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Fatty acid pH titration and the selectivity of interaction with extrinsic proteins in dimyristoylphosphatidylglycerol dispersions. Spin label ESR studies

M.B. Sankaram¹, Peter J. Brophy², Wilco Jordi³ and Derek Marsh¹

¹ Max-Planck-Institut für biophysikalische Chemie, Abt. Spektroskopie, Göttingen (F.R.G.), ² University of Stirling, Department of Biological Science, Stirling (U.K.) and ³ Centre of Biomembranes and Lipid Enzymology, State University of Utrecht, Utrecht (The Netherlands)

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The effects of pH titration on the ESR spectra of 5-(4,4-dimethylloxazolidine-*N*-oxyl) stearic acid in bilayers of dimyristoyl phosphatidylglycerol complexed with either myelin basic protein, apocytochrome *c*, cytochrome *c* or lysozyme have been studied. Binding of the protein increases the outer hyperfine splitting of the stearic acid spin label to an extent which depends on the protonation state of the fatty acid carboxyl group for all proteins studied. The hyperfine splittings have been analysed to determine the relative selectivities of the protonated and unprotonated forms of the fatty acid by assuming that the spectra correspond to fast exchange on the ESR timescale. For myelin basic protein and apocytochrome *c*, the relative association constants are approximately 80% and 30% greater, respectively for the charged state than for the protonated state of the fatty acid. The pK_a of the stearic acid spin label has been determined from the pH titration behaviour of the outer hyperfine splitting in the ESR spectra. The pK_a at the lipid/water interface in the absence of protein is 8.0, and at the lipid/peripheral protein interface is 9.6, 9.2, 8.5 and 8.5 for the case of bilayers complexed with the myelin basic protein, apocytochrome *c*, cytochrome *c* and lysozyme, respectively. The large upward shifts in pK_a are interpreted in terms of a strongly reduced polarity at the lipid/protein interface, suggesting that the dimyristoyl phosphatidylglycerol headgroups become largely dehydrated on binding of either myelin basic protein or apocytochrome *c*. The extent of this dehydration is considerably less for cytochrome *c* and lysozyme.

Introduction

Peripheral proteins are associated with membrane surfaces primarily by electrostatic interactions, although some hydrophobic association may also be possible. In this association, the peripheral proteins may achieve the correct alignment for enzymatic reactions, as for example in the cytochrome *c*-cytochrome oxidase couple, or they may contribute to the structural stability of the

membrane, as for example in the case of the basic protein in the myelin sheath.

The molecular details of the surface association are important for determining the strength of the protein-lipid interactions. For instance, a possible dehydration of the lipid surface on binding of the protein could strengthen both the electrostatic and hydrophobic components of the interaction. The resulting low polarity of the lipid surface would enhance the electrostatic forces via the lower local effective dielectric constant in region of the charged groups, and removal of water would also enhance the hydrophobic association by allowing intimate contact of the hydrophobic groups on the protein and lipid.

One method of probing the surface interactions is via the pH titration of protonatable groups at the lipid surface. On the one hand the pK_a of these groups is partially determined by the local interfacial polarity, which affects the relative stability of the charged and uncharged species. On the other hand, the change in the

Abbreviations: ESR, electron spin resonance; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; 5-SASL, 5-(4,4-dimethylloxazolidine-*N*-oxyl)stearic acid; MBP, bovine spinal cord myelin basic protein.

Correspondence: Dr. D. Marsh, Max-Planck-Institut für biophysikalische Chemie, Abteilung Spektroskopie, D-3400 Göttingen, F.R.G.

net lipid charge on protonation of the lipid headgroup provides a rather direct method of assessing the strength of the electrostatic contribution to the interaction.

In the present work we have investigated the pH titration of a spin-labelled fatty acid incorporated into dimyristoylphosphatidylglycerol bilayers, and have studied the effects of binding of different peripheral proteins on the properties of this pH titration. It is shown that the hyperfine splittings in the ESR spectra of the spin-labelled fatty acid can be used both to determine the pK_a of the fatty acid carboxyl group and to determine the strength and selectivity of interaction of the fatty acid with the peripheral proteins bound at the membrane surface. The results indicate that the charged form of the fatty acid associates more strongly with the protein than does the protonated form, and the large upward shifts in pK_a , observed in the presence of apocytochrome *c* and the myelin basic protein, suggest that the lipid surface is largely dehydrated on binding of the protein.

Materials and Methods

Materials. DMPG was synthesized from DMPC (Fluka, Buchs, Switzerland) by a headgroup exchange reaction catalyzed by phospholipase D [1]. 5-SASL was synthesized as described in Ref. 2. Hen egg white lysozyme was from Serva (Heidelberg, F.R.G.).

MBP Isolation. Bovine spinal cord was obtained fresh from the slaughterhouse and frozen immediately in liquid N_2 . Myelin basic protein was extracted and purified as described in Ref. 3. The protein was further purified by chromatography on a Sephadex G-75 superfine column (3×90 cm) equilibrated with 10 mM HCl. The protein ($M_r = 18400$) was pure as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulphate [4]. Protein concentrations were measured spectrophotometrically by using a value for the absorbance of $A_{1\%}^{276.4} = 5.64$ [5].

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. 6. 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. 7 and subjecting the haem-free protein to a renaturation procedure [8]. The protein was stored at -20°C in 0.5 ml aliquots at a concentration of about 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. DMPG was co-dissolved with 1 mol% 5-SASL spin label in CH_2Cl_2 , the solvent removed with a stream of dry nitrogen, and the samples dried under vacuum overnight. Lipid dispersions were prepared by vortex mixing the dry lipid mixture in the required buffer solution at a concentration of approximately 1 mg/5 ml. For measurements on the lipids alone, the lipid dispersion was incubated at 35°C for 1 h and then pelleted by centrifugation (Beckman Ti65 rotor, 30000 rpm, 45 min, at 4°C). (Since DMPG does not titrate in the pH region studied [12], it is extremely unlikely that the morphology of the dispersions would change over this pH range.) The lipid-protein complexes were formed in each case by adding a solution of the protein to the hydrated DMPG + spin label dispersion in the appropriate buffer and incubated at 35°C for 1 h. The lipid dispersions precipitated either instantaneously (MBP and apocytochrome *c*) or after 15 min incubation (lysozyme and cytochrome *c*) on adding the protein solution. No difference was observed in the ESR spectra if, alternatively, the lipid-protein complexes were prepared by hydrating the dry lipid directly in the protein solution, provided that the incubation step was made. All the buffer solutions contained 10 mM buffer (citric acid/HCl or NaOH (pH 2–5), NaCl/HCl (pH 5–6), sodium borate/HCl (pH 6–7), Tris (pH 7–5), sodium borate/NaOH (pH 8–10), NaCl/NaOH (pH 10–12)), 10 mM NaCl, and 0.1 mM EDTA such that near constancy of ionic strength could be maintained. The protein solutions were dialysed against buffers of the desired pH. Stock solutions of the proteins corresponded to 1 mg/ml MBP, 1.5 mg/ml apocytochrome *c* and 1 mg/ml cytochrome *c* and 1 mg/ml lysozyme. Saturation levels of binding were ensured by adding 2.5 mg of MBP or 3 mg apocytochrome *c* or 3 mg cytochrome *c* or 4 mg lysozyme per mg DMPG. The lipid-protein complexes were isolated as pellets upon centrifugation in a bench top centrifuge. The pellets (lipid or lipid-protein complex) were transferred to 100 μl capillaries which were used for the ESR measurements. The samples were then further concentrated by centrifugation. After the ESR measurements were completed, the pellets were dissolved in a few drops of 1 N NaOH for determining the lipid and protein contents. Phospholipid concentration was determined by the method described in Ref. 9. Protein assays were performed according to the method of Lowry et al. [10].

ESR Spectroscopy. ESR spectra were recorded on a Varian Associates E-line 9 GHz spectrometer. Spectra were digitized and stored on a PDP 11/10 minicomputer equipped with a DEC-LPS system. Temperature was regulated using a nitrogen gas-flow system, and measured with a fine-wire thermocouple located close to the sample at the top of the cavity. The outer hyperfine

splitting constant, A_{\max} , was measured from the separation ($2A_{\max}$) between the low-field maximum and the high-field minimum peaks in the first-derivative spectra of unoriented suspensions (see, for example, Ref. 11).

Results

The pH dependence of the ESR spectra of the stearic acid spin label, 5-SASL, in bilayers of DMPG with saturating amounts of the myelin basic protein bound are given in Fig. 1. The ESR spectra are all very similar at pH values less than 9, and in particular the hyperfine splittings do not change appreciably over the range pH 6 to 8. At pH values of 9 and above, large changes are seen in the ESR spectra. The spectra become broader and the hyperfine splitting between the outer peaks increases greatly as the pH is increased above pH 9. These changes arise from titration of the fatty acid, since the titration of the protein takes place at higher pH, with a pI which is most probably in excess of 10.6 in solution.

The pH titration of 5-SASL both in DMPG alone and in the DMPG-MBP complexes has been followed from the changes in the outer hyperfine splitting constant, A_{\max} . These data are given in Fig. 2. In bilayers of DMPG alone, 5-SASL displays a well-defined titration in the values of A_{\max} with a pK_a of 8.0*. In the DMPG-MBP complexes, the titration of the 5-SASL carboxyl group is shifted to higher pH and has a midpoint at a pK_a of approximately 9.6. The upper part of this titration overlaps with that of the protein, which is evidenced by a sharp decrease in A_{\max} at pH values above 10. This decrease in A_{\max} corresponds to titration of the basic amino acid side chains of the MBP. It has previously been shown that this titration results in a large decrease in the binding of the protein to the lipid, as the isoelectric point of MBP is approached [13]. The decrease in A_{\max} of 5-SASL is therefore caused by the decrease in protein binding. By pH 11, A_{\max} has been reduced to a value not much greater than that observed in the absence of protein at this pH. A similar decrease in A_{\max} has also been observed in MBP-DMPG complexes for spin-labelled phosphatidylglycerol which does not titrate in this pH range [13]. In this latter case, no increase was observed in A_{\max} at pH values above 9, and no titration behaviour was detected either in the presence or absence of MBP over the range pH 6 to 9.

The pH titration of 5-SASL has also been studied in DMPG complexes with saturating amounts of either apocytochrome *c*, cytochrome *c* or lysozyme. The pH

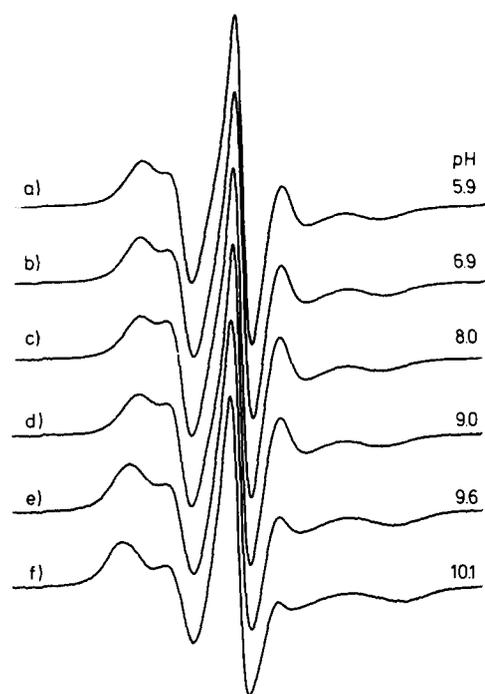


Fig. 1. ESR spectra of the 5-SASL stearic acid spin label in DMPG bilayers with a saturating amount of MBP bound, as a function of the pH of the lipid-protein suspension in 10 mM buffers (containing 10 mM NaCl). $T = 30^\circ\text{C}$; scan width = 100 G.

dependence of the outer splitting, A_{\max} , is given for complexes with these three proteins in Fig. 3. The titration of the carboxyl group of 5-SASL is clearly seen in DMPG-apocytochrome *c* complexes from the sharp increase in A_{\max} centered at a pK_a of approximately 9.2. As for the MBP complexes, the upper part of this titration again overlaps with the titration of the basic amino acids in the protein, which is seen from the decrease in A_{\max} at pH values of 10 and above.

For the complexes with cytochrome *c*, two titrations are seen in the values of A_{\max} for 5-SASL over the pH

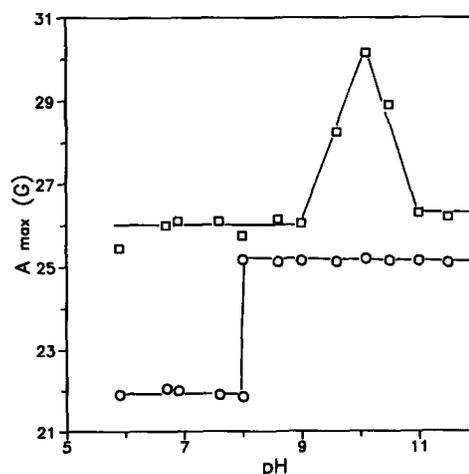


Fig. 2. pH titration of the outer hyperfine splitting constant, A_{\max} , of the 5-SASL spin label in DMPG bilayers in the presence (\square) and in the absence (\circ) of a saturating amount of MBP. $T = 30^\circ\text{C}$.

* The abrupt change shown in Fig. 2 corresponds to the coexistence of spectra from the titration limits which have approximately equal populations at pH 8.0. This therefore corresponds to the pK_a .

range from 6 to 12 (Fig. 3). The first titration, with a pK_a of approximately 8.5, corresponds to the protonation/deprotonation of the 5-SASL spin label. The second titration, with a mid-point in the region of $pK_a \sim 11.5$, corresponds to the titration of the basic amino acid side chains of the protein. The isoelectric point of cytochrome *c* in solution has a value of $pI = 10$ [14], and it is to be expected that this value will be greater at the surface of negatively charged DMPG bilayers, due to the higher local hydrogen ion concentration. The saturation binding of cytochrome *c* to DMPG bilayers decreases steeply at pH values above 10, being reduced to zero at pH 12 (data not shown) which is in good agreement with the high pH titration behaviour of the values of A_{max} for 5-SASL in Fig. 3. The binding remains constant up to pH 9, suggesting only small overlap between the protein and fatty acid titrations.

For complexes of lysozyme with DMPG, two clearly defined titrations are seen over the pH range 6 to 12 in the A_{max} values for 5-SASL (Fig. 3). The values of A_{max} increase abruptly at the pK_a of 8.5, corresponding to the titration of the 5-SASL, and then increase gradually up to pH 11. This is then followed by a sharp decrease in A_{max} , with a pK_a of approximately 11.4, corresponding to the titration of the basic amino acid side chains. The isoelectric point of lysozyme in solution occurs at $pI = 11.3$ [15]. The protein binding increases slightly

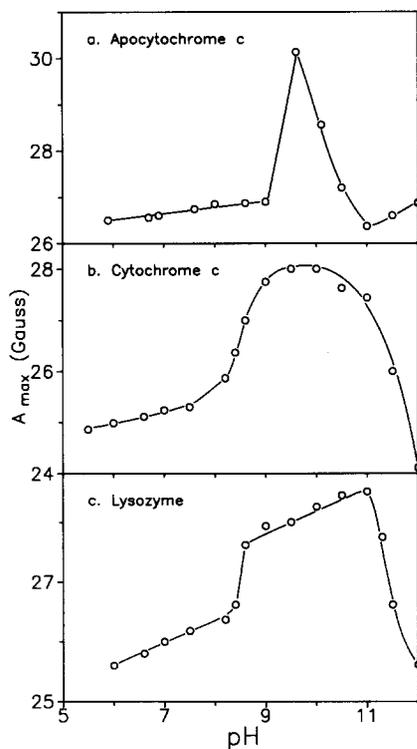


Fig. 3. pH titration of the outer hyperfine splitting constant, A_{max} , of the 5-SASL spin label in DMPG bilayers in the presence of a saturating amount of: (a) apocytochrome *c*, (b) cytochrome *c*, and (c) lysozyme. $T = 30^\circ\text{C}$.

TABLE I

Increases in outer hyperfine splitting constant (ΔA_{max}) at 30°C of 5-SASL in the protonated (SASLH) and unprotonated (SASL⁻) states, on binding peripheral proteins

Estimates of the relative association constant (K_r) in the two protonation states are also given, using Eqn. 3.

Protein	ΔA_{max} (G)		K_r	
	SASLH	SASL ⁻	SASLH	SASL ⁻
MBP	4.05	5.0	1.4 (0.2) ^a	2.6
Apocytochrome <i>c</i>	4.55	5.0	1.9 (0.3) ^a	2.5
Cytochrome <i>c</i>	2.80	2.85	0.7 (0.0) ^a	0.7
Lysozyme	3.55	3.35	1.1 (0.1) ^a	1.0

^a Calculated assuming A^f in Eqn. 3 has the value for SASL⁻ in pure lipid (cf. footnote on p. 67).

over the range pH 9 to 11 and then decreases abruptly beyond pH 11, in parallel with the hyperfine splitting data for 5-SASL in Fig. 3. No change in binding is observed in the region of pH 8.5.

In addition to the effects of peripheral protein binding on the pK_a of 5-SASL in DMPG bilayers, the association of the protein also has a marked effect on the mobility of the spin label. The values of the outer hyperfine splitting in the presence of protein are considerably greater than those in the lipid bilayers alone (Figs. 2, 3). The size of this motional perturbation can be assessed from the differences (ΔA_{max}) of the outer hyperfine splitting in the presence and absence of protein. These values of ΔA_{max} are given, for the different proteins and for the two protonation states of 5-SASL, in Table I. A large effect is seen with all proteins for the protonated state of 5-SASL and, for MBP and apocytochrome *c*, this effect is even greater in the unprotonated (charged) state of 5-SASL.

Discussion

The effects of pH titration on the ESR spectra of the 5-SASL stearic acid spin label in peripheral protein-DMPG complexes yield two important sets of information on the nature of the protein-lipid interaction. One is the extent of perturbation of the chain mobility of the lipid spin label, which reflects the strength or selectivity of interaction with the peripheral proteins. The other is the shift in pK_a of the carboxyl group of the fatty acid at the protein/lipid interface which depends on the polarity in the region of the lipid headgroups, the extent of electrostatic neutralization of the lipid polar groups and on the energetics of the lipid-protein interaction. These two aspects are considered separately below.

Selectivity and protonation state

The dependence of the outer hyperfine splitting, $2A_{max}$, of 5-SASL on protein association may be

analyzed quantitatively, if it is assumed that the ESR spectra correspond to fast exchange on the ESR time-scale. The model assumes that there are n_p specific 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 splitting constant, A^f , which is taken to be that in the pure lipid *. The outer hyperfine splitting constant is then given by the usual expression for fast exchange:

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

Here f is the fraction of the spin-labelled lipids that is associated with the specific sites and which 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 lipid/protein ratio. Combining Eqns. 1 and 2, the relative association constant for the spin-labelled lipid is given by:

$$K_r = (n_t/n_p - 1) \cdot (A_{\max} - A^f) / (A^p - A_{\max}) \quad (3)$$

Values of the relative association constants for 5-SASL in its different protonation states, when interacting with the different peripheral proteins, have been calculated

* The model assigns all changes in A_{\max} to a direct effect of the association of the spin-labelled lipid with the protein. Other effects, such as a longer range influence of the protein on the bulk order of the lipid or a change in the vertical position of the label in the bulk host lipid, are therefore included in the specific interaction. This will result in the lipid association constants derived being upper estimates. For the unprotonated form of the stearic acid spin label, SASL⁻, changes in vertical location other than allowed for specifically in A^p are unlikely to be very significant, since SASL⁻ in the absence of protein has a similar value of A_{\max} to that found previously for the corresponding spin-labelled phosphatidyl glycerol [29], and therefore may be assumed to be positioned vertically in register with the host lipid. However, the protonated form of the stearic acid spin label, SASLH, resides deeper in the bilayer in the absence of protein [18–20], as evidenced by the lower value of A_{\max} at low pH in Fig. 2. Therefore it is possible that the protonated form of the label may move vertically upward in the bulk regions of the bilayer, in response to protein binding. This would give rise to an overestimate of the association constant for SASLH and artefactually diminish the difference in selectivity between the unprotonated and protonated forms. An upper limit for this effect can be obtained by taking the value of A^f for SASLH to be equal to that for SASL⁻ (where the label is in register with the host lipid). The long-range effects are more difficult to estimate, but will most probably lead to comparable overestimates in the selectivities for both protonated and unprotonated forms and therefore will not strongly affect the relative selectivities between the two forms of the fatty acid or the comparison with spin-labelled phospholipids.

in this way and are given in Table I. For the purpose of this calculation, it was assumed that $A^p = A^f + 7.18$ G and that for MBP: $n_p = 17$, where the total lipid/protein ratio at saturation binding is $n_t = 36$. These values have been chosen to be consistent with the higher values of ΔA_{\max} observed for other spin-labelled lipids in MBP/DMPG complexes, and in particular yield a value of $K_r = 1$ for the phosphatidylglycerol spin label in DMPG [29]. It is also interesting to note that the value for n_p is quite close to the net positive charge, $Z = +20$, on the MBP. For the other peripheral proteins, the same value as established for MBP has been assumed for A^p and for the factor $(n_t/n_p - 1) = 1.118$, in order to make a consistent comparison of the relative association constants. The relative values of K_r are not particularly sensitive to small changes in these parameters (see, however, the footnote on this page).

According to Table I, 5-SASL in its unprotonated form displays a significant selectivity relative to phosphatidylglycerol, for interaction with apocytochrome *c* and with myelin basic protein. This is not the case, however, for cytochrome *c* and for lysozyme, which indicates a different pattern of lipid specificity for the different proteins. As discussed in the footnote on this page, uncertainties exist in the estimate of the relative association constant for the protonated fatty acid, because of possible changes in the vertical location of the label. This is probably the reason for the apparent selectivity of SASLH relative to DMPG with MBP and with apocytochrome *c*, when the value of A^f for SASLH is used in the calculation. When the value of A^f for SASL⁻ is used (cf. footnote on this page), a pronounced selectivity of the unprotonated fatty acid relative to the protonated fatty acid is obtained for all four proteins. It is significant, however, that 5-SASL displays an increased selectivity of interaction with both MBP and apocytochrome *c* on titrating to the unprotonated state, irrespective of the choice of A^f . This further illustrates the importance of electrostatic interactions for the association of anionic lipids with the basic peripheral proteins. Similar results have been obtained for the relative selectivities of interaction of the singly and doubly charged species of phosphatidic acid with the MBP [29]. In this latter case, the change in vertical location of the label is much smaller, and probably negligible, on titrating the phospholipid headgroup.

Shifts in interfacial pK_a

The intrinsic pK_a of the stearic acid carboxyl group has a value of $pK_a^0 \approx 5.0$ for the monomer in bulk solution [21]. The interfacial pK_a in the bilayer, pK_a^{int} , is shifted from the intrinsic value, pK_a^0 , by an amount $\Delta pK^{\text{int}} (= pK_a^{\text{int}} - pK_a^0)$ which depends both on the interfacial polarity and the surface electrostatics (see, for example, Ref. 22). In the presence of protein, there will be an additional contribution to the shift in pK_a

arising from the differential interaction of the protein with the two titrating forms, L and LH, of the lipid. Thus, the net shift in pK_a at the interface is given by:

$$\Delta pK^{\text{int}} = \Delta pK^{\text{pol}} + \Delta pK^{\text{el}} + \Delta pK^{\text{LP}} \quad (4)$$

where ΔpK^{pol} is the polarity-induced shift which has been determined to have a value of $\Delta pK^{\text{pol}} \approx 1.1$ at the surface of detergent micelles [23]. This contribution to the shift in pK_a is positive, since the forward reaction $SA^- + H^+ \rightarrow SAH$ is favoured by the lower polarity at the lipid surface. The electrostatic shift in pK_a , ΔpK^{el} , is related to the surface potential, Φ , by the following equation (see, for example, Ref. 22):

$$\Delta pK^{\text{el}} = -e\Phi / (\ln 10 \cdot kT) \quad (5)$$

where T is the absolute temperature and k is Boltzmann's constant. The shift due to the lipid-protein interaction is related to the relative association constants of the protonated and unprotonated lipid species, and is given by [24]:

$$\Delta pK^{\text{LP}} = \log_{10} [K_r(\text{LH})/K_r(\text{L})] \quad (6)$$

where $K_r(\text{LH})$ and $K_r(\text{L})$ are the values of the relative association constants for the interaction of the protonated and unprotonated spin labels with the protein, respectively.

5-SASL has been found to titrate with pK_a values of 6.6 and 6.7 in bilayers of DMPC-cholesterol [25] and of DMPC alone [26], respectively, corresponding to a mean polarity-induced shift of $\Delta pK^{\text{pol}} = 1.65$, relative to the intrinsic pK_a for the carboxyl group. This corresponds to an effective interfacial dielectric constant of $\epsilon \approx 23$, using the calibrations given in Ref. 23. The present measurements with DMPG yield a pK_a of 8.0 for 5-SASL in the negatively charged bilayers without protein. This corresponds to an electrostatic shift of $\Delta pK^{\text{el}} = 1.4$ relative to DMPC-cholesterol, and implies a surface potential of approx. -85 mV for the DMPG bilayers, when calculated according to Eqn. 5.

In the presence of the peripheral proteins, the pK_a shift due to the lipid-protein interaction is estimated from the data in Table I to have values of $\Delta pK^{\text{LP}} = -0.25$ and -0.12 for MBP and apocytochrome c , respectively, and to be negligible for cytochrome c and lysozyme *. The electrostatic shift is expected to be reduced by the neutralization of the surface charge on binding of the protein, by an amount which can be

estimated from electrostatic double layer theory. Substituting the appropriate expression for the surface potential in Eqn. 5 yields (see, for example, Ref. 22):

$$\Delta pK^{\text{el}} = (2/\ln 10) \sinh^{-1}(\sigma/c) \quad (7)$$

where σ is the electrostatic charge density at the lipid surface, and $c = (8000\epsilon\epsilon_0RTI)^{1/2}$, with I being the ionic strength and ϵ the interfacial dielectric constant. The net positive charge on the MBP is $Z = +20$ and this is associated with 36 DMPG molecules, each with net negative charge $Z = -1$ [13]. Therefore the net negative surface charge is reduced by a factor $16/36 = 0.44$ on binding of the MBP. Assuming a value of $\epsilon = 23$ for the interfacial dielectric constant yields a reduction of approximately 25% in ΔpK^{el} , calculated using Eqn. 7. Similar considerations indicate that the surface charge is completely neutralized on binding of apocytochrome c , and also approximately for cytochrome c (cf. Ref. 28). Therefore ΔpK^{el} will be reduced by approximately 100% for saturation binding of these proteins.

The pK_a of 5-SASL in DMPG bilayers is shifted by a net amount of $+1.6$ or $+1.2$ pH units on binding of MBP or apocytochrome c , respectively, and by $+0.5$ pH units for both cytochrome c and lysozyme. Correction for the predicted changes in the electrostatic shift on protein binding, and for the shift in pK_a caused by the lipid-protein interaction, yield values of $+2.2$, $+2.7$ and $+1.9$ pH units for the increase in shift due to the change in interfacial polarity on binding of MBP, apocytochrome c and cytochrome c , respectively **. Clearly, the polarity sensed by the carboxyl group of the fatty acid is considerably lower for the lipid/protein interface than for the lipid/water interface in the absence of protein. The most likely cause of this effect is a reduction in the hydration of the lipid headgroups on binding of the peripheral proteins. An alternative explanation, that the charged fatty acid sinks deeper into the hydrophobic region of the bilayer on binding of the protein, is unlikely in view of the large increase observed in the outer hyperfine splitting. The size of the total polarity-induced shift in pK_a in the presence of saturating amounts of protein is greater than the maximum value given in the calibrations of Ref. 23, which corresponds to a solvent of dielectric constant $\epsilon \approx 9$. Extrapolation of the calibrations indicates that the effective dielectric constant at the protein/lipid interface is $\epsilon \approx 2-3$ and therefore suggests that the lipid may become almost completely dehydrated on binding of the peripheral proteins.

X-ray diffraction studies on complexes of MBP with anionic lipids (different from those used in the present work) have indicated that the MBP is closely associated

* Use of the values for $K_r(\text{LH})$ obtained by taking the value for A^I to be that for SASL^- (see Table I) yields values of $\Delta pK^{\text{LP}} \approx -1$, which serves to make the polarity-induced shift on protein binding even greater and therefore doesn't affect the subsequent conclusions regarding interfacial dehydration.

** See footnote in left-hand column.

with the lipid headgroups [30–32]. This result is consistent with the dehydration of the lipid headgroups by the protein that is deduced from the data presented here. Significantly, a more intimate association of the MBP with the lipid surface was found than for cytochrome *c* [30,31], which correlates rather well with the larger polarity shift in the pK_a of 5-SASL induced by MBP than by cytochrome *c*.

It is interesting to note that, using quite different methods, it was recently concluded that interfacial dehydration takes place on binding of phospholipase A_2 to negatively charged lipid surfaces, whilst in contrast binding of the pro-phospholipase does not alter the lipid hydration [27]. These results are in good accord with the present measurements of the polarity-induced shifts in pK_a of 5-SASL on binding extrinsic proteins. It would appear that dehydration of the lipid surface is an important factor in the interaction of at least some, if not all, extrinsic proteins with biological membranes.

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