The Interaction of Peripheral Proteins and Membranes Studied with \( \alpha \)-Lactalbumin and Phospholipid Bilayers of Various Compositions*

To characterize the interaction of peripheral proteins and membranes at the molecular level, we studied the reversible association of bovine \( \alpha \)-lactalbumin (BLA) with lipid bilayers composed of different molecular forms of phosphatidylycerine or equimolar mixtures of these phosphatidylserine forms and egg yolk phosphatidylcholine. At pH 4.5, almost all BLA (>90\%) associates to negatively charged small unilamellar vesicles. The conformational changes that binding to these bilayers induced on the protein were characterized by circular dichroism and fluorescence spectroscopy. Because binding of BLA to negatively charged vesicles is reverted by adjusting the pH back to >6.0, we also investigated the conformation of the membrane-bound protein by NMR-monitored H-D exchange of the backbone amide protons. The conformation adopted by BLA bound to these bilayers resembles a molten globule-like state but the negative ellipticity at 222 nm and the apparent \( \alpha \)-helix content of the bound protein senses the changes in the physical properties of the membrane. Binding to bilayers in the gel state appears to correlate with an increased amount of \( \alpha \)-helical structure and with a lower extent of integration into the membrane, corresponding to the adsorbed protein, while the opposite is found for BLA bound to vesicles in the liquid-crystalline phase, corresponding to the embedded conformation. A common feature for the membrane-bound conformations of BLA is that the amphipathic helix C (residues 86 to 99) is an important determinant for the adsorption and further integration of the protein into the membrane.

As a part of their functions, some intracellular proteins can reversibly translocate between the cytosol and membrane surfaces, leading to a change in conformation and a consequent variation in activity (1–4). Similarly, extracellular proteins such as apolipoproteins can alternatively exist free in plasma or bound to lipoprotein lipids, in which case a new conformation is induced (5–8). The membrane-bound form of these amphipathic proteins is adsorbed or partially embedded in the lipidic surfaces. Secreted soluble toxins may be inserted through both leaflets of the membrane and in vitro studies have shown a transient membrane-triggered shift of their conformation that is necessary for their insertion in the membrane (9, 10). Among the different factors that modulate the association of proteins with the membrane, the lipid composition seems to be determinant (1, 2, 11, 12). The composition of the lipid bilayer can also act specifically on the conformation of proteins adsorbed or inserted in the membrane (13–16).

The soluble, calcium-binding milk protein bovine \( \alpha \)-lactalbumin (BLA) is a component of the lactose synthase complex. BLA binds to galactosyltransferase, promoting glucose binding and facilitating the synthesis of lactose in the lactating mammary gland (17, 18). BLA can also reversibly associate with lipid membranes under specific conditions. Thus, it has been shown that at pH 4.5, calcium-containing BLA binds to negatively charged liposomes and that the binding is reverted by adjusting the pH back to >6.0 (19). The protein only adsorbs to vesicles made of saturated lipids without disrupting the permeability barrier of the bilayer, whereas it adopts a partial embedded (inserted) state upon binding to vesicles of unsaturated lipids (in the liquid-crystalline phase) able to disrupt the bilayer (20). Our recent NMR studies have lead to a mechanism for the partial insertion of BLA into negatively charged membranes that includes initial protonation of acidic side chains at the membrane interface, which involves helices A and C, and a subsequent conformational change in the protein that adopts a molten globule-like state to maximize the interaction between hydrophobic residues in these helices and the lipid bilayer (21). Svensson et al. (22, 23) have characterized an apoptosis-inducing conformer of human \( \alpha \)-lactalbumin (human \( \alpha \)-lactalbumin made lethal to tumor cells, i.e. HAMLET), that induces the death of tumor cells and immature cells, but does not harm healthy cells. As yet, the mechanism by which a larger protein as the HAMLET conformer of \( \alpha \)-lactalbumin induces apoptosis is unknown, but both a partially unfolded conformation and a specific fatty acid as bound cofactor, oleic acid (18:1), are required for this new function of the protein. Conversion of \( \alpha \)-lactalbumin to the apoptosis inducing form is achieved with both the protein derived from human milk whey and with recombinant protein expressed in Escherichia coli (23). It has also been recently found that the permeabilizing effect of HAMLET on the mitochondria with subsequent cytochrome c release, which

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1 The abbreviations used are: BLA, bovine \( \alpha \)-lactalbumin; DOPG, 1,2-dioleoyl-sn-glycero-3-(phospho-1-glycerol); DSPS, 1,2-distearoyl-sn-glycero-3-(phospho-1-glycerol); EYPC, egg yolk phosphatidylycholine; LUV, large unilamellar vesicles; SOPS, 1-stearoyl-2-oleoyl-sn-glycero-3-(phospho-1-serine); POPES, 1-palmitoyl-2-oleoyl-sn-glycero-3-(phospho-1-serine); PS, phosphatidylyserine; SUV, small unilamellar vesicles.
may lead to activation of the caspase cascade and apoptotic death in transformed cells, is dependent on the oleic acid cofactor of HAMLET (24). The specificity of HAMLET for tumor cells leads the attention to the lipids involved in the recognition mechanism. It appears that the membrane composition is different in healthy cells and its corresponding tumor cells (25–28). In human breast cancer tissue the amount of phospholipid has been measured to be 2.6-fold higher than in non-tumorous breast tissue (25) and tumor cell membranes contain more anionic phospholipids and a different fatty acid composition (29). Moreover, although the negatively charged phospholipids of the plasma membrane are usually segregated to the inner leaflet (30), the earliest sign of apoptosis is translocation of phosphatidylserine (PS) from the inner to the outer leaflet (31).

To further investigate the conformational changes accompanying the binding of α-lactalbumin to membranes, we have studied the interaction of BLA with liposomes of different composition. The conformation of the membrane-bound states of the protein was investigated by fluorescence spectroscopy, circular dichroism (CD), and 1H NMR. The interaction of BLA with the membrane seems to be mostly modulated by the nature, physical state, and charge of the major lipid components of the membranes, the glycerophospholipids (20). In this study we have studied the interaction of BLA with liposomes made of different molecular forms of PS alone or equimolar mixtures of these lipids and egg yolk phosphatidylcholine (EYPC), thus with bilayers of different fluidity and charge density. Most of the previous studies on the interaction of BLA with model membranes have used liposomes containing negatively charged dioleoylphosphatidylglycerol (DOPG), although this phospholipid generally contributes to less than 1% to the total animal cellular phospholipids, except for the 2–5% found in lungs (30). Our results with the more relevant PS are compared with previous results obtained with mixtures of EYPC and DOPG.

**EXPERIMENTAL PROCEDURES**

**Materials—**1,2-Dioleoyl-sn-glycero-3-(phospho-threo-serine) (DSPS), 1-stearoyl-2-oleoyl-sn-glycero-3-(phospho-threo-serine) (SOPS), and 1-palmitoyl-2-oleoyl-sn-glycero-3-(phospho-threo-serine) (POPS) were purchased from Avanti Polar Lipids (Alabaster, AL). EYPC, 1,2-dioleoylphosphatidylglycerol (DOPG), and bovine α-lactalbumin type III (holo-protein, calcium-saturated) were from Sigma. Deuterium oxide (99.9%) was from ICN Biomedicals Inc. (Costa Mesa, CA). Fura-2 was from Molecular Probes (Leiden, The Netherlands).

**Preparation of Unilamellar Lipid Vesicles—**The lipids were dissolved in chloroform and mixed in a round-bottom glass flask to the desired proportions. The solvent was evaporated and the lipids freeze-dried overnight. The dried films were then dispersed in 20 mM citric acid/Na2HPO4, 0.1 M NaCl, pH 4.5, by gently mixing and small unilamellar vesicles (SUV), also referred to as liposomes in the text, were then prepared in a bath sonicator (Branson 1200, Branson, CT), operating at a nominal frequency of 20 kHz during 60–90 min at 4 °C. The temperature was maintained by continuous exchange of the chilled water. A highly homogeneous vesicle preparation with a diameter of 40 nm was obtained, as seen by electron microscopy and quasi-elastic light scattering using a Malvern Zetasizer (Malvern, United Kingdom). Electron microscopy revealed that SUV were unilamellar. Large unilamellar vesicles (LUV) (~1 μm diameter) were prepared by extrusion as described (32).

**Binding of α-Lactalbumin to Liposomes by Ultracentrifugation—**Protein solutions (7 μM) and liposomes were mixed in 1 ml of 20 mM citric acid/Na2HPO4, 0.1 M NaCl, pH 4.5–6.0, at the indicated lipid-protein ratios. Samples were allowed to equilibrate for 30 min at room temperature and were then centrifuged at 105,000 × g for 30 min at 4 °C. The protein concentration in the supernatant was determined spectrophotometrically, using the extinction coefficients of 28,500 M−1 cm−1 at 290 nm, pH 7.0 (33). To account for the sedimentation of the free protein, samples containing the same protein concentration in the absence of liposomes were treated and centrifuged under the same experimental conditions.

**Determination of Free Calcium Content—**Calcium concentration was measured using the Ca2+ indicator fura-2 (34) using a LS-50B PerkinElmer luminescence spectrometer, according to the product information manual from the manufacturer of fura-2 (Molecular Probes) and references therein. Calcium was measured in samples of BLA (0.1 mM) prepared in 20 mM citric acid/Na2HPO4, 0.1 M NaCl, pH 4.5 and 6.0, both in the presence and the absence of liposomes composed of EYPC: DOPG (1:1) and EYPC:SOPS (1:1) (7 μM phospholipid). Calcium was also measured after adjusting the pH of the samples to 6.0 in membrane-free protein fractions and in both membrane- and protein-free fractions prepared by ultrafiltration in Centricon 3 microconcentrators (Amicon) using 1 μM fura-2.

**Differential Scanning Calorimetry—**Measurements were performed on a MicroCal VP-DSC differential scanning calorimeter (MicroCal, Inc.) with cell volumes of 0.5 ml at a scan rate of 80 °C/h. All buffer solutions were degassed under vacuum prior to use. Calorimetric cells were kept under an excess pressure of 207 kPa to prevent degassing during the scan. SUV (5 mM in phospholipid) prepared in a 20 mM citric acid/Na2HPO4, 0.1 M NaCl buffer, at the indicated pH, were used, and thermograms of buffer served as reference. When indicated, BLA was present at a concentration of 50–70 μM. Determination of the transition temperature (Tc) and the half-widths of the transitions (ΔTc) was performed by curve fitting with the Origin TM software (MicroCal, Inc.).

**Fluorescence Spectroscopy—**Fluorescence measurements were performed at 25 °C with a PerkinElmer luminescence spectrometer LS-50B with temperature regulation using quartz cuvettes with a light path of 5 mm. Samples contained 1 μM BLA in 20 mM citric acid/Na2HPO4, 0.1 M NaCl, pH 4.5 to 6.0, in the presence or absence of liposomes (300 μM lipids). The fluorescence emission spectra of the protein were recorded in the 310–500 nm range with excitation at 295 nm using 3- and 5-nm band passes. Protein-free blanks and without liposomes of identical concentration and composition were subtracted. When indicated, the pH of the sample was adjusted from pH 4.5 to 6.0 by the addition of NaOH and incubation up to 30 min at 25 °C.

**Circular Dichroism (CD)—**CD measurements were performed with a Jasco J-310 spectropolarimeter equipped with a FTC-348SI Peltier element for temperature control using quartz cells with path lengths of 1 mm. Samples contained 12.7 μM BLA in 20 mM citric acid/Na2HPO4, 0.1 M NaCl, pH 4.5 to 6.0, in the presence and absence of liposomes (882 μM in lipid) at the indicated temperature. Four consecutive wavelength scans between 195 and 260 nm were recorded for each CD spectrum and buffer blanks were subtracted. Protein thermal denaturation was monitored by following the changes in ellipticity at 222 nm, with a scan rate of 1 °C/min in the 10–95 °C temperature range. Mean residue ellipticity (θ) was calculated from the formula θ = ε(100/ν), where ε is the ellipticity (millidegrees), λ is the path of the cuvette (cm), C is the protein concentration (mol/liter), and ν is the number of amino acid residues.
Table I

Intrinsic fluorescence parameters for BLA bound to SUV of different lipid compositions

Measurements were performed with BLA (1 μM) incubated with SUV (300 μM in phospholipid) for 10 min at pH 4.5.

| Lipid composition | λmax (25 °C) | λmax (37 °C) | IF (25 °C) | IF (37 °C) |
|-------------------|--------------|--------------|------------|------------|
| EYPC:DOPG         | 339.0        | 339.0        | 75.0       | 75.1       |
| EYPC:SOPS         | 338.5        | 338.5        | 53.0       | 52.3       |
| EYPC:POPS         | 340.0        | 339.5        | 34.9       | 34.5       |
| EYPC:DSPS         | 336.0        | 328.0        | 20.4       | 67.0       |
| No bilayer (BLA alone) | 331.0   | 332.5        | 16.7       | 14.4       |

Table II

Temperature for the gel to liquid crystalline phase transition (Tc) and half-width (Tc/2) of the transition obtained by differential scanning calorimetry for the SUV preparations studied

The results are the mean ± S.E. of three measurements and represent the values obtained for the main phase transition. The aim of this table is to describe the thermotropic properties of the lipid mixtures in the absence and presence of the protein, and not to characterize the thermodynamic parameters of the membrane transitions due to perturbed organization of SUV. Measurements were performed in 20 mM citric acid/Na2HPO4, 0.1 M NaCl, pH 4.5. The SUV were 5 mM in phospholipid and the concentration of BLA was 60 μM.

| Sample          | Tc (°C) | Tc/2 (°C) | Tc (°C) | Tc/2 (°C) |
|-----------------|---------|-----------|---------|-----------|
| No BLA          |         |           |         |           |
| SOPS            | 26.7 ± 0.7 | 6.8 ± 0.5 | 28.0 ± 0.6 | 6.9 ± 1.5 |
| EYPC:SOPS       | 8.3 ± 0.5  | 15.8 ± 0.6 | 19.9 ± 0.8 | 16.7 ± 1.9 |
| POPs            | 12.1 ± 0.1 | 1.5 ± 0.1  | 18.6 ± 0.1 | 0.9 ± 0.2  |
| EYPC:POPS       | 4.1 ± 0.8  | 9.5 ± 0.7  | 15.1 ± 0.2 | 7.2 ± 0.5  |
| DSPS*           | 67.6 ± 0.2 | 1.2 ± 0.2  | 69.0*     | 1.7*       |
| EYPC:DSPS       | 51.6 ± 0.1 | 12.9 ± 0.3 | 41.1 ± 0.2 | 10.5 ± 0.7 |

* No apparent binding of the protein as measured by ultracentrifugation or other methods.

RESULTS

Fluorescence and Differential Scanning Calorimetry Measurements—The pH-controlled reversible interaction of BLA with negatively charged liposomes of EYPC:DOPG (1:1) has been characterized by several spectroscopic techniques, including fluorescence spectroscopy (19). BLA free in solution shows an intrinsic fluorescence emission spectrum with λmax at about 330–331 nm both at pH 4.5 and 6.0 (Fig. 1) and on binding to liposomes of EYPC:DOPG at pH 4.5, the fluorescence intensity increases and the λmax red shifts to 339–340 nm (19). These changes have been interpreted as being the result of the transition of at least one of its four Trp residues to a more polar environment and the disappearance of tertiary interactions that quench the fluorescence in the native state (19). No spectral changes occur when liposomes (either LUVs or SUVs) of EYPC:DOPG are added to BLA at pH 6.0 and additional methods, such as ultracentrifugation, have corroborated that no binding takes place at this pH (19, 21). Incubation of the protein with SUVs at protein:lipid molar ratios ranging from 1:70 to 1:300 at pH 4.5 and 37 °C, and consequent ultracentrifugation of the samples, reveals that almost all BLA (>90%) associates to SUV made of EYPC:DOPG, EYPC:SOPS, EYPC:POPS, and EYPC:DSPS. Full binding of BLA was also measured after incubation with the same lipid bilayers at 25 °C, except for those composed of EYPC:DSPS, for which no binding was observed at this temperature.

Liposomes (either LUVs or SUVs) of EYPC alone do not affect the emission fluorescence of BLA at either pH (Fig. 1). A red-shift and an increase in fluorescence intensity are also observed on binding of BLA to liposomes of EYPC:SOPS and EYPC:POPS, whereas a blue shift was observed for the interaction of the protein with liposomes of EYPC:DSPS at 37 °C (Fig. 1 and Table I).

To check the thermotropic properties of these samples they were characterized by differential scanning calorimetry, and in Table II we have summarized the gel to liquid crystalline phase

residues in the protein (123 for BLA). Analysis of the data and determination of midpoint denaturation temperatures (Tm) of the protein were performed using the Standard Analysis program provided with the instrument. The aim of secondary structure elements was estimated with the CDNN program that applies a neural network procedure (35).

Hydrogen-Deuterium Exchange and NMR Spectroscopy—Samples of BLA (1 mM final concentration, 550 μl) were prepared in 99.9% D2O-containing 20 mM citric acid/Na2HPO4, 0.1 M NaCl, pH 4.5. The SUV were 5 mM in phospholipid and the concentration of BLA was 60 μM.
transition temperatures \( (T_c) \) of the bilayers. It should be mentioned that we focused on the characterization of SUVs because the advantageous use of these liposomes over LUV in the spectroscopic experiments, where higher lipid concentrations are required, has been discussed elsewhere (21). The values of \( T_c \) obtained by differential scanning calorimetry for SUV containing pure PS species are consistent with data for the gel to liquid crystalline phase transition for LUV and MLV of the same phospholipids found in the LIPIDAT data base (40). The \( T_c \) values for the mixtures of EYPC and different molecular species of PS in the absence of BLA are lower than for the pure PS species (Table II). The analysis of the pH dependence of \( T_c \), sensitive to the degree of ionization, the surface charge density, and the fluidity (41, 42), indicates that the PS species used in this study are negatively charged in the pH 4.5–6 range.

When the pH was increased from 4.5 to 6.0, the fluorescence spectrum of BLA in the presence of liposomes essentially reverted to that for free BLA (Ref. 21, Fig. 1, and data not shown), in accordance with the release of the protein from the bilayer. Measurement of the calcium content in solutions of BLA bound to liposomes of EYPC:DOPG and EYPC:SOPS at a lipid:protein molar ratio of 300:1 at pH 4.5 using the fluorescence properties of fura-2 (34) showed that no calcium ion is released to the medium from the protein on binding to the bilayer, indicating that membrane-bound BLA most probably remains as holoenzyme. Moreover, BLA released from the membrane by the pH shift to pH 6.0 appears to be recovered largely (80%) as holoenzyme.

The Association of BLA with Liposomes of Different Compositions Studied by CD—The far-ultraviolet spectrum of free BLA shows two minima at 208 and 222 nm, characteristic of proteins with large content of \( \alpha \)-helical structure (Ref. 19 and Fig. 2A). The content of \( \alpha \)-helix and \( \beta \)-extended structures in BLA estimated from the CD spectrum was 27 and 10%, respectively, in agreement with the content estimated from the crystal structure (43). In the presence of liposomes of EYPC:DOPG, EYPC:SOPS, EYPC:POPS, and EYPC:DSPS at pH 4.5 and 10 °C, an increase was observed in the ellipticity of BLA (Fig. 2A), corresponding to larger apparent \( \alpha \)-helix content than in free BLA (Table III). When the CD spectra were acquired at 37 °C, the negative ellipticity at 222 nm of BLA and the apparent \( \alpha \)-helix content was lower for BLA bound to the equimolar mixtures of EYPC and PS (Table III). This effect was better studied by following the temperature-induced changes on the ellipticity at 222 nm (the benchmark for \( \alpha \)-helix). Representative profiles are shown in Fig. 2B. When bound to liposomes of EYPC:DOPG, no significant changes were found for the scans of BLA with respect to that of free protein, whereas remarkable features were observed in the temperature scans of BLA bound to liposomes of EYPC:SOPS (Fig. 2B), EYPC:POPS, or EYPC:DSPS (data not shown). A sudden and significant (about 40%) reduction of \( \alpha \)-helix content was observed for BLA bound to liposomes of EYPC:SOPS in the 25–50 °C temperature range. Complementary experiments intended to follow the kinetics of the cooperative conformational changes associated with the binding of BLA to liposomes of EYPC:SOPS at 25 °C showed that the ellipticity at 222 nm first increased for 10 min up to 10% and then deceased to 60% of its original value in the free protein.

The Association of BLA with Liposomes of Different Composition Studied by NMR—Amide proton (NH) exchange data can be used to probe molecular interactions and conformational changes by NMR. The effect of binding of a ligand to the protein on the time course of signal decay resulting from hydrogen exchange can be assessed by comparison with the exchange rates of the protein alone (44, 45). Thus, the regions involved in the binding to the ligands (with slower exchange rate because of protection by the ligand) and/or the conformational changes, including the extent of protein unfolding necessary to become competent in membrane association, may be identified. Using this method we have recently identified regions of BLA involved in binding to liposomes of EYPC:DOPG (21). We intended to extend this method to study the binding of BLA to liposomes containing different molecular species of PS, and compare the results with those obtained for BLA bound to liposomes of EYPC:DOPG (and consequently released), for which the H-D exchange of about 20% of the NH protons can be followed up to 5 days and be compared with that in control

![Fig. 2](https://example.com/f2.png)

**Fig. 2.** Far-ultraviolet CD spectroscopy of BLA free in solution (——) and bound to liposomes of EYPC:DOPG (-----), EYPC:SOPS (------), and EYPC:POPS (-----). A, CD spectra taken at 10 °C in 20 mM citric acid/Na2HPO4, 0.1 M NaCl, pH 4.5. The protein concentration was 12.7 μM and the liposomes (SUV) of the indicated composition were 300 μM lipid. Spectra were taken after 20 min incubation at 10 °C. [θ] is the mean residue ellipticity. B, temperature dependence of [θ] at 222 nm. The scan rate was 60 °C/h.

| Lipid composition | \( \alpha \)-Helix content at 10 °C | 37 °C |
|-------------------|-------------------------------|------|
| EYPC:DOPG         | 30                            | 28   |
| EYPC:SOPS         | 33                            | 24   |
| EYPC:POPS         | 33                            | 24   |
| EYPC:DSPS         | 32                            | 26   |
| No bilayer (BLA alone) | 27                         | 27   |
samples of BLA free in solution (21). However, NH signals disappeared very quickly when the protein was allowed to exchange in D$_2$O while bound to the liposomes made of EYPC:SOPS, EYPC:POPS, or EYPC:DSPS at pH 4.5 at a temperature of either 4 or 25 °C. Almost no NH signal was detected for exchange time $>1$ h when the spectra were taken after release by pH shift to 6.0. Thus, proper decay curves could not be calculated because the time between pH shift and acquisition (necessary for tuning, matching, shimming, and pulse calibration) was about 40 min. Therefore, we decided to compare the remaining backbone amide protons after H-D exchange for 1 h. To shorten the acquisition time for NMR spectra while still retaining high signal-to-noise ratio and a reasonable resolution, the NMR method chosen was BASHD-TOCSY (36). Experimental parameters were adjusted for the rapidly acquired BASHD-TOCSY spectra so that the majority (about 80%) of the $\alpha$H-NH signals were included in the spectral window for free BLA in solution (10% D$_2$O, pH 4.5). About 80% of the $\alpha$H-NH cross-peaks have been identified and assigned in this spectrum (21) based on previous work by Forge et al. (46) and Alexandrescu et al. (47). When incubation and exchange in D$_2$O was performed at 4 °C, a temperature which is over the $T_c$ for bilayers of EYPC:DOPG (40), but lower than the $T_c$ for the transition of the PS containing SUV (Table II), several $\alpha$H-NH cross-peaks can be detected after 1 h of exchange in control samples of free BLA (Fig. 3A) and for BLA bound to liposomes of EYPC:DOPG (Fig. 3B). On the other hand, most protons in BLA bound to liposomes of EYPC:SOPS were found to be fast-exchanging protons, and were undetected or show less than 0.1 ppm change-rate data taken up to 5 days of exchange (21). Because these residues also show a higher protection than in the acidic molten globule-like form (46), these results indicate that they are involved in membrane binding. When BLA is associated with liposomes of EYPC:SOPS, only residues Lys$^{98}$ and Val$^{99}$ from helix C are more protected from exchange than in the native free protein (Table IV and Fig. 5). The relative peak volume corresponding to Val$^{99}$ was even larger with EYPC:SOPS than with EYPC:DOPG vesicles, suggesting a closer association of the end of the helix C with the membrane. The protected residues in EYPC:DOPG- and EYPC:SOPS-bound BLA with respect to the free enzyme were mapped into the crystal structure of BLA (43) (Fig. 6, A and B).

**DISCUSSION**

The interaction of amphiphilic molecules and amphitropic proteins with membranes has been proposed to be modulated by the surface charge and the physical state of the latter (4, 8, 49, 50). In the present study we have investigated the binding of BLA to PS-containing bilayers with acyl chains of varying unsaturation and length. Combined with analysis of the binding at different temperatures, this allows for a large variability in the chemical composition and physical state of the membranes.

As indicated by CD spectroscopy, the conformation of BLA bound to liposomes of EYPC:DOPG at 37 °C is similar to that

![Figure 3. BASHD-TOCSY spectra of BLA showing the $\alpha$H-NH region.](Image)
obtained at 10 °C. EYPC and DOPG show a high degree of miscibility and are in a liquid crystalline state at temperatures above 0 °C. On the other hand, during temperature scans of the protein bound to EYPC:PS mixtures, the protein experiences a change in the physical state of the bilayers at temperatures around T_C (Table II). Whereas the spectra taken at 4 °C correspond to gel phase-bound protein, at 37 °C both EYPC:SOPS and EYPC:POPS are entirely in the liquid crystalline state and EYPC:DSPS is at the pretransition. In agreement with earlier results by Bañuelos and Muga (20) working with derivatives of BLA, the sudden decrease in ellipticity that the protein undergoes at temperatures right above T_C might be related to the existence of two bound forms of the protein: (i) adsorbed species characterized by a high content of α-helix, and (ii) embedded species, located further into the bilayer. The protein both adsorbs and partially inserts into membranes in the liquid crystalline state, but it cannot penetrate into the hydrocarbon core of the bilayer in the gel phase (20). Our NMR results further contribute to the characterization of these conformers.

TABLE IV

| Residue | Free in solution | Bound to EYPC:DOPG | Bound to EYPC:SOPS |
|---------|-----------------|-------------------|-------------------|
| Arg^{12} | 0.03 ± 0.01    | 0.05 ± 0.02       | ND                |
| Leu^{12} | 0.43 ± 0.06    | 0.66 ± 0.09       | 0.10 ± 0.04       |
| Glu^{79} | 0.18 ± 0.03    | 0.04 ± 0.01       | 0.11 ± 0.3        |
| Val^{79} | 0.60 ± 0.07    | 0.08 ± 0.03       | 0.33 ± 0.09       |
| Tyr^{36} | 0.05 ± 0.01    | 0.08 ± 0.03       | 0.05 ± 0.02       |
| Lys^{60} | 0.16 ± 0.02    | 0.25 ± 0.06       | ND                |
| Ile^{75} | 0.53 ± 0.08    | 0.28 ± 0.05       | 0.34 ± 0.06       |
| Ly^{84}  | 0.22 ± 0.04    | 0.04 ± 0.02       | ND                |
| Asp^{72} | 0.88 ± 0.10    | 0.19 ± 0.04       | 0.24 ± 0.04       |
| Lys^{86} | 0.33 ± 0.04    | 0.40 ± 0.07       | 0.07 ± 0.04       |
| Asp^{77} | 0.12 ± 0.02    | 0.09 ± 0.02       | ND                |
| Ly^{88}  | 0.04 ± 0.01    | 0.44 ± 0.06       | 0.20 ± 0.03       |
| Val^{96} | 0.06 ± 0.01    | 0.67 ± 0.08       | 1.81 ± 0.24       |
| Trp^{104}| 0.10 ± 0.01    | 0.13 ± 0.03       | 0.03 ± 0.01       |

ND, non-detectable.

FIG. 4. BASHD-TOCSY spectrum of BLA with liposomes of EYPC:SOPS showing the α-NH region. BLA (1 mM) was prepared in 20 mM citric acid/Na_2HPO_4, 0.1 M NaCl, pH 4.5, 10% D_2O with SUV of EYPC:SOPS, and the spectrum was taken 48 h after incubation at 4 °C and subsequent increase of the pH to 6.0 to release the protein from the liposomes. Spectra were taken at a probe temperature of 308 K.
protected from the solvent, only two residues being more protected from exchange than in the embedded form (Lys\(^{98}\) and Val\(^{99}\)). This would be in agreement with a more superficial and exposed localization of the adsorbed form with respect to the embedded form. A further NMR investigation of the change in protein conformation associated with the insertion of the protein into the membrane expected during the gel to liquid crystalline transition is hindered by the large H-D exchange rate constants at temperatures >4 °C (51). Common to the association of BLA with liposomes made of EYPC:DOPG and EYPC:SOPS, helix C seems to be involved in the interaction. This helix has a typical amphipathic character (Fig. 6C), and its involvement in binding may possibly be related to the increased apparent helicoidal content of membrane-bound proteins (1, 52). Put together, our results are in agreement with a higher structuration or stabilization of the interacting helix (helices) in the membrane-adsorbed BLA and a larger unfolding of the membrane-embedded protein.

The fluorescence data corresponding to BLA bound to EYPC:DOPG, EYPC:SOPS, and EYPC:POPS are consistent with the exposure of (a) Trp residue(s) to the solvent (or the polar membrane interface) as well as the disappearance of tertiary interactions that quench the fluorescence in the native state (19), in agreement with a molten globule-like form. As seen by NMR, Trp\(^{104}\) seems to be an appropriate candidate for being located at the polar solvent-membrane interface (Table IV and Ref. 21). However, BLA bound to EYPC:DSPS bilayers at 37 °C, which shows the characteristic increase in fluorescence of the molten globule-like membrane-bound conformations, displays a blue-shifted fluorescence emission spectrum, probably indicating a different chemical environment, more apolar, for the Trp residue in this conformation bound to an ordered membrane.

Although BLA is a Ca\(^{2+}\) containing protein, the increase in \(T_c\) that accompanies the interaction of the protein with bilayers composed only of EYPC:SOPS and EYPC:POPS (Table II) cannot be merely related to thermotropic effects of the cation binding to the membrane, because at a similar Ca\(^{2+}\):lipid molar ratio as the protein:lipid ratio utilized in this study, no effect on \(T_c\) is observed (41).\(^2\) Nevertheless, it is interesting to note that in our proposed scheme, in which helix C (residues 86–98) is involved in membrane binding, the Ca\(^{2+}\) ion (coordinated by Lys\(^{79}\), Asp\(^{82}\), Asp\(^{87}\), and Asp\(^{88}\) in the holoprotein (43)), would be brought close to the surface of the membrane where the negatively charged head groups of the phospholipids can compete with the protein for the cation. Moreover, the affinity of the molten globule for Ca\(^{2+}\) is lower than that of the native form (53), which would facilitate calcium release from the membrane-bound form of BLA, and its possible binding to the membrane. Accordingly, a minor (20%) but significant proportion of BLA is recovered from the bilayer.

\(^{2}\) A. V. Agasseter, E. Fuglebakk, H. Holmsen, and A. Martinez, unpublished results.
as apoprotein in solution after the pH shift to 6.0 (Ref. 21 and this work), which seems to be relevant for understanding the finding by Håkansson et al. (54) and Svensson et al. (22, 23) that fatty acid (18:1) containing folding variant forms of human α-lactalbumin are calcium-elevating and apoptosis-inducing agents, with cytotoxic activity for transformed, embryonic, and lymphoid cells. Peptides with antibacterial and apoptotic activity on tumor cells (Ref. 55 and references therein) are characterized for their high content of basic amino acids, a motif that is also found in helix C of BLA. It seems clear that the apoptotic character of HAMLET is connected with its special folding state that must be able to both transverse the membranes of the vulnerable cells and induce the DNA fragmentation noted by Håkansson et al. (56). A possible relevant observation is that HAMLET is isolated from the casein fraction when purified from milk at pH 4.6, 40 °C (22). This contrasts with the fact that native α-lactalbumin is purified from the whey fraction (57). Caseins have been described as “naturally occurring molten globules,” and attempts have been made to explain their membrane. Further studies on the effect of protein concentration, and a specific phospholipid composition of the membrane, with its oleic cofactor bound, which could stabilize the tensegral arrangement. Thus, the necessary conditions for translocation of the protein across the bilayers, however, is expected to require large scale disruption of the phospholipid molecular arrangement. Thus, the necessary conditions for translocation may be offered by a combination of the large HAMLET complex with its oleic cofactor bound, which could stabilize the tensegral structure, and a specific phospholipid composition of the membrane. Further studies on the effect of protein concentration and membrane composition on the conformation adopted by membrane-bound BLA and its degree of insertion into the membrane will contribute to understand the selectivity of some folding variants of human α-lactalbumin to induce apoptosis in tumor cells (22, 23).

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The Interaction of Peripheral Proteins and Membranes Studied with α-Lactalbumin and Phospholipid Bilayers of Various Compositions
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