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α-Lactalbumin binding and membrane integrity—effect of charge and degree of unsaturation of glycerophospholipids

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Abstract

Several studies have shown that the physical state of the phospholipid membrane has an important role in protein–membrane interactions, involving both electrostatic and hydrophobic forces. We have investigated the influence of the interaction of the calcium-depleted, (*apo*)-conformation of bovine α-lactalbumin (BLA) on the integrity of anionic glycerophospholipid vesicles by leakage experiments using fluorescence spectroscopy. The stability of the membranes was also studied by measuring surface tension/molecular area relationships with phospholipid monolayers. We show that the degree of unsaturation of the acyl chains and the proportion of charged phospholipid species in the membranes made of neutral and acidic glycerophospholipids are determinants for the association of BLA with liposomes and for the impermeability of the bilayer. Particularly, tighter packing counteracted interaction with BLA, while unsaturation—leading to looser packing—promoted interaction and leakage of contents. Equimolar mixtures of neutral and acidic glycerophospholipids were more permeable upon protein binding than pure acidic lipids. The effect of lipid structure on BLA–membrane interaction and bilayer integrity may throw new light on the membrane disrupting mechanism of a conformer of human α-lactalbumin (HAMLET) that induces death of tumour cells but not of normal cells.

Keywords: Alpha lactalbumin; Protein–membrane interaction; Liposome; Langmuir; Vesicular leakage; Fluorescence

1. Introduction

There has been an increasing interest for the study of amphitropic proteins such as α-synuclein, cytochrome *c* and α-lactalbumin, all of which interact reversibly with phospholipid membranes [1–4]. Furthermore, there have been a few reports on how membrane systems modulate the conformation of embedded proteins, and vice versa [5,6]. In recent works, we investigated the structural determinants for the interaction of the amphitropic protein α-lactalbumin with model phospholipid bilayers and how the phospholipid composition modulated the conformation and degree of insertion of the membrane-bound protein [7,8]. Much work has been done on the structural characterisation of peptides bound to membranes [9,10]; also, the ability of different compounds to modulate the phase-behaviour of non-biological surfactants has been thoroughly investigated and the literature offers numerous examples of a wide range of such phenomena, e.g., micellar size increase, morphology changes, depression of critic micelle concentration (CMC) and phase-change behaviour [11,12]. There are also several studies on the mechanisms for membrane-interaction of amphitropic proteins [8,13,14]. However, the available knowledge on how amphitropic proteins influence the packing and the integrity of *biologically* relevant membranes is more limited.

In the present study, we have investigated the influence of the calcium-depleted, *apo*-conformation of bovine α-lactalbumin (BLA) on anionic phospholipid membranes. BLA is a 14.2-kDa globular calcium-binding protein serving as the

Abbreviations: BLA, bovine α-lactalbumin; CMC, critic micelle concentration; HAMLET, human lactalbumin made lethal to tumour cells; DOPG, 1,2-dioleylphosphatidylglycerol; DSPS, 1,2-distearoyl-sn-glycero-3-[phospho-L-serine]; EYPC, egg yolk phosphatidylcholine; LUV, large unilamellar vesicles; SOPS, 1-stearoyl-2-oleyl-sn-glycero-3-[phospho-L-serine]; SUV, small unilamellar lipid vesicles

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component of the lactose synthase complex that binds to galactosyltransferase, promotes glucose binding and facilitates the synthesis of lactose in the Golgi lumen of mammary secretory cells [15]. As a milk extracellular protein, BLA is also very well characterised and is well suited for membrane–protein interaction studies since it associates reversibly with negatively charged lipid membranes under specific conditions; the binding of *holo*-BLA occurs at mild acidic conditions (pH $\leq$ 4.5) since it requires protonation of key acidic residues on the protein and a loosening of the tertiary structure, and the protein is released at pH 6–7 [1,2,16,17]. Thus, previous in vitro studies have shown that BLA adopts a molten globular like conformation when it interacts with membranes [1,2,7,8]. The studies also showed that *apo*-BLA is capable of binding to negatively charged model membranes at pH 5.0, a value slightly higher than the protein isoelectric point (4.5).

A conformer of α-lactalbumin (HAMLET) that induces death of tumour cells but does not harm healthy cells has recently been characterised [18,19]. The mechanism of how this conformer induces apoptosis is poorly understood; both a partially unfolded conformation and a specific fatty acid (oleic acid, C18:1), which seems to stabilize the conformation, are reported required for this activity of the protein. Recent results on the topic show that the protein interacts with histones and chromatin in the tumour cell nuclei, but it is unclear how the protein gets into the nuclei of these cells [20]. It is not unlikely that lipids may have a role in this targeting. An analogous conformer of BLA (BAMLET) has the same effect as HAMLET [18].

In order to elucidate (1) the effects of membrane charge and fluidity on the binding of *apo*-BLA to phospholipid membranes, and (2) the responses of the different membranes to the bound protein, we have studied the interaction of *apo*-BLA with liposomes of different compositions by several approaches. We monitored binding by following the intrinsic fluorescence spectroscopy of the protein, and the vesicle integrity was studied by leakage experiments. The Langmuir technique was also used to study how binding of protein influences the packing of monolayers.

2. Materials and methods

2.1. Materials

Bovine α-Lactalbumin type III (*apo*-protein, calcium free), bovine α-Lactalbumin type I (*holo*-protein, calcium saturated), egg yolk

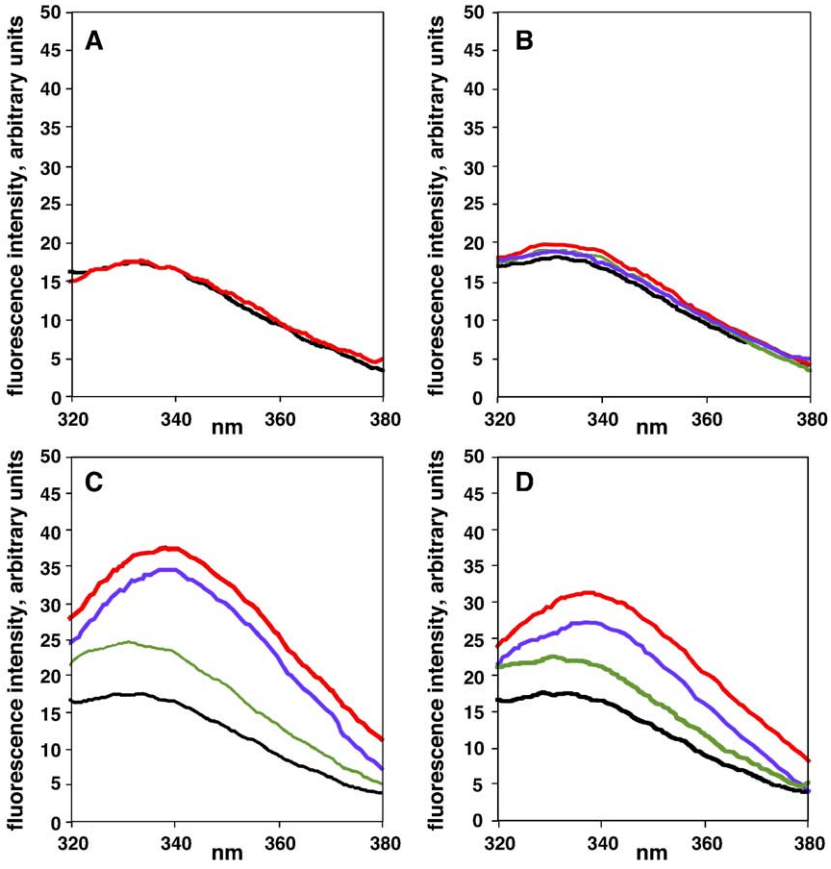

Fig. 1. Effect of SUV of different composition on the intrinsic emission fluorescence spectrum of *apo*-BLA. Measurements were performed with 1 μM *apo*-BLA at 37 °C, pH 5.0, in the presence of liposomes made of (A) EYPC, and of (B) DSPS:EYPC, (C) DOPG:EYPC and (D) SOPS:EYPC at a negative phospholipids:EYPC ratio of 1:3 (—), 1:1 and pure EYPC for (A) (—), and 3:1 (—). Control spectrum for the protein in the absence of liposomes (—). The lipid:protein ratio was 300:1 (μM/μM). Each curve in this figure is representative for three parallel measurements.

phosphatidylcholine (EYPC) and 1,2-dioleylphosphatidylglycerol (DOPG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-stearoyl-2-oleyl-*sn*-glycero-3-[phospho-L-serine] (SOPS) and 1,2-distearoyl-*sn*-glycero-3-[phospho-L-serine] (DSPS) were from Avanti Polar Lipids Inc. (Alabaster, AL, USA). 8-aminonaphtalene-1,2,3-trisulfonic acid (ANTS) and p-xylene-bis-pyridinum bromide were from Molecular Probes (Eugene, Oregon, USA). Analysis of EYPC by reverse phase HPLC showed that 1-palmitoyl, 2-oleoylphosphatidylcholine (POPC) was the major molecular species, i.e., 64.4% 16:0/18:1 PC and ~20.5% 16:0/18:2 PC.

2.2. Preparation of liposomes

Large unilamellar vesicles (LUV) were freshly prepared as described [21] by dissolving the lipids in chloroform, mixing them in a round-bottom glass flask to the desired proportions, and evaporating the solvent with a rotavapor for 30 min at temperatures above the phase transition point. The resulting lipid films were then frozen rapidly in liquid nitrogen and freeze-dried overnight. The dried films were then dispersed in 20 mM citric acid/Na_2HPO_4, 0.1 M NaCl, with selected pH (usually pH 5.0) at room temperature. This freeze–thaw process was repeated seven times, to increase the inclusion of buffer solvents into the vesicles [22]. LUVs of ~1 μm diameter were then obtained by extrusion as described [23]. Small unilamellar vesicles (SUV) were prepared as described [8].

2.3. Intrinsic fluorescence spectroscopy measurements

Intrinsic fluorescence spectroscopy measurements were performed at 37 °C in a Perkin Elmer luminescence spectrometer LS-50B with temperature regulation using quartz cuvettes with a light path of 5 mm. The samples contained 1 μM *apo*-BLA and 0.1 M NaCl in a 5-mM citrate-/10 mM Na_2HPO_4-buffer, 1 mM EDTA, pH 5.0. SUV were then added in a final lipid:protein ratio of 300:1 (μM:μM). The fluorescence emission spectra were recorded in the 320–400 nm range in the presence or absence of liposomes with an excitation of 295 nm using 3- and 5-nm bandwidths in the excitation and emission pathways, respectively. Protein-free blanks of identical composition with and without liposomes were subtracted.

2.4. Release of vesicular contents to the medium

Release of vesicular contents to the medium was monitored by the ANTS/DPX assay [24] at 37 °C. 5 mM LUV containing 12.5 mM ANTS, 45 mM DPX, 0.1 M NaCl and 5 mM citrate-/10 mM Na_2HPO_4-buffer, 1 mM EDTA, pH 5.0, were prepared as described above and freed from uncapsulated material by gel filtration on a Sephadex G-75 column eluted with buffer. Lipid concentrations were determined by a phosphate assay [25] and diluted to 100 μM LUV. Fluorescence measurements were performed as above but with excitation at 355 nm, and emission at 520 nm. After measuring fluorescence intensity of the liposomes, protein was added in a final lipid:protein ratio of 300:1 (μM:μM). Zero % leakage corresponded to the fluorescence before adding BLA to the solution; 100% leakage was the fluorescence value obtained after addition of 300 μM Triton-X-100; fluorescence % increase was calculated as $(F/F_t)\times 100$, where F is fluorescence intensity after protein addition and F_t is fluorescence intensity after addition of Triton X-100. The fluorescence measurements were customarily performed 8 min after the addition of BLA (or Triton X-100).

2.5. Surface pressure measurements

Surface pressure measurements were carried out with a KSV Minitrough (Helsinki, Finland) using the manufacturer's software at 37 °C. The Teflon trough (75 mm × 364 mm × 5 mm) was filled with 0.1 M NaCl and 5 mM citrate-/10 mM Na_2HPO_4-buffer, 1 mM EDTA, pH 5.0, with or without 3 nM BLA. The surface was swept and the possible impurities removed from the air/water interface with a Pasteur pipette. 15 μl of glycerophospholipid in chloroform was carefully spread on the surface with a Hamilton syringe, and the chloroform was allowed to evaporate before starting the measurements. Compressions of the lipid monolayers were done at 5 mm/min while an electrobalance recorded the surface tension with a Wilhelmy plate. The surface tension of the film-free solution was taken as a reference. For each condition, at least three surface pressure/area curves were recorded, and the most representative shown in the figures. Control experiments to measure possible adsorption of BLA to the Teflon trough were performed by the Bradford [26] dye-binding method with reagent from Bio-Rad (Bio-Rad, Hercules, CA).

3. Results

3.1. Binding of apo-BLA to liposomes of different composition as measured by intrinsic fluorescence spectroscopy

The binding of BLA to liposomes of negatively charged membranes can be measured by following the changes in the intrinsic fluorescence of the protein [1,8,17]. Some previous studies have exposed an effect of the size of the lipid vesicles on the degree of interaction or the conformation of bound proteins [27,28]. However, for the interaction of BLA with phospholipid bilayer systems, similar results have been obtained with either SUV or LUV of comparable composition [7,8]. The use of SUV is beneficial over LUV in spectroscopic assays, such as fluorescence measurements from 320 to 400 nm where SUV show no intrinsic fluorescence at these conditions. In order to investigate the effect of the negative charge of glycerophospholipids on the

Table 1
Leakage of the contents of LUVs with different lipid compositions upon *apo*-BLA binding

Liposome composition	% Increase in fluorescence intensity
EYPC	4±2
DOPG	40±5
SOPS	20±4
DSPS	5±3
DOPG:EYPC (3:1)	46±3
DOPG:EYPC (1:1)	79±6
DOPG:EYPC (1:3)	13±6
SOPS:EYPC (3:1)	28±5
SOPS:EYPC (1:1)	26±6
SOPS:EYPC (1:3)	24±5
DSPS:EYPC (3:1)	7±1
DSPS:EYPC (1:1)	7±3
DSPS:EYPC (1:3)	3±2

Data are given as mean±S.D. Recordings were performed 8 min after addition of 1 μM BLA to samples of liposomes containing 12.5 mM ANTS and 45 mM DPX in 0.5 ml 5 mM citrate-/10 mM Na_2HPO_4-buffer with 0.1 M NaCl, pH 5.0. The lipid: protein ratio was 300:1 (μM:μM) and the temperature 37 °C. At least three parallels were done for each sample.

interaction of *apo*-BLA with membranes, we thus prepared SUV composed of each of the negatively charged lipids DOPG, SOPS and DSPS and (neutral) EYPC in ratios 1:1, 1:3, 3:1 and 1:0, where the ratio 1:0 designates SUVs of pure anionic lipid species. EYPC, DOPG and SOPS are all in a liquid crystalline state at room temperature [29–31]. On the other hand, the transition temperature of DSPS in SUV is about 69 °C, and this phospholipid tends to form a gel phase at room temperature [7,32]. DOPG and SOPS both contain unsaturated fatty acids, 18:1–18:1 and 18:0–18:1, respectively, while in DSPS, both fatty acids are saturated (18:0–18:0). EYPC is a heterogeneous mix of several molecular species of PC, but analysis by reverse phase HPLC showed that 1-palmitoyl, 2-oleoylphosphatidylcho-

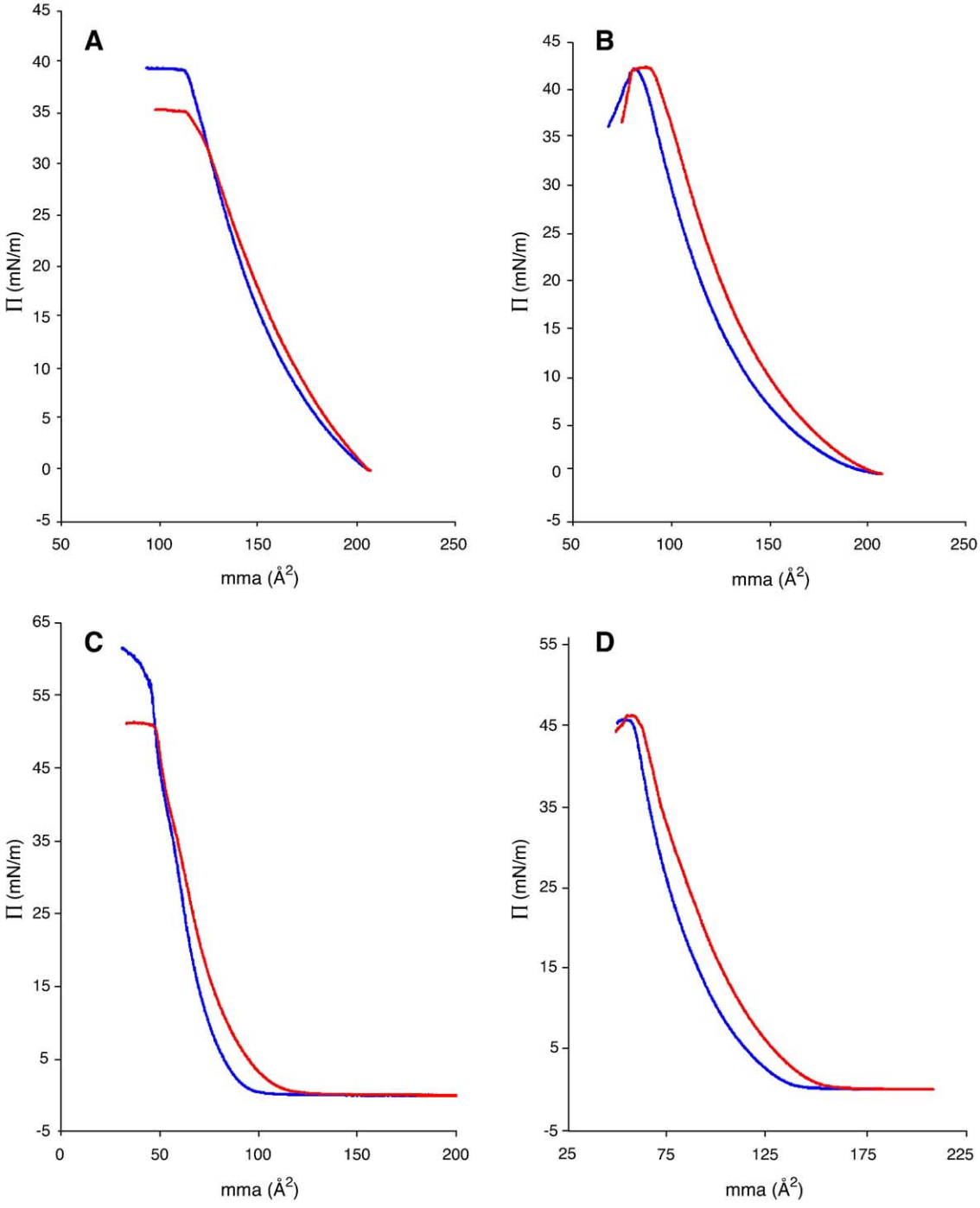

Fig. 2. Surface pressure isotherms. Surface pressure vs. apparent molecular area (mma) at 37 °C of monolayers of (A) EYPC, (B) DOPG, (C) DSPS and (D) SOPS, in the absence (—) and the presence (—) of *apo*-BLA (at a final concentration in the subphase of 3 nM), at pH 5.0. Each of the isotherms given in the figure represents three parallel measurements.

line (16:0–18:1) was the major molecular species (data not shown). Regarding the charge of the phospholipids, PG has an intrinsic pK_a value of 2.3, while PS has intrinsic pK_a-values<1 for the phosphate group, 3.2–5.5 for the serine carboxyl group and 11.0–11.5 for the amino group [33,34]. Nevertheless, calorimetric analysis of the pH dependence of the transition temperature of the bilayers have shown that the PS species integrated in the SUV used in this study are negatively charged in the pH range pH 4.5–6.0 [7].

Thus, representative fluorescence spectra of BLA bound to SUV of different ratios and of different lipid species are presented in Fig. 1. *Apo*-BLA has an intrinsic fluorescence emission spectrum with λ_{max} at about 332 nm with excitation at 295 nm. This spectrum mainly reflects the environment of the Trp residues [1,35]. SUV made of neutral lipids like EYPC have little or no influence on the spectrum of *apo*-BLA (Fig. 1A), as previous studies have shown for the *holo*-BLA [1,8]. EYPC was therefore used as a negative control. Adding SUV composed of mixtures of DOPG:EYPC or SOPS:EYPC, as well as pure anionic SUV, at saturating proportions to *apo*-BLA solutions at pH 5.0 [2], gave an increase in fluorescence intensity and a red shift of λ_{max} (Fig. 1C, D and below summarized data in Fig. 4). The increase in fluorescence intensity has been interpreted as a weakening of tertiary interactions present in the native state, and a red shift of λ_{max} reflects that at least one of the Trp residues in the protein is exposed to a more polar environment [1,8]. As seen by comparative measurements with other methods, the extent of increase in fluorescence intensity of the protein correlates well with the degree of binding [7,8]. Both changes support the association of BLA with the liposomes in a molten-globule-like conformation. For SUV of DOPG:EYPC, the changes of the spectrum were maximized in ratios 1:1>3:1>1:3>1:0. For SUVs of SOPS:EYPC, the changes were maximized in ratios 1:1>3:1>1:0>1:3. The overall fluorescence changes were less marked for SOPS:EYPC than for DOPG:EYPC. No significant changes were observed on the fluorescence spectrum of BLA when adding SUV composed of DSPS:EYPC (Fig. 1B). Again, there appears to be no difference in the behaviour of this system when exchanging SUV for LUV [1,7].

3.2. The ability of apo-BLA to penetrate bilayers of different composition studied by leakage experiments

The fluorescence binding experiments give an indication on the conformational change on the protein upon binding, but they do not clearly determine the degree of penetration of the protein into the bilayer. In contrast, leakage experiments distinguish between adsorption and absorption of the protein [17]. Preliminary experiments showed that LUV were superior to SUV for these leakage experiments due to the higher concentrations of reactants entrapped in the liposomes and the consequent higher sensitivity of the method. LUV of different composition were therefore used in these experiments.

There was no significant leakage for liposomes composed of either EYPC or DSPS, or the mixtures DSPS:EYPC, upon addition of *apo*-BLA above the <10% leakage measured in the absence of protein and which is likely to be caused by natural instability of the liposomes (Table 1). This is consistent with the lack of association inferred from the fluorescence binding assays described above. For LUV composed of varying amounts of EYPC and DOPG, the largest increase in fluorescence intensity (i.e., degree of leakage) took place when the ratio between the lipids was 1:1 (Table 1). This corresponds, at least qualitatively, with the fluorescence binding assays. For LUVs composed of different proportion of SOPS versus EYPC, the leakage pattern was different to the inferred association of the protein, and as shown in Table 1, these LUV gave approximately the same increase in fluorescence intensity after adding BLA to the samples. We also observed that the rate of the increase in fluorescence intensity was slower for these liposomes compared to corresponding LUV with DOPG (data not shown), further indicating differences in the way BLA penetrate bilayers of different phospholipid molecular species.

3.3. The effect of apo-BLA on the packing of different negatively charged glycerophospholipids studied by the Langmuir technique

Further insights on the perturbation of lipidic layers by *apo*-BLA binding were obtained from compression studies of monolayers studied by the Langmuir technique, which has been successfully used to study the penetration of other proteins on phospholipids membranes [36,37]. BLA appears to adsorb to solid interfaces, like polystyrene nanobeads adopting a molten globule-like conformation [38]. We therefore investigated if BLA adhered to the Teflon trough of the Langmuir instrument, which could affect the reliability of the results. As seen by control experiments intended to measure protein concentration in solution after dispensing the protein sample in the through, BLA does significantly interact with the solid surface.

Addition of BLA to the subphase with monolayers composed of single species of EYPC, DOPG, SOPS or

Table 2
Alteration (%) of the molecular area at molecular area at which the surface pressure begins increasing and at 30 mN/m, and change in surface pressure at the collapse point for the isotherms given in Figs. 2 and 3

Lipids	% increase in mma at surface pressure increase	% increase in mma at 30 mN/m	Δ mN/m at the collapse point
EYPC	0	0	4
DOPG	0	9	0
SOPS	12	11	1
DSPS	17	2	11
DOPG:EYPC (1:1)	18	11	1
SOPS:EYPC (1:1)	9	7	3
DSPS:EYPC (1:1)	6	1	0

DSPS appears to influence the packing of all molecular species (Fig. 2). The isotherm of EYPC monolayers showed no significant increase in surface area after adding BLA to the subphase, which indicates that BLA does not absorb significantly into this monolayer. Yet, BLA seemed to influence this monolayer by making it more unstable, as seen by the decrease of the surface pressure of the collapse point (Fig. 2A). Addition of BLA to the subphase under SOPS or DOPG monolayers resulted in an increase in molecular area at constant surface tensions, indicating interactions between the protein and lipid molecules (Fig. 2B, D). The isotherms of SOPS and DOPG showed much the same pattern of packing since both had a direct transition to condensed phase and an unaltered surface pressure at the collapse. In contrast, with DSPS monolayers, the isotherm with BLA in the subphase was the same as the isotherm without BLA when increasing the surface pressure, although with a lowering of the collapse point (Fig. 2C). The compression results are summarized in Table 2.

The fluorescence studies indicated that a ratio of acidic lipid and EYPC at 1:1 was optimal for interaction with *apo*-BLA (see above). Thus, monolayers of two molecular species at 1:1 proportions were also investigated with the Langmuir technique. The packing patterns for monolayers containing DOPG:EYPC or SOPS:EYPC were quite similar, but a larger increase in surface area was observed for the monolayer of DOPG:EYPC when *apo*-BLA was present in the subphase (Fig. 3A and C). This corresponds well with the leakage data showing that the integrity of the membrane is more

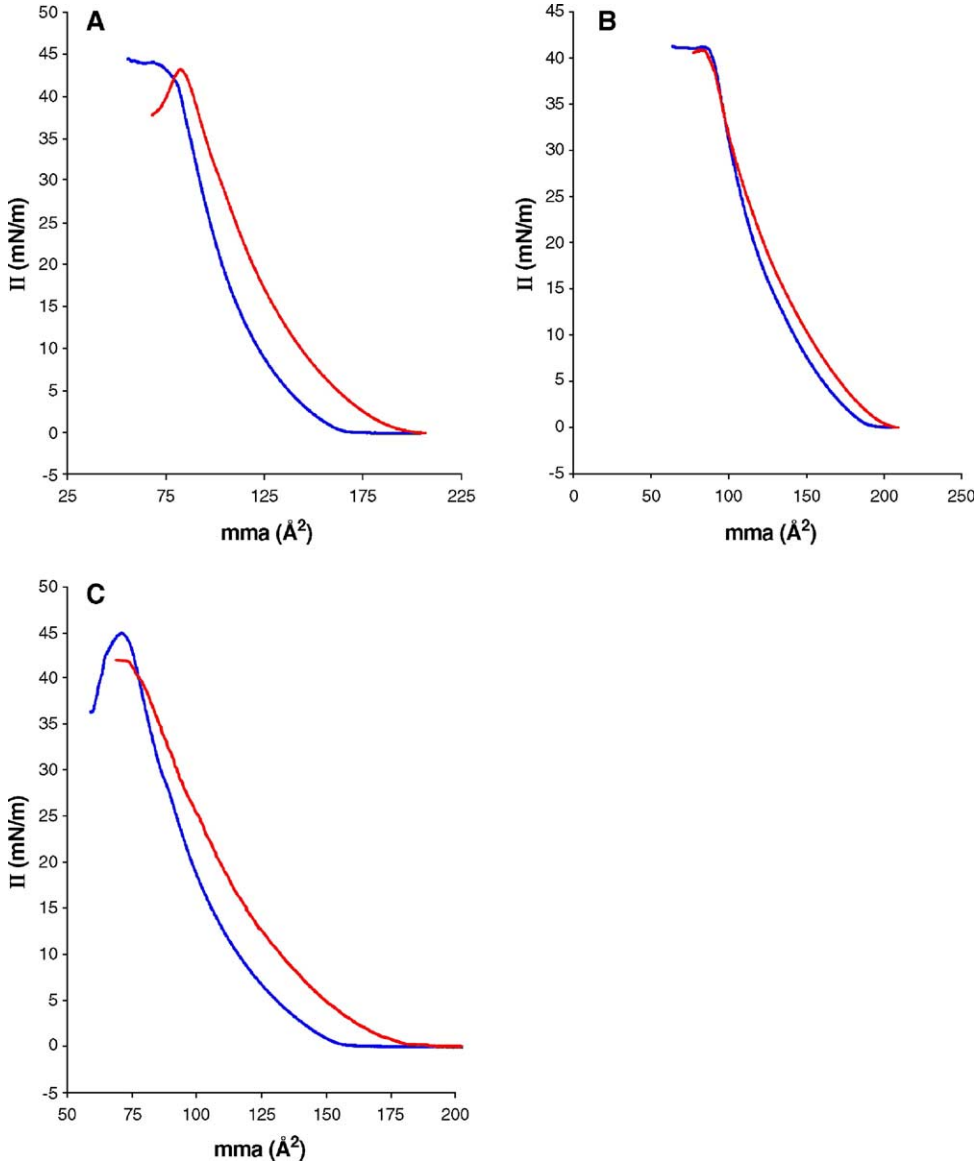

Fig. 3. Surface pressure isotherms. Surface pressure vs. apparent molecular area (mma) at 37 °C of monolayers of equimolar amounts of EYPC and (A) DOPG, (B) DSPS and (C) SOPS, respectively, in the absence (—) and the presence (—) of *apo*-BLA (at a final concentration in the subphase of 3 nM), at pH 5.0. Each isotherm given in the figure represents three parallel measurements.

affected by the protein binding on 1:1 mixtures of EYPC:DOPG than of EYPC:SOPS (Table 1 and Fig. 4, where the phospholipid selectivity for binding was recapitulated by the specificity for induction of leakage). The isotherm for monolayers of EYPC:DSPS with BLA was similar to that without BLA in the subphase at surface pressures higher than 30 mN/m (Fig. 3B).

4. Discussion

The physical state of phospholipid membranes plays important roles in protein association [39–49], and anionic lipids are often necessary for insertion and translocation of proteins in membranes [7,39,41,42,45,48]. The mechanisms of such interactions are complex and involve both electrostatic and hydrophobic forces [8,13,49]. The interactions appear to take place in stages, starting with protonation of key acidic head groups followed by loosening of the protein tertiary structure, which exposes hydrophobic areas that can intercalate into the membrane [8]. In spite of a recent report stating that BLA binds to negatively charged SUV in a native conformation [50], previous work has largely proven that the protein adopts a molten globule-like state upon binding to both SUV and LUV [8]. Moreover, the structural determinants, including the residues in helix A and C important for interaction with negatively charged SUV, appear to be similar *both* using the native and the molten globule conformers as reference [8]. In fact, the membrane-bound state of the protein is best represented as a continuum of conformers, where different degrees of tertiary structure can be present depending on variables, such as the physical state of the bilayer [7,17]. This is also related to the inherent, high flexibility of BLA, which appears to have a structure very sensitive to micromolar concentrations of surfactants and is prone to form a molten globule-like state [51].

We show here that the degree of unsaturation of the acyl chains at 37 °C and the proportion of charged phospholipid species in the mixed membranes are also determinants for the association of *apo*-BLA with liposomes. Our fluorescence and Langmuir results indicate that there is interaction between BLA and liposomes composed of either DOPG:EYPC or SOPS:EYPC, while there is no significant interaction between BLA and liposomes of DSPS:EYPC, although DSPS also has the same negatively charged headgroup at the experimental conditions. These results strongly suggest that the acyl chains play active roles in binding the protein, for example anchoring apolar parts of the protein, thus stabilizing the association. Because of its long, saturated fatty acids, DSPS gives a highly dense membrane that can be difficult for a protein to penetrate. EYPC did not facilitate the binding of BLA to DSPS liposomes, which agrees with the tendency of such mixtures to segregate into domains [52].

Since BLA requires negatively charged lipids for membrane interaction, one would expect that liposomes composed of acidic and neutral lipids at 3:1 ratio, or pure anionic lipids, would give the optimal interaction. Nevertheless, we show here that a 1:1 ratio gives the best interaction with BLA, both for SOPS and DOPG. The sea anemone toxin Sticholysin interacts optimally with

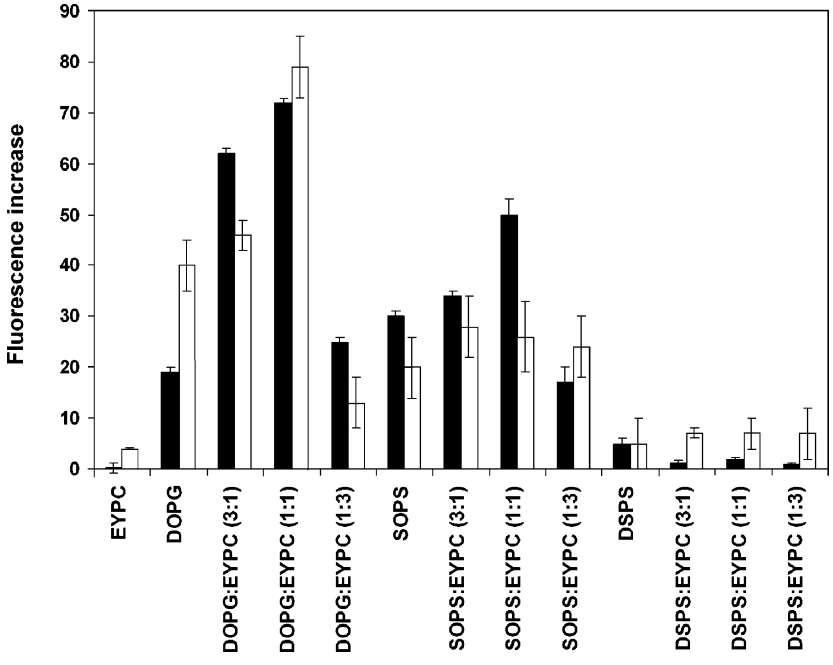

Fig. 4. *apo*-BLA binding measured by intrinsic florescence and ANTS/DPX leakage. Relation between BLA binding to LUV measured as increased fluorescence intensity (black bars) and leakage from the vesicles, measured as % fluorescence increase in the ANTS/DPX system (white bars).

vesicles containing only 5 mol% of acidic lipids [53] while chlorpromazine has an interaction optimum at a membrane composition around 2:3 PS:PC, and the degree of unsaturation plays a definite role [54]. Thus, the presence of neutral lipids appears to promote binding to acidic lipids in liposomes and an optimal neutral:acidic lipid proportion for BLA might be explained in terms of an optimal position of hydrophobic areas for interaction with the molten-globule intermediate in the membrane binding process [8].

Leakage experiments show that vesicles with DOPG and EYPC in a 1:1 ratio are clearly most susceptible to loss of bilayer integrity during interaction with BLA, and correlated with the extent of interaction as seen by intrinsic fluorescence analysis (Fig. 4). The disruption of liposomes made of single DOPG by BLA absorption was elevated, and in fact higher than both induced on those of DOPG:EYPC (1:3) and than expected from the fluorescence binding analysis. With SOPS as the acidic lipid, BLA appeared to have almost the same ability to absorb and disrupt these vesicles independent of the EYPC:SOPS ratio, while the intrinsic fluorescence measurements indicated a more marked binding to SOPS and EYPC in equimolar amounts. Thus, it seems that the ability of BLA to penetrate further and hence disrupt SOPS containing vesicles differs from that of DOPG containing vesicles, in agreement with previous results [7]. Furthermore, our compression studies show that BLA can influence the packing of monolayers as seen by the increase in molecular area of the monolayers of both SOPS and DOPG. BLA did not affect the molecular area of EYPC monolayers, but lowered the surface pressure of the collapse point, indicating that the protein makes the monolayer more unstable (Table 1). In contrast to the monolayers of the other acidic lipids studied, the isotherm of DSPS was little influenced by BLA in the subphase, demonstrating that the less flexible acyl chains in DSPS are harder to penetrate than those in SOPS. Thus, the partition coefficient is lowered and the work of insertion increases as saturation is introduced in the bilayer. At a surface pressure of 30 mN/m, BLA caused less increase in surface area in monolayers of DSPS than in those of SOPS or DOPG. This is close to the surface pressure found in biological membranes and in liposomes [55], and thus might explain the results of the binding- and leakage fluorescence experiments showing no interaction of BLA and liposomes of DSPS. The monolayer studies did, however, show interaction between DSPS and BLA at lower surface pressures. This is to be expected since DSPS and SOPS have the same headgroup, and at large molecular areas the acyl chains should play a minor role. If the results are viewed in light of linking cell membrane composition to protein interaction, a cell state—pathological or otherwise—that has a more fluid membrane would promote binding of BLA.

The surface pressure/molecular area isotherms for the mixtures indicates that BLA associated more easily with DOPG:EYPC and SOPS:EYPC than with DSPS:EYPC, in accordance with the binding/leakage experiments. On the other hand, the DOPG:EYPC and SOPS:EYPC mixtures (1:1) gave results similar to those obtained with the corresponding homogenous monolayers and a larger protein-induced increase in surface area than in the corresponding homogenous monolayers—as was expected from the fluorescence analyses—was not observed. A possible explanation for this finding might be that in a monolayer the interaction of acyl chains in opposite direction (as in the bilayered liposomes) is absent. The close packing of hydrocarbon chains in bilayers induces van der Waals interactions that contribute to stabilizing the membrane.

A decisive step for the function of the tumour apoptotic conformer of α-lactalbumin, HAMLET, is the transport of the protein into the nucleus, where it apparently interacts with histones and irreversibly disrupts the chromatin organization leading to cell death [20]. The mechanism for the protein co-localization to these components is not understood, as BLA has no known mechanism for transport to the nucleus, and could occur by an energy-independent pathway. PG is an intermediate in the synthesis of cardiolipin and mainly present in the mitochondria [56]. However, both PG and cardiolipin have been shown to modulate nuclear transcription in model experiments [57]. Thus, our results on the binding of BLA to PG may suggest a role for targeting of BLA to nuclear PG. The selective uptake and apoptosis of tumour cells may also be explained by a change in the lipid compositions of the nuclear membranes, which is known to be altered during tumour development [58–61]. In a study of phospholipid composition of the nuclei and nuclear membranes from rat liver and hepatoma, the proportion of acidic phospholipids was much higher in the hepatoma nuclear membranes than in those of the normal liver [62]. Other reports of increased content of negative charge in the outer membrane leaflet exist, including the increase of PS headgroups in cancerous and undifferentiated cells [63,64]. Furthermore, the increased occurrence of other sources of negative charge, like O-glycocylated mucine, has been reported for cancer cells [65]. If the increase of negative charge and fluidity in the outer membrane leaflet is a general trend in tumour cells, the results from the present study might contribute to explain the nuclear uptake and the membrane disruption of HAMLET. Importantly, it has recently been shown that both human α-lactalbumin and BLA interact as efficiently with histones independently of the oleic acid cofactor [66]. Thus, the HAMLET conformation is likely irrelevant regarding the proposed action by which the membranes are disrupted and the cells killed, but the conformation may be more effective in membrane accumulation at neutral pH. In conclusion, we propose that the selectivity towards cancer cells can be explained in terms of the greater membrane affinity of HAMLET in general and its affinity towards pathological membrane compositions in particular.

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