be obtained from the percentage of motionally restricted lipids found with spin-labelled phosphatidylglycerol in DMPG-apocytochrome *c* complexes [14]. Similar values have also been obtained for MBP/DMPG complexes [13]. A boundary condition is that n_p/n_t must yield values of ΔA_{\max}^0 from Eqn. 4 which are greater than the largest experimentally observed values of ΔA_{\max} . This condition is certainly fulfilled for the data of Table I. A further boundary condition is that ΔA_{\max}^0 cannot be greater than $(A_{zz} - A^f)$, where A_{zz} is the principal element of the nitroxide hyperfine tensor. This condition is also fulfilled. To give a direct comparison between the different proteins, the value of $n_p/n_t = 0.5$ will be used in all cases.

Values of the relative association constants for the different spin-labelled lipids, calculated from Eqn. 5, are given in Table II. Only the value of K_r obtained for 5-PISL interacting with apocytochrome *c* is very sensitive to the choice of the parameter n_p/n_t , since its value for ΔA_{\max} lies close to ΔA_{\max}^0 and therefore small changes in the latter will produce a large effect on K_r . In this latter case, it is clear that the value of K_r is considerably greater than in all the other cases, although the precise value cannot be specified very ex-

* The assumption is made that the value of $A^p - A^f$ is the same for all spin-labelled lipids. This is likely to be the case for phospholipid labels which all have the same value of A^f in DMPG alone, and strictly this assumption regarding $A^p - A^f$ defines the model for the lipid-protein association. For the diglyceride, 5-DGSL, A^f is smaller than for the phospholipids and this means that the values derived for K_r are probably an upper limit. The selectivities for 5-DGSL relative to the phospholipids deduced from ΔA_{\max} may be somewhat in error, for this reason. However, this does not invalidate the comparison between 5-DGSL in the different protein/DMPG systems.

TABLE II

Relative association constants, K_r , of 5-C atom position spin-labelled lipids with different peripheral proteins in DMPG bilayers, estimated relative to 5-PGSL from Eqn. 5 with $n_p/n_t = 0.5$

Spin label	K_r			
	apocyt. c	cyt. c	lysozyme	polylysine
5-PSSL	2.3	0.55	0.60	1.1
5-CLSL	2.5	0.90	1.1	1.7
5-PGSL	1.00	1.00	1.00	1.00
5-PISL	14.4	1.2	0.55	0.85
5-PESL	0.65	0.40	0.70	0.15
5-PCSL	1.2	0.55	0.65	0.40
5-DGSL	2.5	0.75	0.95	0.27

actly. The other values of K_r are relatively insensitive to small changes in the choice of parameters. For these cases, the association constants relative to DMPG take more modest values: the largest value is 2.5 (5-CLSL and 5-DGSL with apocytochrome *c*) and the smallest is 0.4 (5-PESL with cytochrome *c*). (An exception to the latter is the very small degree of association of the zwitterionic 5-PESL and the neutral 5-DGSL with the polypeptide polylysine.) This indicates that the differences in energetics for association of a single lipid with the protein sites in bulk DMPG bilayers are significant. With the exception of 5-PISL interacting with apocytochrome *c* and 5-PESL/-DGSL with polylysine, the maximum differential free energies of association ($\Delta G = -RT \ln[K_r/K_r(\text{PG})]$) are of the order of 2.3–2.5 kJ·mol⁻¹ per lipid, or 7–11 kJ·mol⁻¹ per protein, respectively. The total free energy of association per protein is, of course, contributed mainly by the DMPG host lipids and the net binding energy is enhanced by an amount $Ze\Phi_s$, where Φ_s is the electrostatic surface potential of the bilayer.

In conclusion, it should be emphasized that selectivities determined in the present work refer to the different lipids in protein complexes with DMPG and do not necessarily reflect the relative strengths of binding of these proteins to the single isolated lipid species. Previous comparative binding studies of apocytochrome *c* and cytochrome *c* in monolayer [18] and bilayer [14,19] systems of both homogeneous and heterogeneous lipid

composition, have revealed different selectivity patterns, with a special emphasis on the role of cardiolipin.

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