α-Lactalbumin:Oleic Acid Complex
Spontaneously Delivers Oleic Acid to Artificial and Erythrocyte Membranes

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Abstract

Human α-lactalbumin made lethal to tumor cells (HAMLET) is a tumoricidal complex consisting of human α-lactalbumin and multiple oleic acids (OAs). OA has been shown to play a key role in the activity of HAMLET and its related complexes, generally known as protein–fatty acid (PFA) complexes. In contrast to what is known about the fate of the protein component of such complexes, information about what happens to OA during their action is still lacking. We monitored the membrane, OA and protein components of bovine α-lactalbumin complexed with OA (BLAOA; a HAMLET-like substance) and how they associate with each other. Using ultracentrifugation, we found that the OA and lipid components follow each other closely. We then firmly identify a transfer of OA from BLAOA to both artificial and erythrocyte membranes, indicating that natural cells respond similarly to BLAOA treatment as artificial membranes. Uncomplexed OA is unable to similarly affect membranes at the conditions tested, even at elevated concentrations. Thus, BLAOA can spontaneously transfer OA to a lipid membrane. After the interaction with the membrane, the protein is likely to have lost most or all of its OA. We suggest a mechanism for passive import of mainly uncomplexed protein into cells, using existing models for OA’s effect on membranes. Our results are consistent with a membrane destabilization mediated predominantly by OA insertion being a significant contribution to PFA cytotoxicity.

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Introduction

The phenomenon of cytotoxic protein–oleic acid (OA) complexes has received continuous attention in recent years, in particular, human α-lactalbumin made lethal to tumor cells (HAMLET) and its related complexes [1–5]. Such complexes are more generally known as protein–fatty acid (PFA) complexes and the focus these have received are partly due to their possible clinical potential—HAMLET has been through several trials of medical relevance [6–8]. The precise nature of the complexes and exact toxic mechanism has been subject to scrutiny. The protein–OA complexes are prepared from bovine α-lactalbumin (BLA; BAMLET) [1], equine lysozyme [equine lysozyme complexed with OA (ELOA)] [2], goat α-lactalbumin [9] and other proteins that are made to bind OA using a variety of approaches, including passage of the protein through a column equilibrated with OA [2,4], direct titration of OA into a protein solution [3,10] and heat treatment [9,11]. HAMLET was initially thought to consist of one OA or only a few OAs initially described as “cofactors” [12]. It has later become clear that the complex consists of many OAs per protein molecule (ratios of up to 1:48 has been reported) [2,3,10] and that the main cytotoxic component is the fatty acid [13]. When bound to the protein, the fatty acid appears to be predominately in its deprotonated oleate form and the binding has a significant electrostatic component [14,15]. It is uncertain whether the protein part of the complex has a role beyond being a solubilizer and carrier of OA, which is insoluble but have toxic properties. HAMLET and ELOA complexes do form annular oligomers in the presence of lipid monolayers more effectively than the native protein that also forms these under the same conditions to some extent [2,16]. These pore-like formations are associated with cell death and the destruction of cellular membranes, at least in systems related to

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neurodegeneration [17,18]. Ion fluxes and large effects on the cellular membranes have been observed for HAMLET-treated cells [19], and one of the key characteristics of the complex appears to be its high affinity for the lipid membrane and the destabilizing effect it has upon it [20].

Recently, we published a paper focusing on the effects that one such PFA complex has on the lipid membrane [21]. We prepared bovine α-lactalbumin complexed with OA (BLAOA), a complex similar to BAMLET, using a simple incubation of BLA in the presence of OA under mild conditions favoring partial unfolding (Ca²⁺ depletion and 37 °C) [22]. This approach provides the opportunity to form BLAOA with varying amounts of OA included in the complex. Our subsequent analysis showed that both the complex ability to induce leakage and cell death correlated well with the amount of OA bound in the complex. We also noted large changes in the packing environment of lipid molecules in large unilamellar vesicles (LUVs) treated with BLAOA relative to controls. Earlier studies performed by Nielsen et al. on ELOA suggested that OA is transferred to the target membrane [23]. Although there have been numerous studies that label and track the protein component of HAMLET and similar complexes at membranes and in cells [2,23,24], there is a lack of information about what happens to the OA component. Very recently, the transfer hypothesis formulated by Nielsen et al. for the ELOA complex has been forwarded again in the context of studying erythrocyte treated with BAMLET, which causes eryptosis by a detergent-like action [25]. The authors find it likely in their discussion that OA (or oleate) is transferred to the erythrocyte membrane as part of the eryptosis mechanism, but they did not proceed to investigate this possibility further. In the current work, we relieve the lack of information on the fate of OA by identifying a transfer of OA from the complex to artificial membranes. The chemical shift of the carboxylate carbon of OA is a useful tracker of how saturated the BLAOA complexes are with respect to the fatty acid component. We use this shift dependency to track the effect that membrane has on the BLAOA complex, and we conclude that the changes observed can only be explained by a transfer to the membrane. Finally, we show that a similar transfer also occurs when erythrocytes are treated with BLAOA, and we isolate the purified erythrocyte membrane fraction and show that it contains OA that cannot be ascribed to any remaining BLAOA. Transfer and embedment of OA into the cellular membrane would lead to its destabilization and, for large doses, their complete disruption. Using existing frameworks for understanding OA effect on membranes [26], we propose an explanation for how the protein component of PFA complexes can be detected in the cytosol and many other places in the cell without invoking active transport as an explanation. Our results also have implications for how the many proposed cellular targets of HAMLET is interpreted, as the protein is likely to enter the cell without much OA attached.

Results

Chemical shifts of OA as a function of BLAOA complex saturation

To characterize the BLAOA complex used in this study and how increasing the amount of OA relative to BLA affects the chemical environment of the carboxylate of the fatty acid, we performed NMR spectroscopy, BLAOA was prepared by OA labeled with 13C at the carboxyl carbon (1-13C OA), and NMR spectra of BLAOA were obtained as a function of BLA:OA molar ratios (Fig. 1A). All the spectra show only one peak from the OA carboxyl carbon, although there may be small amounts of excess OA present after the preparation of BLAOA. For comparison, reported parts per million (ppm) values for bound olate and OA HAMLET prepared using a column pre-incubated OA are indicated (Fig. 1A) [27]. The peak in the carboxyl region of the 13C NMR spectrum of BLAOA is bound OA, since fatty acids in aqueous solution self-assemble into high-order structures and have extremely broad carboxyl resonances that cannot be observed (Fig. 1A, inset, and Ref. [28]). The peak positions are plotted against OA-to-BLA molar ratios in Fig. 1B. The peak moves upfield when increasing the amount of OA used to prepare the complex. For example, the peak for BLAOA 1:80 is at 179.1 ppm, 1.2 ppm upfield from its position at 180.3 ppm for BLAOA 1:2. This overall trend toward lower ppm values is, however, not unbroken. There is a local ppm maximum at BLAOA 1:20. Also, for BLAOA 1:5, 1:10 and 1:20, the peak position was prone to fluctuation in parallels. For larger ratios, the standard deviation was much lower.

OA is associated with the membrane after BLAOA–membrane interaction

We have shown earlier that BLAOA and HAMLET interact strongly with lipid membranes and perturb the membrane with respect to both its lateral packing and its integrity [20,21]. To investigate to what extent the fatty acid component, the protein component or the complex as a whole associates with the membrane, we performed ultracentrifugation on egg yolk phosphatidylcholine (EYPC):porcine brain phosphatidylserine (PBPS) LUVs treated with BLA, OA and BLAOA, using a self-generating iodixanol gradient [29]. Such self-generated gradients provide a straightforward way of fractionating membrane
vesicles and macromolecular complexes. Components that have different densities but are strongly associated with each other will end up in the same fraction. The distribution profiles of lipids (in the form of LUVs and detected by the fluorescent dye Dil embedded in the membrane) for different samples are shown in Fig. 2A. One can see that only BLAOA caused the membrane distribution in different fractions to change. Compared with the distribution profile for LUVs only, BLAOA-treated LUVs ended up more in the denser fractions (fractions 1 and 2) while less in those of lower density (fraction 9). This indicates that BLAOA treatment caused the lipid membranes to become denser. Similar to the membrane distribution profile in Fig. 2A, after the ultracentrifugation, most of the OA also ends up in the lighter fractions (solubilized in methanol and detected by 13C NMR spectroscopy), as shown in Supplementary Information Fig. S1a. For LUVs treated with OA alone, OA was not detected by NMR in any of the fractions (but later detected on the test tube walls after centrifugation; see Fig. S3). Unlike the membranes and OA, the protein component (detected by NanoOrange), after the ultracentrifugation, is evenly distributed in every fraction for both BLAOA- and BLA-treated LUVs (Supplementary Information Fig. S1b), except for a slight rise in amount in the fraction of lowest density (fraction 10), which is not seen for BLAOA or BLA centrifuged alone. In Fig. 2B, the distribution profiles of the membrane, protein and OA for BLAOA-treated LUVs are plotted together for easier comparison. As can be seen, OA and the lipid membranes have very similar distribution profile; that is, OA follows the membranes in all the fractions. The protein component is not strongly associated with the membranes on the timescale of the experiment (3 h), and OA and protein are not associated with each other. The association of the OA component of BLAOA with the membrane was also confirmed using size-exclusion chromatography (Supplementary Information Fig. S2).

Changes of BLAOA complex in response to LUV treatment

It was earlier established by several laboratories that PFA complexes have a high affinity to membrane, but these studies did not examine the fate of the fatty acid component separately [20,21,23]. Very
recently, erythrocytes were reported to undergo eryptosis-like behavior upon BAMLET treatment, and the authors of this paper again discuss transfer of OA from complex to the membrane [25]. The results presented above indicate that the fatty acid component of the complex follows the membrane closely. We are interested in knowing whether this is the result of a wholesale transfer or just an association between the BLAOA complex and the membrane. Since the chemical shift of OA carboxyl
carbon is sensitive to the BLA:OA molar ratio in BLAOA, it can be used as a tracker to access the complexation state of BLA to OA in the complex during membrane interactions. We monitored the change in the $^{13}$C NMR chemical shift of OA carboxyl carbon in BLAOA (BLA:OA 1:40, 160 μM) during titration by EYPC:PBPS LUVs and the results are shown in Fig. 3. One can see that, upon adding LUVs, the peak moves upfield from 179.67 ppm for the complex alone to 179.16 ppm as the lipid concentration reaches 600 μM. In addition, there are clear changes in the signal linewidth and intensity during the titration, further indicating a change in the environment of the OA. The chemical shift change and line broadening is consistent with a transfer of the OA to a more hydrophobic and slow-tumbling environment.

Interaction of BLAOA with erythrocytes

The titration of BLAOA with LUVs showed significant changes in the chemical environment of the carboxylate carbon of the OA factor that were not compatible with a simple stripping of the complex, which would have led to the peak moving downfield. Membrane effects have been linked to the efficacy of HAMLET-like complex on target cells [20,23]. We are interested whether natural cell membranes elicit similar responses, and we choose erythrocyte membranes. Erythrocytes are easily isolated and, unlike most other cell types, they have a simplified outer membrane structure and they lack nucleus and other organelles. We proceed to titrate BLAOA 1:40 with erythrocytes instead of LUVs and record the $^{13}$C NMR spectrum of BLAOA during the interactions (Fig. 4A). Instead of a continuous movement upfield as was observed in response to LUVs treatment, the addition of erythrocytes to BLAOA results in a slight downfield movement of the OA carboxyl carbon peak from 179.27 ppm before movement upfield to 179.52 ppm at the titration endpoint (Fig. 4B). Moreover, the signal intensity decreases upon erythrocyte titration.

The drop in signal intensity was not seen as clearly for the LUV titration. There is also a question in both the LUV and erythrocyte instance about the fate of the OA. Clearly, the OA signal is perturbed in a manner consistent with an association with the membrane, but whether the disappearance of the signal is due to a transfer and embedment into the membrane is unclear. It is to be expected that the OA signal is difficult to detect when the molecule is completely embedded in a large aggregate such as a LUV and even more so in the case of the very large (50-fold larger) erythrocyte. To investigate whether we could find evidence for a complete transfer to erythrocyte membranes, we performed $^{13}$C NMR on isolated cell membranes from erythrocytes treated with BLAOA 1:40 (Fig. 5A). Indeed, we observe a
clear signal from OA, which is rather low when the sample is scanned without further effort to resolve it but which is significantly boosted when methanol and SDS are added, dissolving OA completely and dissolving lipid and OA molecules into a solution of mostly monomers. We also show that the sample is practically free of BLA, which is only detected by anti-BLA in the supernatant after BLAOA treatment (Fig. 5b). The control experiments subjecting erythrocytes to similar amounts of uncomplexed OA show no detectable OA in any fraction (Fig. 5A and inset), confirming that BLA complexation of OA is necessary for this behavior. Further control experiments relocate the OA in the test tube after spinning down the erythrocyte control sample after the 4-h exposure to OA or BLAOA, as described in Materials and Methods (Supplementary Information Fig. S3).

Discussion

Tracking the chemical environment of OA in complex with BLA and lipid membranes required us to first investigate the relationship between BLA:OA molar ratios used to prepare the complex and the OA carboxylate chemical shift. As expected, the OA carboxyl carbon chemical shift is dependent on the BLA:OA ratio in BLAOA (Fig. 1), indicating that the magnetic microenvironment of OA changes with OA content in the complex. In general, the chemical shift of OA carboxyl carbon moves upfield upon addition of OA to BLA, indicating a slightly more shielded environment for the carboxyl group as the complex becomes more saturated with OA. The 13C NMR methodology used here has been used in earlier works to identify different types of binding sites of OA to bovine serum albumin where multiple carboxyl peaks appeared [28]. There are also reports available for the more closely related complex HAMLET. The HAMLET 13C NMR spectrum shows clear peaks for oleate and OA at about 180.9 ppm and 178.1 ppm, respectively [27]. Similarly, the work of Forato et al. shows that OA storage proteins can bind OA using either a predominantly electrostatic mode or a predominantly hydrophobic mode [30]. In contrast, in this study, only a single, broad carboxyl peak, whose position is dependent on the BLA:OA ratios in the complex, was observed. This is best explained by fast exchange
taking place between a range of closely related chemical environments, where electrostatically dominated oleate binding on one hand and hydrophobic OA binding on the other hand define the extremes. That a distribution rather than two distinct environments are observed underlines the fact that BLAOA is not exactly the same as HAMLET or BAMLET. BLAOA does retain HAMLET key properties, however, such as being highly cytotoxic and having large effects on membranes [20,21]. For column-prepared HAMLET [12], bound fatty acid is predominantly in its oleate form, that is, analogous to the electrostatic mode reported by Forato et al. for bovine serum albumin [30]. For BLAOA prepared at low OA:protein ratios, the $^{13}$C chemical shift of bound OA is closest (180.3 ppm for BLAOA 1:2; Fig. 1B) to the values reported by Ho et al. for oleate binding. However, the average environment becomes more similar to that of OA binding as ratios increase (179.1 ppm for BLAOA 1:80; Fig. 1B).

Our earlier results indicate that BLAOA may incorporate into the lipids upon membrane interaction and cause a tighter lipid packing in the membrane [21]. The membranes become denser after being treated with BLAOA (Fig. 2A), indicating that the complex and/or its components have interacted with and altered the properties of the membranes. BLA or OA alone did not cause obvious changes to the LUV distribution profiles, suggesting little effect on the membranes under these conditions. Furthermore, from Fig. 2B, one can see that it is the OA component of BLAOA that is associated with the membranes after the complex–membrane interaction. The protein component, on the other hand, is strongly associated neither with the membranes nor with OA. This suggests that the complex transfers fatty acid from protein component to lipid membranes upon membranes interactions. This is consistent with previous findings for BLAOA and ELOA reporting at least partially protein disassociated from the fatty acid [21,23]. Although the protein component is rather evenly distributed in all the fractions after the ultracentrifugation, indicating that the protein is well dissolved in the sample solution and therefore could not be fractionized, there is a small but notable increase of protein component in the lightest fraction where most of the LUVs also reside, showing that the protein does associate weakly with the lipid membranes. This is also observed for BLA-treated LUVs (Fig. S1b), suggesting that this weak interaction of protein with lipid membranes is a general property of the protein.
and can happen with or without the help of OA. Indeed, the calcium-depleted, apo-BLA has been reported to bind weakly to lipid membranes at neutral pH values [31,32]. This is inline with our findings and is inline with the protein component's tendency to interact weakly with many substances [22]. In the control experiments where BLA or BLAOA was centrifuged alone without any LUVs being added, the protein distribution was the same in every fraction.

The disassociation of BLA from OA caused by membrane interactions was tracked by following the chemical shift of OA carboxyl carbon (Fig. 3), which is dependent on the BLA:OA ratio in BLAOA (Fig. 1). During the titration of BLAOA with LUVs, if more and more OA is stripped away from the complex to the membranes, one would expect that the chemical shift of OA carboxyl carbon would move downfield (Fig. 1). However, we observed the opposite trend (Fig. 3); that is, the peak moved upfield upon adding LUVs. We interpret this as OA being inserted wholly or partially into the lipid membranes while remaining detectable by NMR. The slow tumbling and dipolar–dipolar relaxation in the membrane is expected to broaden the OA resonance, but it seems that we were still able to observe a membrane environment for OAs associated with LUVs of 110 nm hydrodynamic diameter (as determined by dynamic light scattering). The broad nature of the signals involved means that one cannot distinguish OA bound to the membrane from OA bound to BLA. The peak observed has contributions from mainly protein-associated and the mainly lipid-associated OA that in sum cause the average signal to move upfield as more vesicles are added. As can be seen from Fig. 1A, the peak of OA in BLAOA is relatively sharp. However, the addition of LUVs makes the OA resonance weaker and broader (Fig. 3), consistent with a transfer of OA from BLA to membranes. While LUVs have a hydrodynamic diameter of about 110 nm, erythrocytes are much larger (1000–5000 nm in diameter), and thus, NMR signals from factors that are embedded in their membrane are expected to be unobservable. Therefore, a stripping of OA from the complex during erythrocytes treatment will cause the chemical shift of OA carboxyl carbon to move downfield and the signal to become weaker as we were left only with the signal from the remainder of the complex. This is exactly what is observed (Figs. 1A and 4A). Moreover, we isolate the BLAOA-treated (1:40) erythrocyte membranes, washed away excess complex and showed that labeled 13C carboxylate could be identified in the membranes (Fig. 5). This behavior is only observed when erythrocytes are treated with BLAOA. Control experiments using equal amounts of OA (8 mM) is not transferred to the membrane in this manner. All experiments performed points toward BLAOA effectively being able to transfer OA to both LUV and membranes, while OA in the absence of the protein is not able to do so. This is indicative of a situation where the main role of the protein is to keep the OA soluble and available for effective interaction with the membrane, inline with what other authors have
suggested earlier [9,13,24], and recently also for PFA action on erythrocyte cells [25].

In the case of experiments tracking the OA chemical shift, the resulting trends recorded can be explained in terms of observable (in the case of LUVs) and non-observable (in the case of the much larger erythrocytes) NMR signals. We conclude that BLAOA can effectively transfer OA to lipid membranes using a mechanism that is independent of active transport. That is not to say that cells other than the simple erythrocytes may not respond actively to PFA treatment, merely that it is not necessary to postulate involvement of cellular machinery to achieve transfer as a first step of PFA's action. The LUV experiments where we see an average signal of both protein-associated and lipid-associated signals open for the question about what the lipid-associated situation is like. Are the OA molecules completely embedded in the membrane, and is the membrane packing much perturbed by this? The final experiment where we relocate the OA signal in the membrane fraction of erythrocytes only after we treat the sample with methanol and SDS indicates that the OA is embedded in the membrane.

Insertion of OA into a bilayer would be disruptive to its packing. One important determinant of membrane shapes is the difference in tensions between the inner and outer membrane leaflets [26,33]. This difference is caused by the molecular area in each leaflet and the geometry of the components and the forces acting between them. If OA or oleate is inserted in the outer leaflet, this would strain the configuration of the bilayer until a new equilibrium is reached by transfer of molecules between leaflets [34]. In addition to spontaneous and relatively slow flip-flop behavior of membrane components, it has been proposed that transient membrane defects contribute to the transfer to the inner leaflet in a tension-dependent manner [35,36]. That is, the more tension there is between inner and outer leaflets due to BLAOA action, the stronger this effect would be. BLAOA adversely affects the integrity of the membrane in a manner that is proportional to the amount of OA in the complex, and a concomitant tightening packing of the leaflet structure is observed [21]. Thus, BLAOA seems to provide both the necessary tension and the necessary defects for this mechanism to be effective. The proponents of this mechanism also suggest that other molecular species can be transferred in this way. The spontaneous import of HAMLET observed in many studies [20,37] may actually be due to such a tension-driven influx. Intercalation of oleate in particular would favor the formation of structures with positive curvature. Simple exposure to slurry of OA at slightly (pH 8.8) basic conditions can greatly affect the membrane structure of giant unilamellar vesicles [26]. Among effects seen are rapid invagination and eventual collapse of vesicles [26], in a manner very similar to those reported by us when exposing giant unilamellar vesicles to BLAOA at physiological conditions [21].

This observation may explain why cells treated with HAMLET experience broadly increased ion fluxes that are only partially inhibited by known blockers of ion channels [19]. Our results also indicate that the OA component of the BLAOA and similar complexes tend to end up in the membrane, rather than remaining in the complex. There are many reports, mostly based on tracing of the protein component of HAMLET, that it enters the cell and associates with a wide range of organelles and individual protein components [37–39]. Our current finding indicates that much, if not all, of the OA gets stripped away to a large extent. At least some of the effects and interactions reported for HAMLET and similar complexes could conceivably be caused by the protein component alone, since the apo-form of human α-lactalbumin that remains after full or partial shedding of OAs will be partially unfolded and thus prone to interact to some extent with multiple targets [22]. It is also not always clear whether any given interaction implied in HAMLET action is actually causing cell death or whether the cell is already dying from another primary cause. It is clear, however, that complex binding to the lipid membrane has significant disruptive effects [16,20,21,23]. For some experimental setups, for instance, those utilizing complexes saturated with OA such as here, this may be sufficient to kill the cell outright.

Materials and Methods

Materials

BLA [type III, calcium-depleted, ≥85% (PAGE)], deuterium oxide (99.9%), Wilmad NMR sample tubes (5 mm, 500 or 600 MHz, L7) and OptiPrep Density Gradient Medium [60% (w/v) solution of iodixanol in water] were all purchased from Sigma. We obtained 99% EYPC and PBPS lipids from Avanti Polar Lipids, Inc. We obtained 1.3C OA (1.3C OA, oleic acid labelled with 13C in position 1) from Cambridge Isotope Laboratories, Inc. We acquired NanoOrange Protein Quantitation Kit and Dil Stain from Molecular Probes (Invitrogen). Anti-α-lactalbumin antibody specific for bovine was acquired from Abcam. Blood with K3EDTA (EDTA, ethylenediaminetetraacetic acid) as an anti-coagulant was donated to us by Haukeland University Hospital Department for Immunology and Transfusion Medicine, Bergen, Norway, and used for isolating erythrocytes. All other chemicals were from Merck.
**Erythrocyte isolation**

Erythrocytes were isolated from blood provided by Haukeland University Hospital. Briefly, the blood was stored for 72 h at 4 °C to allow reticulocytes to mature. Then, the blood was centrifuged (650g, 5 min), and plasma, buffy coat and 30% of the erythrocytes were removed by aspiration. The remaining cells were washed four times. The washing buffer consisted of 10 mM Tris–HCl (pH 7), 0.9% NaCl and 5 mM glucose. The cells were either used directly for titrations against BLAOA monitored by 13C NMR or treated with BLAOA prior to isolation of their membranes.

**Isolation of erythrocyte membranes**

Erythrocytes treated with BLAOA or untreated cells were centrifuged (650g, 5 min), and subsequently, supernatant was removed by aspiration. Then, the cells were resuspended in ice-cold 5 mM Tris–HCl (pH 7) to allow complete lysis and centrifuged (9000g, 20 min). This process was repeated until the hemolyse appeared to be colorless; most times, two washes were enough. The isolated ghost membranes were treated with 0.1 mM EDTA and 5 mM Tris–HCl (pH 7) for 30 min at 37 °C, then the ghost membranes were centrifuged (20,000g, 30 min). Then the supernatant was removed by aspiration and the isolated membranes were resuspended in 5 mM Tris–HCl (pH 7) or 5 mM Tris–HCl (pH 7), 50% MeOH and 1% SDS and the presence of OA was identified by 13C NMR. Supernatants collected throughout the procedure were analyzed by dot blotting with anti-BLA to track the location of BLA.

**Preparation of the BLAOA complexes**

BLA was added to phosphate-buffered saline (10 mM, pH 7.5) to make stock solutions at 400 μM. All the protein concentrations are given in terms of monomeric protein and the isolated membranes. This is noted in the pertinent figure texts.

**Preparation of the phospholipid vesicles**

LUVs were prepared from EYPC and PBPS. The prerequisite amount of chloroform-dissolved 1:1 molar ratio mixture of EYPC/PBPS was added to a glass tube wrapped in aluminum foil. The chloroform solution was dried to a thin lipid film using a nitrogen stream and residual chloroform was removed under vacuum for 4 h. The sample was then mixed with phosphate-buffered saline (10 mM, pH 7.5) and left to hydrate overnight on a shaker at 37 °C. To prepare LUVs, we subjected the solution to seven freeze/thaw cycles using liquid nitrogen and a warm water bath. The sample was then extruded through a 100-nm pore-size membrane (Nuclepore, Whatman) 11 times using Avanti Mini-Extruder (Avanti Polar Lipids, Inc.) and transferred to a clean tube.

**NMR spectroscopy**

NMR experiments were performed on either a Bruker AV600 instrument equipped with a QCi CryoProbe or a Bruker AV500 equipped with a BBO probe. For a typical pulse-and-acquire 13C experiment, 96 scans were accumulated with a recycling delay of 1 s. The receiver gain was set to 14,200, and a short delay of 100 μs was inserted between the end of pulse sequence and FID accumulation to avoid baseline distortions. Processing in Topspin in all instances used exponential multiplication as a window function with a line broadening of 30 Hz. All spectra were baseline adjusted using the manual interface provided for this by Topspin. In some cases, the number of scans and receiver gain setting was not the same for all experiments. In these cases, the data were scaled to match the other data presented. For identification of 1,13C OA when the signal was completely obscured by line-broadening effects, methanol was added to the samples to completely dissolve all the OA molecules (final concentration of up to 67% v/v methanol/water). In this way, the resonance peak height could be used to compare the OA content in different samples. This was performed for experiments employing ultracentrifugation, the PD10 column and for the final detection of OA embedded in the isolated erythrocyte membranes. This is noted in the pertinent figure texts.

**Ultracentrifugation**

The self-generating gradient (10%) was made by diluting Optiprep Density Gradient Medium with DI water. All the samples were centrifuged in 5-mL centrifuge tubes in a Beckman Coulter SW 55 Ti swinging bucket rotor in a Beckman Coulter ultracentrifuge (Optima LE-80K) at 50,000 rpm for 3 h, 24 °C. The rotor was allowed to decelerate naturally with the brake off. The fractions (300 μL each) were collected from dense end first. The density profile was calculated from blank gradients with known concentrations using a refractometer (Hanna Instruments, Inc.).

**Fluorescence assays**

Fluorescence assays for protein and lipid quantifications were performed on Typhoon FLA 9000 laser scanner (GE Healthcare Life Sciences). Protein quantification assay was conducted using NanoOrange Quantitation Kit (Invitrogen) according to the product’s protocol. Briefly, 10 μL of each sample was diluted in the working solution to achieve a final volume of 250 μL. The mixture was then incubated at 95 °C for 10 min. All the samples were cooled down to room temperature before being transferred to a 96-well microplate. The fluorescence was measured with excitation at 485 nm and emission at 590 nm. The protein content in different samples was assayed by comparing their fluorescence intensity (reagent blank
was subtracted). For lipid quantification assay, a lipophilic membrane stain Dil (1%) was added to the chloroform-dissolved EYPc:PBPS mixture for preparation of LUVs. The lipid content in different samples was then compared by measuring the Dil fluorescence (excitation at 549 nm and emission at 565 nm) in each sample.

**Accession number**

Swiss-Prot or National Center for Biotechnology Information: bovine α-lactalbumin P00711.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2015.08.009.

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fatty acids; cell death; membrane; HAMLET; PFA complexes

**Abbreviations used:**

BLA, bovine α-lactalbumin; BLAOA, bovine α-lactalbumin complexed with OA; ELOA, equine lysozyme complexed with OA; EYPc, egg yolk phosphatidylcholine; HAMLET, human α-lactalbumin made lethal to tumor cells; LUV, large unilamellar vesicle; OA, oleic acid; PBPS, porcine brain phosphatidylserine; PFA, protein–fatty acid.

**References**


