Interaction of α-Lactalbumin with Phosphatidylglycerol. Influence of Protein Binding on the Lipid Phase Transition and Lipid Acyl Chain Mobility†

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ABSTRACT: The mobility of spin-labeled lipids has been studied in dioleoyl and dimyristoyl phosphatidylglycerol bilayers and in their complexes with α-lactalbumin at pH 4.0, by using electron spin resonance (ESR) spectroscopy. The ESR spectra of phosphatidylglycerol spin-labeled at position 5 of the *sn*-2 chain indicate that association of α-lactalbumin with dimyristoyl phosphatidylglycerol bilayers increases the chain mobility at temperatures in the lipid gel phase, restricts the chain mobility at temperatures corresponding to the lipid fluid phase, and abolishes the cooperative lipid chain-melting transition. The ESR spectra of phosphatidylglycerols spin-labeled at eight different positions in the *sn*-2 chain show that binding of α-lactalbumin to dioleoyl phosphatidylglycerol bilayers at pH 4.0 causes a motional restriction throughout the full length of the lipid acyl chain. For phosphatidylglycerols spin-labeled at the terminal methyl end of the chains, a population of motionally restricted lipids that directly contacts membrane penetrant portions of the protein is detected. This population corresponds to 6.3 ± 0.7 lipids/α-lactalbumin at saturation binding, and the high degree of motional restriction (maximum hyperfine splitting ≈ 60 G) suggests that the protein may traverse the lipid bilayer. A small selectivity of phosphatidylglycerol over zwitterionic phospholipids for interaction with α-lactalbumin is found at subsaturating levels of binding at pH 4.0. Binding of α-lactalbumin also strongly restricts the motion of lipids spin-labeled in the polar head group region. These results are of direct relevance to the insertion and translocation of a protein in the molten globule state across lipid membranes.

Partial unfolding is an essential feature in the post-translational membrane translocation of proteins (Eilers & Schatz, 1988). This finding is based on studies of the maltose-binding protein precursor in *Escherichia coli* (Randall & Hardy, 1986), precursors of F_1-ATPase β-subunit and cytochrome c_1 in *Neurospora* (Schleyer & Neupert, 1985), and mouse dihydrofolate reductase fused to a mitochondrial presequence (Eilers & Schatz, 1986). Some soluble globular bacterial toxins can penetrate and translocate the lipid membrane when they suffer partial unfolding and adopt the molten globule conformation at acidic pH (Parker & Pattus, 1993). The molten globule is a conformational state in which the protein has the native secondary structure, but the specific interactions of the tertiary structure are largely absent. The protein is partially unfolded but remains relatively compact (Dolgikh et al., 1981, 1984; Goto & Fink, 1989; Kuwajima, 1989). Bychkova et al. (1988) have proposed that the molten globule is the partially unfolded conformational state competent for membrane translocation. The molten globule is a kinetic intermediate between the native and the unfolded structure; when it is relatively stable compared to the native state, it can be trapped as an intermediate (Ikeguchi et al., 1986; Kuwajima et al., 1985). The study of this structure is relevant to understanding the folding process, membrane translocation, and the insertion or translocation of soluble proteins into lipid membranes.

The molten globule conformation of α-lactalbumin (α-LA)[1] has been studied extensively (Alexandrescu et al., 1993; Dolgikh et al., 1981, 1985; Kuwajima, 1989). The interactions of α-LA with lipid membranes have also been studied (Hanssens et al., 1980, 1983, 1985; Hanssens & Van Cauwelaert, 1978; Herreman et al., 1981; Kim & Kim, 1986, 1989; Van Cauwelaert et al., 1983). It has been shown, both in monolayers at the air−water interface and in bilayer membranes (Hanssens et al., 1980, 1985), that α-LA interacts with the polar head groups of PC in the native state at pH 7. At acidic pH, however, α-LA also interacts hydrophobically with PC membranes. At low pH, α-LA binds to vesicles composed of 1:1 PE:PS and induces their fusion (Kim & Kim, 1989). These authors have also shown that the segment from residue 80 to 108 is protected from trypsin digestion when the protein is bound to the lipid membrane. A large portion of this segment is found to be in an α-helical conformation (Acharya et al., 1989). In the bound state, at pH 4.0, only one side of this helix was labeled with a photoactivatable label that partitions into the hydrophobic core of the membrane (Kim & Kim, 1986), suggesting that the protein lies parallel to the plane of the membrane.

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[1] Abbreviations: α-LA, α-lactalbumin; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; *n*-PGSL, *n*-PCSL, *n*-PESL, and *n*-PSSL, 1-acyl-2-[*n*-(4,4-dimethyl-*N*-oxyoxazolidinyl)]stearoyl-*sn*-glycero-3-phosphoglycerol, -phosphocholine, -phosphoethanolamine, and -phosphoserine, respectively; C_9-N^+SL, 4-(*N*′,*N*′-dimethyl-*N*′-nonylamino)-2,2,6,6-tetramethyl-*N*-oxypipiderine bromide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance.

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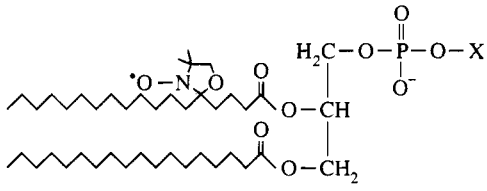
Scheme 1: Chemical Structures of Spin-Labeled Lipids[a]

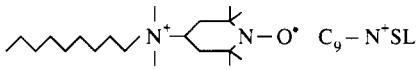


X = $-(CH_2)_2\overset{+}{N}(CH_3)_3$ 5-PCSL

$-CH_2CH(OH)CH_2OH$ 5-PGSL

$-CH_2CH(COO^-)NH_3^+$ 5-PSSL

$-(CH_2)_2NH_3^+$ 5-PESL



C_9-N^+SL

[a] The phospholipids shown bear the spin label on the 5-position of the *sn*-2 chain.

In the present work, we have studied the interaction of α-LA with phosphatidylglycerol bilayers at a bulk pH of 4.0 by using ESR spectroscopy. The ESR spectra of spin probes attached to different positions of the phospholipid *sn*-2 acyl chain have been used to determine the effects on the lipid chain mobility and to assess how deeply α-LA perturbs the membrane hydrophobic core. The temperature dependence of the spin label spectra has been studied to determine the influence of α-LA on the chain-melting lipid phase transition of dimyristoyl phosphatidylglycerol bilayers. The spectra from different spin-labeled phospholipids were also used to investigate whether α-LA shows a selective interaction with a particular phospholipid headgroup.

MATERIALS AND METHODS

Materials. α-LA type I and III from bovine milk was obtained from Sigma (St. Louis, MO). The protein ran as a single band in 14% polyacrylamide SDS-PAGE electrophoresis. DMPG, DMPC, and DOPG were obtained from Avanti Polar Lipids (Alabaster, AL). Spin-labeled phospholipids were prepared as described in Marsh and Watts (1982). The C_9-N^+SL spin label was obtained from Molecular Probes (Eugene, OR). The chemical structures of the various spin labels are given in Scheme 1.

Sample Preparation. One milligram of DMPG in 2:1: 0.1 chloroform:methanol:water (v/v/v) or 1 mg of DOPG in 2:1 chloroform:methanol (v/v) was mixed with 0.01 mg of spin-labeled phospholipid in dichloromethane solution. The lipid mixture was dried under a nitrogen stream and kept under vacuum overnight. The dry lipid was hydrated in a buffer containing 10 mM acetic acid, 10 mM NaCl, and 0.1 mM EDTA at pH 4.0 or in the same buffer containing different amounts of α-LA. The samples were incubated for 30 min at room temperature (DOPG-containing samples) or at 35 °C (DMPG-containing samples). The lipid or the lipid/protein complexes were gently removed with a Teflon spatula and sedimented in a bench top centrifuge. The lipid-protein complexes required 5 min of centrifugation, whereas the lipids alone or lipids in the presence of protein at pH 7.0 required at least 30 min to sediment. The pellets were transferred to 1-mm glass capillaries and further concentrated in a bench top centrifuge. Samples containing the lipid alone were further concentrated by centrifugation at 15000*g*.

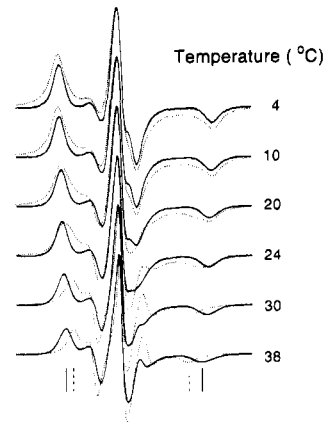


FIGURE 1: ESR spectra of the 5-PGSL spin label in DMPG/α-LA complexes with a protein/lipid ratio of 0.07 mol/mol (continuous line) and in DMPG dispersions (dotted line) at the temperatures indicated. The outer hyperfine splittings, $2A_{max}$, for the spectra at 38 °C are indicated by the solid and dashed vertical lines for the DMPG/α-LA complex and DMPG dispersion, respectively. Buffer: 10 mM acetic acid, 10 mM NaCl, 0.1 mM EDTA, pH 4.0. Total scan width: 100 G.

ESR Spectroscopy. ESR spectra were recorded on a Varian E-12 Century Line 9 GHz spectrometer with nitrogen gas flow temperature regulation. The capillaries containing the samples were placed in a 4 mm quartz tube which contained silicone oil for thermal stability. The temperature was measured with a thermocouple placed inside the quartz tube just above the ESR cavity. ESR spectra were recorded at a microwave frequency of 9 GHz, with a field scan of 100 G centered at 3245 G. Spectral subtractions and other ESR analysis were performed as described in Marsh (1982).

After ESR measurements, the samples were dissolved in 0.2 mL of 10% SDS and 0.5 M NaOH. Aliquots of these solutions were taken to quantify lipid phosphate using the method of Bartlett (1959) and protein by the method of Lowry et al. (1951).

RESULTS

At pH 7.0, the presence of α-LA did not produce any significant change in the ESR spectra from the 14-PGSL or 5-PGSL spin labels in DOPG or DMPG membranes. The macroscopic appearance of the lipid dispersions did not change either, upon addition of the protein. At pH 4.0 and below, however, lipid/protein complexes precipitated from the dispersion upon addition of α-LA. All the results reported here were obtained from dispersions at a pH value of 4.0 in the bulk solution. The pH at the surface of the negatively charged lipid bilayers is, however, much lower than this for electrostatic reasons (see Discussion). α-LA preparations contain Ca^{2+} (Hiraoka et al., 1980) that binds with an association constant on the order of nM^{-1} (Bryant & Andrews, 1984; Mitani et al., 1986; Permyakov et al., 1987; Van Ceunebroeck et al., 1985). At acidic pH, H^+ ions compete for the Ca^{2+} binding site and the metal ion is released to the aqueous medium (Permyakov et al., 1981). Control experiments were performed to check that the changes in the lipid spin label ESR spectra observed in the presence of α-LA were caused by interaction with the protein and not by Ca^{2+} that could be released at low pH.

Temperature Dependence with DMPG. Figure 1 shows the ESR spectra of phosphatidylglycerol spin-labeled on the 5-C atom of the *sn*-2 chain (5-PGSL) in DMPG/α-LA

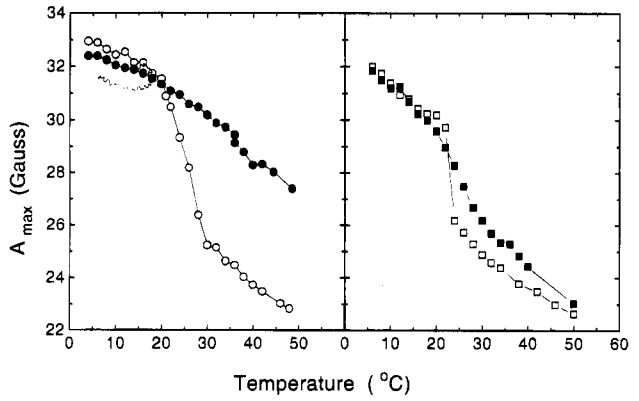


FIGURE 2: (Left) Dependence on temperature of the maximum hyperfine splitting constant, A_{max}, of 5-PGSL in DMPG/α-LA complexes with a protein/lipid ratio of 0.07 mol/mol (●) and in DMPG dispersions (○). α-LA was mixed with DMPG at a lipid/protein ratio of 1:2 w/w to produce these precipitated complexes. (Right) Temperature dependence of A_{max} for 5-PCSL in 1:2 w/w DMPC/α-LA mixtures (■) and in DMPC dispersions (□). Precipitated complexes were not formed with DMPC. Buffer: 10 mM acetic acid, 10 mM NaCl, 0.1 mM EDTA, pH 4.0.

complexes and in dispersions of DMPG alone at different temperatures. At temperatures below the chain-melting phase transition of DMPG dispersions (i.e. up to 20 °C), the protein does not produce large changes in the ESR spectra, the main effects being the removal of the slight spin−spin broadening evident in the spectra from the gel phase lipid alone and a slight reduction in outer hyperfine splitting. Both of these effects, the better mixing of the spin label and the increase in its rotational mobility, are consistent with a loosening of the gel phase lipid packing upon complexation with α-LA. At temperatures above the phase transition of the pure lipid (i.e. 30 °C and above), α-LA causes a large increase in the outer hyperfine splitting of the ESR spectra and a broadening of the spectral line shapes. These effects indicate that a considerable restriction in motion of the fluid lipid chains is induced by α-LA in the lipid/protein complexes.

The temperature dependence of the outer hyperfine splitting, $2A_{max}$, of the 5-PGSL spin label in DMPG/α-LA complexes and in DMPG bilayers alone is given in Figure 2. In the absence of protein, the cooperative lipid chain-melting transition is evidenced by the steep decrease in A_{max} at temperatures above 20 °C. In the lipid/protein complexes, containing a saturating amount of protein, no abrupt change in A_{max} as a function of temperature is observed (Figure 2). The cooperative lipid phase transition is abolished by interaction of the lipids with α-LA. Below 20 °C, the values of A_{max} are decreased slightly relative to those for the lipid alone. Above this temperature, the values are much higher in the lipid/protein complexes than in the fluid lipid bilayers, indicating an intermediate state of lipid chain mobility with a considerably reduced temperature dependence in the DMPG/α-LA complexes. In contrast, the effects of association of α-LA at pH 4.0 with phosphatidylcholine (DMPC) bilayers are much smaller (see Figure 2). Unlike with DMPG, α-LA does not form precipitated complexes with DMPC at pH 4.0. The data given in Figure 2 are for α-LA added to DMPC at the same protein/lipid ratio as it was added to form the complexes with DMPG. The outer hyperfine splitting, A_{max}, of the 5-PCSL spin label is changed very little in DMPC bilayers in the gel phase and is increased

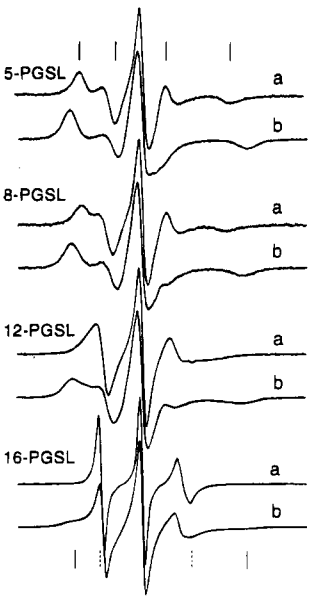


FIGURE 3: ESR spectra of different positional isomers of the *n*-PGSL spin probe labeled at the *n*th C atom of the *sn*-2 chain. (a) *n*-PGSL in DOPG membranes, from upper to lower: 5-PGSL, 8-PGSL, 12-PGSL, and 16-PGSL. (b) *n*-PGSL in DOPG/α-LA complexes with a protein/lipid ratio of 0.07 mol/mol. For 5-PGSL in DOPG alone, the total spectral anisotropy (defined by the difference between the outer and inner hyperfine splittings) is indicated by the outer and inner pair of solid vertical lines. For 16-PGSL, the positions of the outer hyperfine splittings of the motionally restricted and fluid components in the presence of α-LA are indicated by the solid and dashed vertical lines, respectively. Buffer: 10 mM acetic acid, 10 mM NaCl, 0.1 mM EDTA, pH 4.0. Temperature: 20 °C. Total scan width: 100 G.

only by a small amount at higher temperatures in the fluid phase. The principal effect of α-lactalbumin on DMPC bilayers is broadening of the chain-melting phase transition.

Chain Positional Dependence. Figure 3 shows the ESR spectra from phosphatidylglycerol spin probes, *n*-PGSL, with the spin label at different positions, *n*, in the *sn*-2 chain, both in DOPG/α-LA complexes and in dispersions of DOPG alone at pH 4.0. The spectra are recorded at a temperature of 20 °C which is in the fluid phase of DOPG bilayers. The axial anisotropy of the spectra from the lipid alone shows a gradual decrease with position down the chain toward the terminal methyl group that is characteristic of the chain flexibility profile in fluid lipid bilayers (Görrisen et al., 1986; Rottem et al., 1970). A somewhat similar progression is seen for the spectra from the lipid/protein complexes, except that the spectral anisotropy is much larger than in the absence of protein and, for label positions closer to the lipid polar groups, the spectra are typical of slow motions that approach the limits of sensitivity of conventional spin label ESR spectroscopy. Significantly, it can be seen from Figure 3 that α-LA induces a restriction in mobility along the full length of the lipid chain. A further feature is that the spectra from spin labels at positions close to the end of the chain (n = 12 and 16) consist of two components that, in the case of n = 16, are well-resolved. One component has a relatively small anisotropy characteristic of fluid lipids. The second component that is visible in the outer wings of the spectrum from 16-PGSL has a much larger anisotropy and corresponds to lipids for which the chain motion is strongly restricted by direct contact with the protein [cf. Görrissen et al. (1986)].

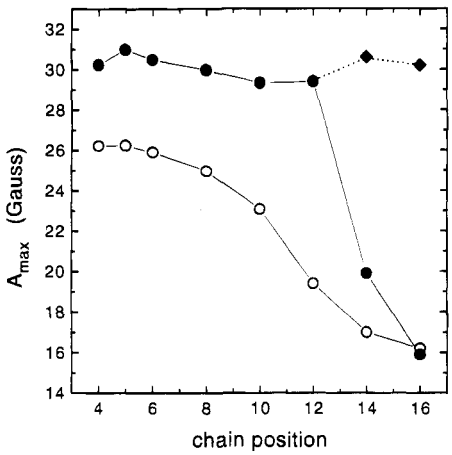


FIGURE 4: Dependence of the maximum hyperfine splitting constant, A_{max}, of the *n*-PGSL nitroxide spin probes on the position of labeling, *n*, in the *sn*-2 acyl chain. (○) *n*-PGSL in DOPG dispersions. (●) *n*-PGSL in DOPG/α-LA complexes (protein/lipid ratio, 0.07 mol/mol). (◆) Value of A_{max} for the motionally restricted spectral component induced by α-LA in spectra where two components are resolved. Buffer: 10 mM acetic acid, 10 mM NaCl, 0.1 mM EDTA, pH 4.0. Temperature: 20 °C.

The dependence of the outer hyperfine splittings, $2A_{max}$, of the *n*-PGSL spin labels in DOPG dispersions and in DOPG/α-LA complexes on the label position, *n*, in the *sn*-2 chain is given in Figure 4. In bilayers of DOPG alone, the hyperfine splittings remain reasonably constant for positions of labeling closer to the polar head group region ($n = 6-8$) and then decrease steeply upon proceeding further down the chain ($n = 10-12$) toward the terminal methyl group ($n \leq 16$). This behavior is characteristic of the chain flexibility gradient for phospholipids in a fluid liquid−crystalline phase [see e.g. Marsh and Watts (1981)].

In the DOPG/α-LA complexes, the outer hyperfine splitting remains approximately constant over a longer section of the chain, from $n = 4$ to $n = 12$. There are two reasons for this difference from the behavior in the lipid bilayers alone. The first is that the hyperfine splitting, $A_{max} \approx 30$ G, is close to the maximum possible for these spin labels in a hydrophobic environment. Therefore, as a result of interaction with the protein, the spectra lie in a regime that is not very sensitive to rotational motion. The second reason is that the spectra are most likely composed of two unresolved components, as evidenced by the spectra from labels close to the methyl end of the chain where the two components are resolved (cf. Figure 3). The result of this is that the effective splittings measured at intermediate chain positions (e.g. $n = 12$) are not truly representative of the component from the more fluid lipids but are biased toward those of the more motionally restricted lipid component. This is seen clearly for the positions of chain labeling where the two spectral components are resolved ($n = 14$ and 16). The outer hyperfine splittings of the fluid lipid component then drop steeply, displaying the characteristic flexibility gradient. Those of the motionally restricted spectral component, however, remain high at close to the maximum value, $A_{max} = 30-31$ G, that is found for the unresolved spectra from label positions close to the polar group end of the chain.

That the outer hyperfine splitting of the motionally restricted lipid component induced by α-LA at pH 4.0 is so large is noteworthy. The value found here is typical of that found for lipids interacting with integral membrane proteins

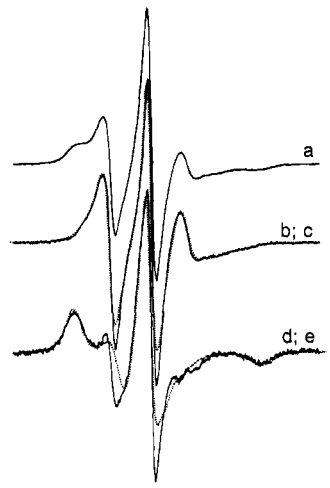


FIGURE 5: Spectral subtraction of the ESR spectrum of 14-PGSL in DOPG/α-LA complexes. (a) Spectrum from 14-PGSL in the protein/lipid complexes with a protein/lipid ratio of 0.074 mol/mol, pH 4.0, at 20 °C. (b, continuous line) Fluid difference spectrum obtained after subtraction of 45% of the double-integrated intensity from spectrum a by using spectrum e. (c, dotted line) Fluid spectrum used in the subtraction, 14-PGSL in DOPG membranes, pH 4.0, at 3 °C. (d, continuous line) Motionally restricted difference spectrum obtained after subtraction of 64% of the double-integrated intensity from spectrum a by using spectrum c. (e, dotted line) Motionally restricted spectrum used in the subtraction, 5-PGSL in DOPG/α-LA complexes, pH 4.0, at 24 °C. Total scan width: 100 G.

such as the acetylcholine receptor (Marsh & Barrantes, 1978) or rhodopsin (Pates & Marsh, 1987). It is considerably larger than that found for peripheral proteins that penetrate the membrane, such as apocytochrome *c* (Görrisen et al., 1986) or the myelin basic protein (Sankaram et al., 1989a). The ESR spectra therefore reflect a considerably greater degree of direct interaction of α-LA with the lipid chains than in the latter two cases.

Two-Component ESR Spectra. The ESR spectra of the 14-PGSL spin label in DOPG/α-LA complexes and in DOPG bilayers alone are given in parts a and c of Figure 5, respectively. The two components are well-resolved in the spectrum from the lipid/protein complex. The spectrum from the lipid bilayers alone was recorded at a lower temperature (3 °C) than that for the lipid/protein complex (20 °C). This was the temperature at which the spectrum from the lipid alone best matched the fluid component in the lipid/protein complex (cf. Figure 4). The two-component nature of the spectrum from the lipid/protein complex (Figure 5a) is revealed very clearly by subtraction of a matching single-component motionally restricted spectrum (Figure 5e) to yield a fluid component difference spectrum (Figure 5b) that is very similar to the single-component lipid bilayer spectrum (Figure 5c). Correspondingly, subtraction of the lipid bilayer spectrum (Figure 5c) from that of the lipid/protein complex (Figure 5a) yields a motionally restricted difference spectrum (Figure 5d) that is very similar to that of the single-component spectrum in Figure 5e. The spectrum in Figure 5e was chosen empirically from a range of experimental single-component spectra as that which best matched the motionally restricted component in the two-component spectrum of interest (i.e. Figure 5a). From the point of view of spectral subtraction, the origin of this single-component spectrum is unimportant; the significant feature is its line shape. However, because this best-matching spectrum

corresponds to a label at the 5-position of the chain, the choice does indicate that the degree of motional restriction of the lipid chains at the 14-position by α-LA is very considerable (cf. Figure 4).

By quantitation of the double-integrated intensities of the spectral components required to achieve the subtraction end points (cf. legend to Figure 5), it is found that the motionally restricted component in the spectrum of the lipid/protein complex contributes a fraction $f = 0.41 \pm 0.05$ to the total spectral intensity. The number of motionally restricted lipids per protein, n_b, can be calculated according to $n_b = fn_t$, where n_t is the total number of lipids per protein in the lipid/protein complex (Marsh & Watts, 1988). From the subtractions shown in Figure 5, a value of $n_b = 5.5 \pm 0.6$ motionally restricted lipids per protein is obtained. This number remains approximately constant within the range from 13 to 26 for the total number of lipids per protein, at a mean value of $n_b = 6.3 \pm 0.7$ lipids per protein. At a higher lipid/protein ratio of $n_t = 52$ mol/mol, however, a value of $n_b = 13$ motionally restricted lipids per protein is found.

In contrast to the situation with anionic phosphatidylglycerol bilayers, no detectable second motionally restricted component is observed in the ESR spectrum of the 14-PCSL spin label in bilayers of the zwitterionic lipid dioleoyl phosphatidylcholine in the presence of α-LA at pH 4.0 (spectra not shown). In this case, $n_b \approx 0$, paralleling the only small effect of α-LA on the outer hyperfine splitting, A_{max}, of the 5-PCSL spin label in DMPC bilayers in the fluid phase at pH 4.0 (see Figure 2). For the 14-PCSL spin label in DOPG bilayers, however, the proportion of motionally restricted spectral component induced by α-LA is only slightly less than that induced with 14-PGSL, paralleling the limited selectivity found with 5-PGSL over that with 5-PCSL in DOPG bilayers (see next paragraph).

Spin Labels with Different Polar Head Groups. ESR spectra were recorded for phospholipid probes with different polar head groups spin-labeled on the 5-C atom of the *sn*-2 chain (5-PCSL, 5-PESL, 5-PSSL, and 5-PGSL) in DOPG/α-LA complexes. The dependence of the outer hyperfine splitting, $2A_{max}$, for the different phospholipid spin labels on the amount of α-LA added to the lipid dispersion is given in Figure 6. The outer hyperfine splitting increases with increasing protein content at different rates for the different spin-labeled phospholipids. Only at very high protein contents do the splittings achieve approximately the same saturating value for all labels. At intermediate protein concentrations, a small selectivity of interaction with the protein is exhibited by the different phospholipids [cf. Sankaram et al. (1989b)]. This lipid selectivity found for the increase, $2\Delta A_{max}$, in outer hyperfine splitting (above the value in the absence of protein) that is given in Table 1 is in the order PG > PS ≈ PC > PE. It will be noted that, at pH 4.0, the carboxyl group of PS should be fully protonated (Cevc et al., 1981).

Lipid Head Group Spin Label. The ESR spectra of a lipid probe bearing the spin label reporter at the head group region and incorporated in DOPG dispersions containing increasing amounts of α-LA at pH 4.0 are given in Figure 7. With increasing amounts of protein added, the spectra broaden progressively, and then the maximum outer hyperfine splitting increases. At a protein/lipid ratio of 0.04 mol/mol, the maximum hyperfine splitting constant is $A_{max} = 30.3$ G at 20 °C, which is comparable to that found for lipids labeled in the chain at a position close to the head group region (cf. Figure 4). The spectra therefore indicate that α-LA also interacts strongly with the lipid head groups at pH 4.0, as well as interacting directly with the lipid chains. These results complement those with the chain-labeled lipids that indicate some selectivity in the interaction with phospholipids of different head group species.

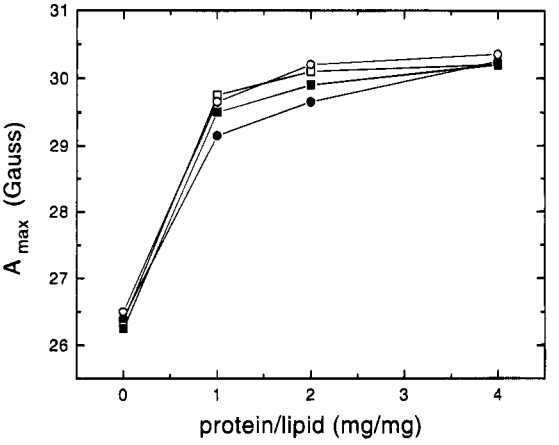


FIGURE 6: Dependence of the maximum hyperfine splitting constant, A_{max}, of different phospholipids spin-labeled at the C-5-position of the *sn*-2 chain in DOPG/α-LA complexes on the amount of α-LA added to the lipid. (■) 5-PCSL, (●) 5-PESL, (○) 5-PSSL, (□) 5-PGSL. Buffer: 10 mM acetic acid, 10 mM NaCl, 0.1 mM EDTA, pH 4.0. Temperature: 20 °C.

Table 1: Increase in Outer Hyperfine Splitting, $2\Delta A_{max}$ (G), at 20 °C for Phospholipids Spin-Labeled at the 5-Position of the *sn*-2 Chain in DOPG/α-LA Complexes at pH 4.0 with Different Amounts of α-LA Added[a]

	α-LA/DOPG (w/w)				
spin label	0.5/1	1/1	1.5/1	2/1	4/1
5-PGSL	2.5	6.8	6.9	7.5	7.7
5-PSSL	1.4	6.3	6.6	7.4	7.7
5-PCSL	1.9	6.5	6.7	7.3	7.9
5-PESL	1.8	5.5	6.6	6.5	7.7

[a] $2\Delta A_{max}$ is defined as the difference in outer hyperfine splitting, $2A_{max}$, in the presence and absence of the given amount of α-LA.

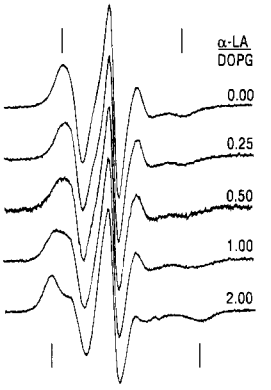


FIGURE 7: ESR spectra of the C_9-N^+SL head group spin label in DOPG dispersions with increasing amounts of α-LA added. From upper to lower, α-LA/DOPG = 0:1, 0.25:1, 0.5:1, 1:1, and 2:1 mg/mg. Buffer: 10 mM acetic acid, 10 mM NaCl, 0.1 mM EDTA, pH 4.0. Temperature: 20 °C. The outer hyperfine splittings, $2A_{max}$, for the sample in the absence of α-LA and with the largest amount of added α-LA (2:1 mg/mg) are indicated by the pairs of solid vertical lines in the figure. Total scan width: 100 G.

DISCUSSION

The spin label ESR measurements reported here refer to the interaction of α-LA in its molten globule form at low pH with negatively charged phosphatidylglycerol bilayers. In solution, α-LA has a pI ≈ 4.5−5 (Robbins et al., 1967) and undergoes a transition to the molten globule state at pH 4.2−3 (Kuwajima, 1989). At the surface of negatively charged bilayers, the pH is enhanced relative to that in the bulk solution by an amount $\Delta pH = e\psi/(kT \ln 10)$, where ψ is the electrostatic surface potential [see e.g. Cevc and Marsh (1987)]. From electrostatic double-layer theory, the latter is given by $\psi = (2kT/e) \sinh^{-1}(\sigma/c)$, where σ is the surface charge density and $c = (2\epsilon RTI/1000\pi)^{1/2}$, with ϵ the dielectric constant and I the ionic strength of the (assumed 1:1) electrolyte [see e.g. Cevc and Marsh (1987)]. For an ionic strength of $I \approx 0.02$, this yields a surface potential of $\psi \approx -180$ mV and a decrease in surface pH relative to the bulk solution by $\Delta pH \approx -3.0$ units. Therefore, in the region of higher proton concentration at the anionic lipid surface, for a bulk pH of 4.0, the protein will be well below its isoelectric point and at a pH (of 2.0 or lower) that corresponds to formation of the molten globule state. Under these conditions, α-LA interacts strongly with phosphatidylglycerol membranes and directly perturbs the lipid chain mobility and packing. Experiments at different pH values also confirmed that, at a bulk pH of 4.0, the perturbation of the lipid mobility by α-LA had achieved its maximum value. On the other hand, at neutral pH, the lipid chain mobility is unchanged by the presence of α-LA in its native state, and precipitated lipid/protein complexes are not formed. Also, for bilayers of the zwitterionic lipid DMPC, which has no surface charge and for which the surface pH is close to the bulk pH, the perturbation of the lipid mobility by α-LA is relatively small at a bulk pH of 4.0 (see Figure 2). This confirms that the strong binding of α-LA to anionic phosphatidylglycerol bilayers and the accompanying perturbation of the lipid chain mobility arises from the enhanced proton concentration at the charged bilayer surface and consequent transition of α-LA to the molten globule form.

Association with α-LA at pH 4.0 abolishes the chain-melting phase transition of DMPG bilayers. This is caused partly by a disruption of the cooperative chain packing in the gel phase which is evidenced by the decrease in hyperfine splitting and elimination of spin−spin interactions for the spin-labeled lipid (Figures 1 and 2). By contrast, at temperatures in the fluid phase of the lipid, the protein induces a large restriction in mobility of the lipid chains. The increase in maximum outer hyperfine splitting constant, $\Delta A_{max} \approx 4-5$ G, of the 5-PGSL spin label is comparable to that found also for DOPG bilayers in the fluid phase. Effects of this size, accompanied by removal of the lipid phase transition, are consistent with penetration of the protein into the lipid bilayer.

The most striking observation is that the binding of α-LA at pH 4.0 induces a strong restriction in the mobility of the lipid acyl chains along their entire length. This extends to the terminal methyl region of the chains, for which a second, motionally restricted population of lipid chains is resolved. The spectroscopic characteristics of this motionally restricted spin-labeled lipid component that is observed with 14- and 16-PGSL are similar to those found with integral membrane proteins [see e.g. Marsh and Watts (1982)]. The degree of restriction in the chain mobility is considerably greater than that found with peripheral proteins such as myelin basic protein and apocytochrome *c* which are known to penetrate negatively charged lipid membranes (Sankaram et al., 1989a; Görrissen et al., 1986). In these latter cases, the proteins are in a random coil, rather than in a molten globule conformation, when free in solution. This comparison suggests that α-LA in its molten globule state penetrates more effectively into the hydrophobic interior of lipid membranes than do peripheral proteins studied hitherto. It is possible that apocytochrome *c* and the myelin basic protein penetrate only partially into the membrane or disturb the membrane surface such that contact is achieved with terminal methyl ends of the lipid chains, whereas α-LA in the molten globule state may penetrate beyond the bilayer midplane in a manner more similar to that of integral membrane proteins. As already mentioned, Kim and Kim (1986) have suggested on the basis of hydrophobic labeling that the segment of α-LA from residues 80 to 108 lies parallel to the bilayer surface in PS:PC (1:1) vesicles. It is possible that different modes of association occur with membranes of different lipid compositions. Such an effect has been observed with apocytochrome *c* (Snel et al., 1994).

The stoichiometry of the motionally restricted spin-labeled lipid component is rather low ($n_b \approx 6$ lipids/α-LA) in the DOPG complexes with a lipid/protein ratio of 13−26 mol/mol. It therefore seems likely that the protein is aggregated in DOPG complexes with these relatively high protein contents. In support of this, it is observed that the stoichiometry increases to $n_b \approx 13$ mol/mol at a lipid/protein ratio of 52 mol/mol. Even so, this latter value still only corresponds to approximately the number of lipids that would be accommodated around a transmembrane α-helical dimer [see Marsh (1993)].

The experiments with different spin-labeled phospholipid species indicate a small selectivity of interaction of α-LA with different polar head groups, at levels of binding below saturation. The results with a lipid spin-labeled in the head group region also demonstrate a strong interaction of α-LA with the polar surface of the lipid membrane. This presumably involves, at least in part, polar regions of the protein that are not involved in penetration of the lipid membrane.

In summary, α-LA in the molten globule state inserts deeply and efficiently into phosphatidylglycerol bilayers. The inserted portions of the protein interact strongly with the lipid chains, and the more polar parts of the protein interact strongly with the lipid head groups. These results may be generally of relevance to the translocation of proteins across membranes, and particularly to those bacterial toxins that enter the cell via the early endosome pathway.

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