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HAMLET Forms Annular Oligomers When Deposited with Phospholipid Monolayers

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Recently, the anticancer activity of human α-lactalbumin made lethal to tumor cells (HAMLET) has been linked to its increased membrane affinity *in vitro*, at neutral pH, and ability to cause leakage relative to the inactive native bovine α-lactalbumin (BLA) protein. In this study, atomic force microscopy resolved membrane distortions and annular oligomers (AOs) produced by HAMLET when deposited at neutral pH on mica together with a negatively charged lipid monolayer. BLA, BAMLET (HAMLET's bovine counterpart) and membrane-binding Peptide C, corresponding to BLA residues 75–100, also form AO-like structures under these conditions but at higher subphase concentrations than HAMLET. The N-terminal Peptide A, which binds to membranes at acidic but not at neutral pH, did not form AOs. This suggests a correlation between the capacity of the proteins/peptides to integrate into the membrane at neutral pH—as observed by liposome content leakage and circular dichroism experiments—and the formation of AOs, albeit at higher concentrations. Formation of AOs, which might be important to HAMLET's tumor toxic action, appears related to the increased tendency of the protein to populate intermediately folded states compared to the native protein, the formation of which is promoted by, but not uniquely dependent on, the oleic acid molecules associated with HAMLET.

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Abbreviations used: AFM, atomic force microscopy; ANTS, 8-aminonaphtalene-1,3,6-trisulfonic acid; AO, annular oligomer; α-La, α-lactalbumin; BLA, bovine α-lactalbumin; DPX, *p*-xyelene-bis-*N*-pyrimidium bromide; HAMLET, human α-lactalbumin made lethal to tumor cells; LUV, large unilamellar vesicle; PBPS, porcine brain phosphatidylserine; EYPC, egg yolk phosphatidylcholine; TFE, trifluorethanol; 3D, three dimensional.

Introduction

Within the standard pharmacological framework, emphasis is put on the action of drugs via activation of receptors; the drug elicits a discrete conformational change in the receptor that triggers a cellular signaling response that, in turn, has the intended medical effect. While the success and explanatory power of the receptor-focused paradigm is very real, there exist a number of independent reports on prospective protein and peptide-based therapeutics that do not easily fit into this framework. The fungal ribotoxin protein α-sarcin has cytotoxic and anticancer properties that are thought to be mediated mainly through interaction with the plasma membrane, independently of receptors and endocytosis pathways.[1–3] As is well established, many pharmaceutically interesting peptides also work through perturbation of membrane integrity,[4–8] and these are under intense scrutiny for their cytotoxic action on cancer cells, as well as for their antibiotic properties.[6,9–12] What both protein and peptide phenomena have in common is that they imply an affinity for membranes that is invariably enhanced by the presence of negatively charged bilayer components.[2]

A promising candidate for a membrane-targeting therapeutic is human α-lactalbumin (α-La) made lethal to tumor cells (HAMLET).[13,14] This protein–oleic acid complex selectively kills a broad range of tumor cell lines,[13,15] and its pharmacological potential has been successfully realized in clinical trials.[16,17] From a folding perspective, the HAMLET conformer is also very interesting. It resembles the α-La molten globule folding intermediate,[18] which has been characterized in detail for both the bovine[19] and the human[20] varieties, and appears to be locked in this configuration by the oleic acid «cofactors» that are needed for activity.[21] A broad range of effects is seen in the cell as HAMLET rapidly kills susceptible targets. HAMLET is reported to accumulate in the nucleus, to interact with histones,[22,23] mitochondria,[24] lysosomes[25] and proteasomes.[26] While cell death mediated by HAMLET shows some hallmarks of apoptosis, including loss of membrane potential and phosphatidylserine exposure in the membrane,[21,27] the apoptotic process is not slowed by caspase inhibition or overexpression of Bcl-2.[27,28] Recently, elevated expression levels of c-Myc, an oncogene that binds to a significant fraction of all known gene promoters, were identified as determinants for HAMLET-sensitive phenotypes. Cells with elevated c-Myc levels are sensitive to HAMLET, and short hairpin RNA-mediated knockdown of the c-Myc gene desensitizes normally susceptible cells to treatment.[29] Despite this discovery, questions regarding HAMLET's mechanism of action remain unanswered, including details of the initial HAMLET–cell interaction and how the sensitive phenotype differs from the resistant in this and subsequent steps. The individual roles of the protein and oleic acid components in causing cell death are also unclear.

An interesting quality of HAMLET is its ability to bind to and at the same time perturb bilayers, cell remnants and whole cells under physiological conditions.[30] α-La, in contrast, binds weakly to tumor cells and causes little further effect on the outer membrane or cell viability.[21] We have recently shown that, compared with bovine α-La (BLA), HAMLET causes increased leakage of vesicle contents at neutral pH, in parallel with its increased membrane binding ability.[30] This increased affinity of HAMLET for membranes appears important to understand its selectivity, cellular import and cause of cell death, although the particular mechanism for membrane destabilization lacks detail. We here show by atomic force microscopy (AFM) that, under physiological conditions, HAMLET readily distorts the lipid monolayer at very low concentrations (7 ng/mL) and pore-like oligomeric structures in lipid monolayers at higher concentrations. The pores resemble annular oligomers (AOs), toxic aggregate states described in other protein systems under scrutiny for their role in disease.[31,32] Further, BLA produces essentially the same pore-like AOs, but effective protein concentrations higher than those for HAMLET are required. Moreover, one out of two peptides derived from BLA membrane binding motifs caused leakage and formed similar pores at elevated peptide concentrations. These finding emphasize the enhancement of intrinsic properties of the α-La polypeptide chain in HAMLET, coupled to a local weakening of the membrane.

Results

Membrane distortions and formation of AOs by HAMLET studied by AFM

In order to investigate the mechanism and details of membrane disruption, we explored the membrane interaction of the active, oleic-acid-associated HAMLET by AFM. The protein was co-deposited using the Langmuir–Blodgett technique onto mica chips with lipid monolayers consisting of a zwitterionic extract, egg yolk phosphatidylcholine (EYPC), mixed 1:1 with a negatively charged phospholipid extract, porcine brain phosphatidylserine (PBPS), a composition referred to as EYPC:PBPS. This mix is considered a good mimic of negatively charged physiologically relevant membranes.[30,33]

Deposition of mixed polypeptide–lipid monolayer films on hydrophilic mica results in an orientation of the lipid head groups toward the mica surface.[34]

Thus, the interacting polypeptides are expected to be trapped between the mica and the lipid monolayers or, alternatively, intercalated in the lipid film. Throughout the study, reference AFM images of mica only exposed to polypeptide-containing subphase in the Langmuir tray (Fig. S1a) and lipid monolayers deposited in the absence of polypeptide were prepared and in all instances produced no distortions (Fig. S1b). We investigated the effect of HAMLET on monolayer topology at neutral pH, where HAMLET is biologically active. As seen by surface plasmon resonance and vesicle leakage content experiments, HAMLET strongly binds to immobilized large unilamellar vesicles (LUVs) at low protein concentrations.[30] We observed membrane distortions that formed readily at subphase concentrations as low as 7 ng/mL (Fig. 1c) and built-up in the monolayers in a dose-dependent manner (Fig. 1c, e and g). At the highest HAMLET subphase concentration (142 ng/mL; Fig. 1i), additional and larger distortions of approximately 4 nm in height relative to the lipid film baseline appeared. These structures typically spanned 100–300 nm, were evenly distributed in the monolayer and were characterized by a depression down to the image baseline in their centre. The distortions are immediately suggestive of AOs, polypeptide structures forming pores in membranes.[32,35] Such pores are reported to form from disordered and usually aggregated protein states and show a large range of dimensions, from around 20 nm[35,36] to 70–90 nm[35,37] and 100–250 nm.[32] The latter pore size, very similar to that obtained here for HAMLET-induced distortions (Fig. 1i), was reported in an AFM investigation of α-synuclein on protein–lipid monolayer films, where the occurrence of the AOs was also linked to membrane destabilization.[31]

Ability of native BLA to influence the monolayer compared to HAMLET

We then investigated the interaction of native BLA with EYPC:PBPS monolayers by AFM at pH 5.0, where BLA binds to membranes with high affinity. Under these conditions and at a subphase polypeptide concentration of 43 ng/mL, both HAMLET and BLA caused distortions of the monolayer (Fig. 1a and b, respectively), which were smaller than those observed for HAMLET at neutral pH, and did not have the characteristic AO topology found under neutral conditions. Under acidic conditions, BLA and HAMLET showed similar effects on the monolayer with respect to affinity and topological changes. At neutral pH values, where the affinity of the native protein is much lower,[30,38] we also observed concentration-dependent AO formation by BLA (Fig. 1d, f and h). As was the case for HAMLET at the lowest concentration (7 ng/mL), the effect induced by BLA on the monolayer was limited to smaller (<40 nm) distortions (Fig. 1c and d). These might be AO precursors, as reported prior to the formation of AOs by other proteins.[35] To verify that the bovine HAMLET counterpart (prepared using BLA), that is, BAMLET, also has the ability to form AOs at comparable concentrations, we performed the same experiments using BAMLET, both at 43 ng/mL and at 142 ng/mL. Indeed, BAMLET also formed AOs, albeit at slightly attenuated efficiencies (Fig. S2). The results with HAMLET, BAMLET and BLA thus indicate that AO formation is an intrinsic property of the polypeptide chain that is enhanced—but not dependent on—complexation with oleic acid (the fatty acid component of HAMLET).

Ability of Peptide A and Peptide C to influence the lipid monolayer

To test whether there are segments of the protein that contribute to the formation of AOs, we also studied the effect of Peptides A and C, corresponding to helices A (residues 1–18) and C (residues 75–100) in BLA (Fig. 2). We have previously identified these segments as structural determinants involved in the reversible, pH-dependent membrane binding of BLA.[39] At pH 5.0, conditions where the native apoprotein binds with high affinity,[38] 3 nM Peptides A (7 ng/mL) and C (9 ng/mL), did not perturb the membrane to the same extent as equimolar concentrations of HAMLET and BLA at pH 5.0 (Fig. S1c and d). Under these conditions, the peptide-induced effect on the monolayer is again limited to the smaller (<20 nm) distortions of low incidence, notably for Peptide C (Fig. S1d).

Since it appears that the formation of AOs in lipid monolayers is promoted in a dose-dependent manner at neutral pH (Fig. 1), we proceeded to examine the effect of the peptides under neutral conditions and at highly elevated subphase concentrations compared with those used with HAMLET and BLA. This was justified as the peptides induced some monolayer perturbations (Fig. S1c and d), similar to those observed for both BLA and HAMLET at lower subphase concentrations. Also, small peptides are less surface active overall than full-length proteins.[40] At 10 nM concentrations, both peptides (22 ng/mL for Peptide A and 30 ng/mL for Peptide C) perturbed the monolayer to some extent (Fig. 3a and b) but failed to form AOs. At subphase concentrations of 200 nM, we observed AO formation in the case of Peptide C (591 ng/mL; Fig. 3d and inset). Clearly, the propensity for AO formation is much lower than for the full-length protein but still present to some degree. Peptide A showed no AO formation in monolayers even at 200 nM (440 ng/mL; Fig. 3c). Both helices A and C are implicated in membrane binding,[39] and we were thus interested in determining whether the two corresponding

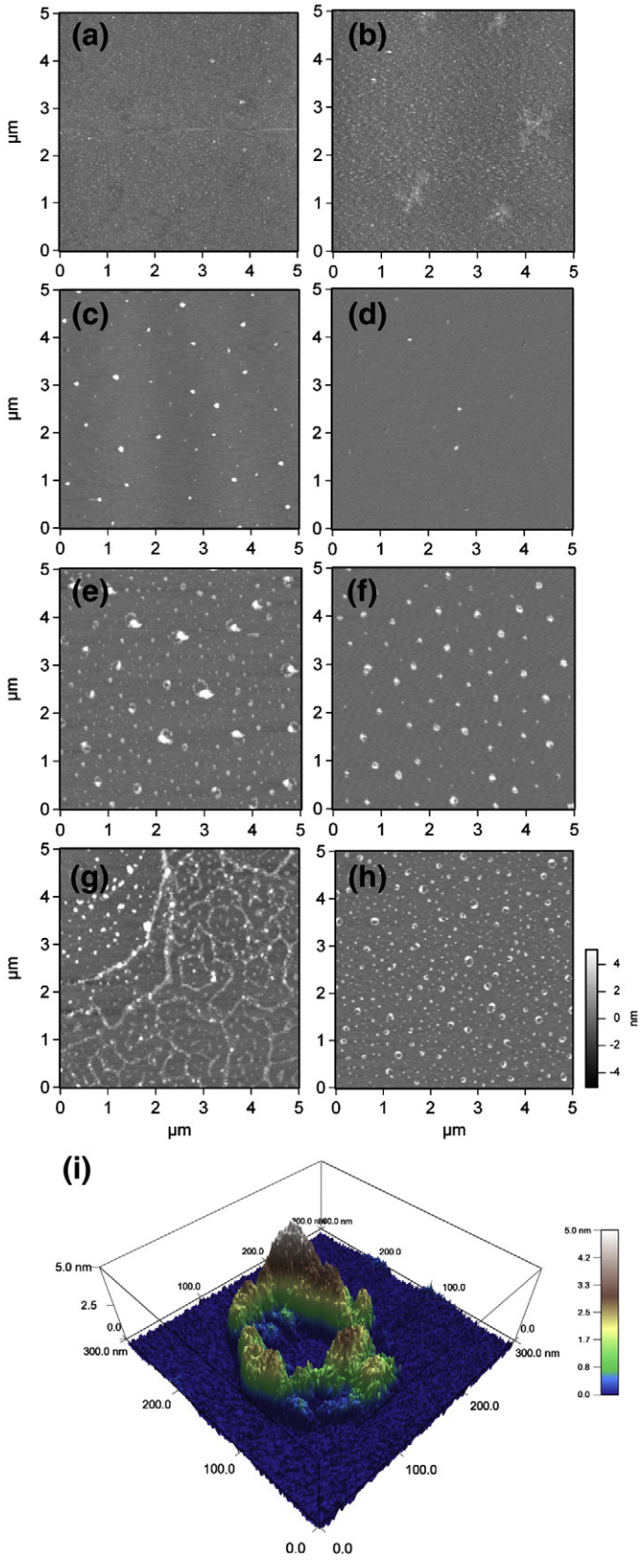

Fig. 1. AFM images acquired on tapping mode of HAMLET (left column) and BLA (right column) deposited with EYPC:PBPS monolayers on mica chips. HAMLET (a) and BLA (b) prepared at 3 nM subphase concentration, pH 5.0. (c–h) EYPC:PBPS monolayers and either HAMLET (c, e and g) or BLA (d, f and h) prepared at pH 7.4, subphase protein concentrations at 7 ng/mL, 43 ng/mL and 142 ng/mL (0.5 nM, 3 nM and 10 nM), respectively. (i) 3D representation of an AO formed by HAMLET in an EYPC:PBPS monolayer.

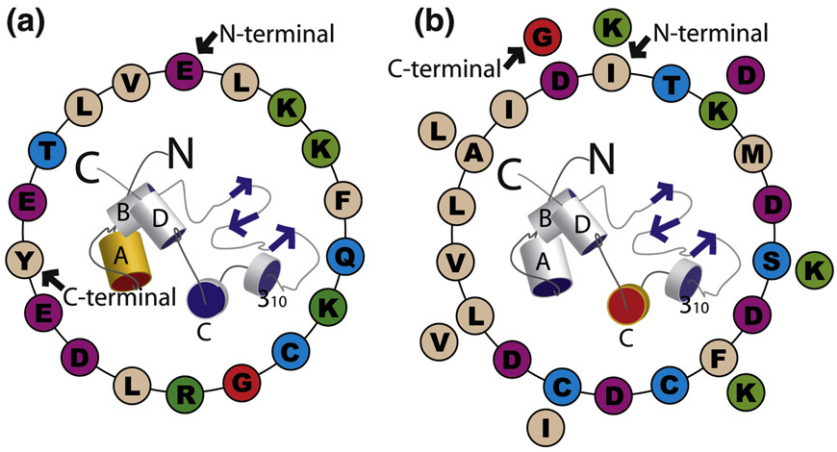

Fig. 2. Helical wheel projections of (a) Peptide A (residues 1–18 from BLA) and (b) Peptide C (residues 75–100 from BLA). Amino acids are denoted using their standard one-letter abbreviations. Positively charged residues are colored green; negatively charged residues, magenta; polar residues, blue; glycines, red; and hydrophobic residues, light ochre. The schematic representations of BLA inside the wheels highlight (in yellow) the position of the corresponding A (a) and C (b) peptides in the 3D structure of BLA. Helices are labeled A, B, C and D, as well as a short 3_{10}-helix, reading from the N-terminus to the C-terminus of the protein.

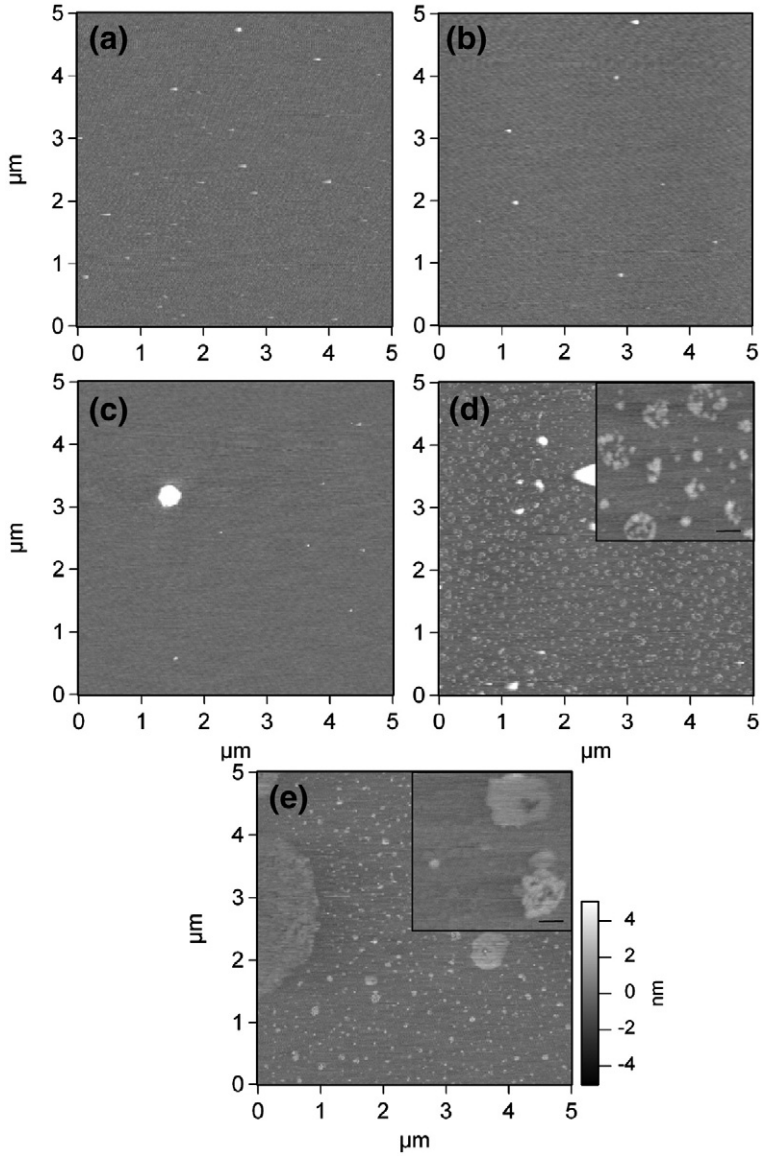

Fig. 3. AFM images acquired on tapping mode of Peptide A (left column) and Peptide C (right column) and Peptides A and C combined (center bottom) deposited with EYPC:PBPS monolayers on mica chips. Peptide A (a) and Peptide C (b) at pH 7.4, 22 ng/mL and 29 ng/mL subphase concentrations, respectively (10 nM). Peptide A (c) and Peptide C (d) at pH 7.4, 440 ng/mL and 589 ng/mL subphase concentrations, respectively (200 nM). Inset: scale bar represents 100 nm. (e) Peptide A and Peptide C at 100 nM subphase concentration (for each peptide, total peptide mass is 515 ng/mL), pH 7.4. Inset: scale bar represents 100 nm.

peptides behave synergistically with respect to AOs' formation and monolayer perturbation. As can be seen (Fig. 3e), the peptides added together in equimolar amounts formed a significant amount of distortions but no clear AOs (515 ng/mL, 200 nM total peptide concentration).

Destabilization of the membrane by HAMLET, BLA, Peptide A and Peptide C

Taken together, the results above show that HAMLET, BLA and Peptide C form pore-like structures that, in other systems, have been associated with cytotoxic activity. Formation of these structures requires binding, accumulation and aggregation at the membrane. HAMLET shows the highest ability to generate these structures at very low concentrations, but in fact, BLA was also effective. Previous investigations from us and others have shown that, at neutral pH, BLA has low affinity for membranes, both negatively charged and zwitterionic.[41,42] There are, however, some reports that BLA and the human variety bind weakly to cell membranes[30] and to some model membranes[43] at neutral pH. We therefore comparatively reinvestigate the membrane binding of the proteins and asses the binding of the peptides. We have recently shown a good correlation between the concentration dependencies for the adsorption to bilayers—as measured by surface plasmon resonance—and induction of leakage content to liposomes, for both BLA and HAMLET.[30] Leakage is caused by the perturbation of the hydrophobic core of the bilayer by the protein.[38,42,44] The assay of membrane disruption by the 8-aminonaphtalene-1,3,6-trisulfonic acid (ANTS)/*p*-xyelene-bis-*N*-pyrimidium bromide (DPX) leakage fluorescence assay thus represents a simple way to estimate binding coupled to membrane perturbation. We investigated the efficiency of HAMLET, BLA, Peptide A and Peptide C in inducing leakage of liposome content by using LUVs prepared from EYPC:PBPS with encapsulated ANTS/DPX and monitored the ANTS fluorescence in response to increasing amounts of proteins and peptides.[30,45] BLA and HAMLET destabilized the bilayers under acidic conditions (~ pH 5) at similar concentrations (Fig. 4a). Moreover, Peptide C accounted for ~50% leakage relative to the full-length, native protein, while Peptide A caused negligible leakage (Fig. 4a).

At neutral pH (Fig. 4b), both BLA and peptides caused some leakage, although effective concentrations much higher than those for HAMLET were needed (Fig. 4b). Peptide C was again particularly effective, and under neutral conditions, it caused higher content release than the native protein, while Peptide A showed a low ability to disrupt the vesicles. Thus, we conclude that helix C (corresponding to Peptide C; see Fig. 2b) is important for the overall membrane-disruptive properties of BLA and most probably HAMLET (sequence identity for this peptide section between human and BLA is 84.6%). As in the case of the AFM experiments, attempts to evaluate a possible concerted action of the peptides in leakage promotion by simultaneous addition of both peptides did not provide conclusive evidence for synergy (Fig. 4). Although the binding and membrane disruption of HAMLET cannot be explained in terms of the properties of the two peptides—since they cannot account for HAMLET ability to cause the extent of vesicle disruption at neutral pH and low concentrations—it was interesting that Peptide C can both cause leakage at elevated concentrations and form AOs. We thus proceeded to explore the conformational propensities of this peptide in membrane-mimicking systems compared to Peptide A.

Membrane binding and destabilization associated with high propensity for helix formation

We compared the propensity of Peptides A and C to adopt secondary structure as they interact with liposomes at pH 5.0, where interaction with the membrane is effective (Fig. 4a). As seen by circular dichroism (CD) and secondary structure estimation by neural network analysis,[46] both peptides formed helices upon binding (Fig. 5a and b), as is usual for peptides interacting with membrane bilayers.[47] Helix formation was more pronounced for Peptide C, which at the endpoint of the titration was more than 60% α-helical in the presence of LUVs made of EYPC:PBPS (Fig. S3). The helix-forming abilities of the peptides were further tested in trifluorethanol (TFE), and this tendency was also found to be lower for Peptide A compared to Peptide C. Peptide C fulfilled most of its potential to form a helical configuration in this medium (Fig. 5d), suggesting that its high helical propensity in response to binding charged lipids—both in absolute terms and compared to Peptide A and in TFE—may be linked to a penetration into (and disruption of) the membrane (Fig. 4).

Discussion

AO formation by HAMLET, BLA and Peptide C

HAMLET is the most prolific AO-forming polypeptide of those examined here and readily causes leakage at very low concentrations. For Peptide C, AOs appear at 200 nM (591 ng/mL) peptide concentration in the subphase. At neutral pH, there is in fact a good correlation between the ability to cause leakage, which in turn correlates well with

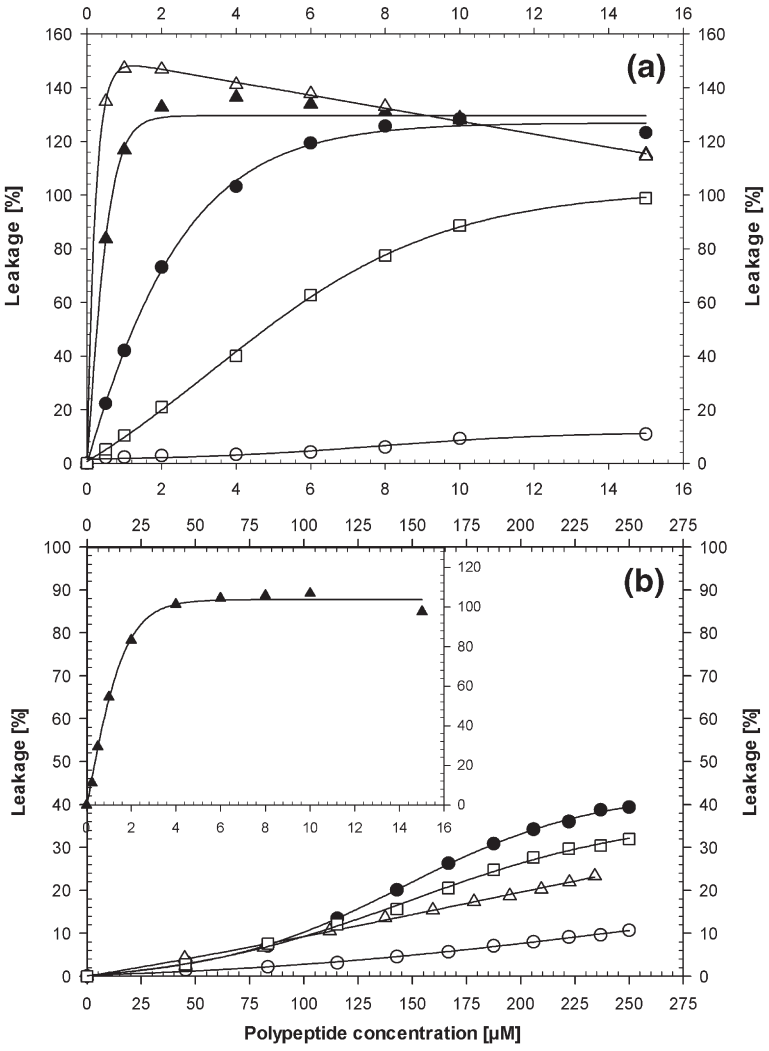

Fig. 4. Disruption of membrane integrity. Leakage of fluorophore (ANTS) and its quencher (DPX) from LUVs made of EYPC:PBPS induced by Peptide A (○), Peptide C (●), Peptide A and C together (□), BLA (△) and HAMLET (▲). Briefly, the dose-dependent increase of fluorescence at 510 nm (excitation at 355 nm) is benchmarked relative to unperturbed LUVs (set to 0% leakage) and LUVs disrupted by addition of Triton X-100 (set to 100% leakage). For details, see Materials and Methods. (a) Leakage induced by Peptide A and Peptide C at pH 5.0 compared to HAMLET and BLA. (b) Leakage induced by Peptide A and Peptide C at pH 7.0 compared to HAMLET and BLA. Inset: leakage induced by HAMLET at 0–16 μM concentration, from Mossberg *et al.*[30]

the membrane binding affinity of BLA and HAMLET,[30,41] and the appearance of AOs as seen by AFM. Similar AOs are observable for both BLA and HAMLET at neutral pH and low concentrations (3–10 nM, 43–142 ng/mL protein concentration in the subphase). Thus, the very high HAMLET ability to interact with membranes and form pores under physiological conditions could be interpreted as a propensity already present in the bovine and human α-La polypeptide chains that is enhanced by the oleic acid component in the complex. An important step in the membrane binding of BLA is the loosening of the protein tertiary structure,[41] which is facilitated by the proton gradient near the membrane and marginal folding barrier of BLA.[38,39,48] This step would not be required for HAMLET, which is already loosely folded.[21] Other proteins that also form AOs upon membrane association are intermediately folded as well.[32,49] HAMLET was first reported to be multimeric,[13] and BLA also appears to form multimers under conditions known to promote the population of intermediate folds.[50,51] Thus, conditions that drive membrane binding, with a significant associated disturbance of the monolayer as seen by leakage experiments, also appear to be strong drivers for the formation of AOs and *vice versa.* Moreover, oligomerization reaction rates are concentration dependent,[52] and as a protein migrates from bulk [three dimensional (3D)] to the membrane (approximately two-dimensional array), there is a great effective increase in the protein concentration.[53] Conceivably, if a protein—or its multimeric form—diffuses to a membrane domain border in the lipid membrane, the membrane will experience a further up-concentration of protein at a location where there is a mismatch in structure and physical state between the two domains. We propose that the initial distortions and pore-like AO structures observed by AFM may represent such a localized up-concentration and spatial restriction of oligomeric BLA/HAMLET (and BAMLET). HAMLET could,

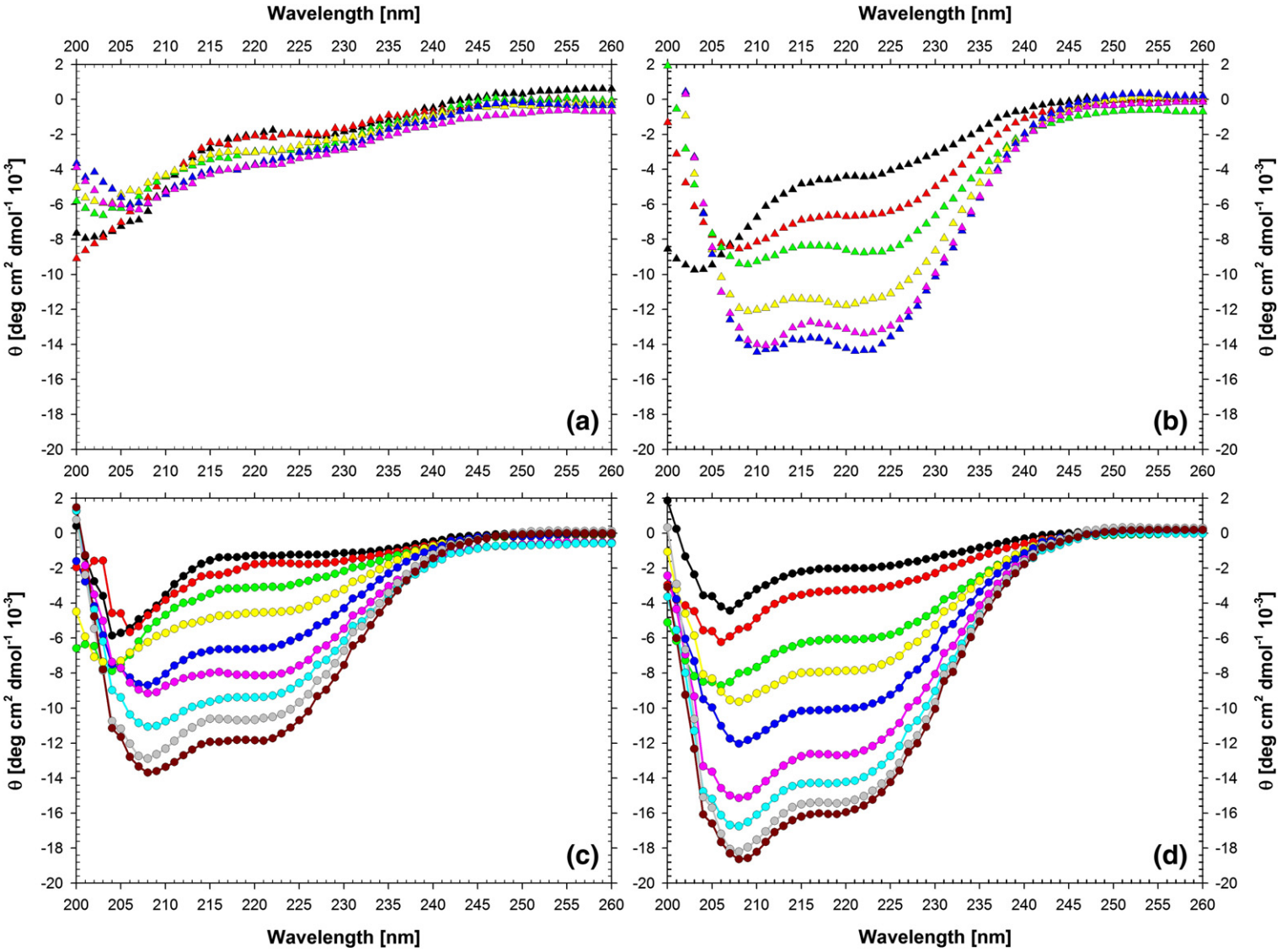

Fig. 5. Structural changes in Peptides A and C upon membrane binding monitored by CD. CD spectra of Peptide A (a) and Peptide C (b) in the presence of increasing amounts of LUVs made of EYPC:PBPS at pH 5.0. Phospholipid concentration was 0 mM (●), 0.17 mM (●), 0.33 mM (●), 0.50 mM (●), 0.67 mM (●) and 0.83 mM. CD spectra of Peptide A (c) and Peptide C (d) in increasing amounts of TFE at pH 5.0. TFE concentration was 0% (●), 4.8% (●), 9.1% (●), 13.0% (●), 16.7% (●), 20.0% (●), 23.1% (●), 25.9% (●) and 28.6% (●).

hypothetically, assemble preferentially at domain borders and in this way facilitate pore formation. Of possible relevance to this is the finding that both HAMLET and BLA show a hitherto unexplained enhanced activity when ~1:1 mixes of negatively charged and zwitterionic lipids are used to prepare the vesicles for leakage assays.[42] Earlier studies have also indicated that bound BLA greatly influences the T_C and $T_{C½}$ (the gel-to-liquid crystalline phase transition temperature and transition half-width, respectively) of lipid mixes while the effect on lipid vesicles made of only one lipid is small.[38] Langmuir monolayer studies show that the miscibility of EYPC:PBPS 1:1 mixes is low but can be altered by adsorbing species.[54]

It is tempting to directly link disruptions in planar monolayers and leakage in bilayer vesicle systems (with an estimated available surface area per LUV of 125,000 nm^2), but this cannot be performed in a straightforward manner; any such interpretation should be made with caution. There exist empirically determined relationships between the ability of a protein to intercalate in a lipid monolayer and leakage experiments, see in particular work by Wijewickrama *et al.*[55] Briefly, leakage in the Wijewickrama study drops to zero if the protein mutants cannot affect monolayers precompressed to lateral pressures of about 30 mN/m. The monolayers in this study were not precompressed, but we observed extensive leakage, strongly indicating HAMLET's ability to intercalate into monolayers also at elevated lateral pressures. As used here and based on previous studies,[30,44] leakage can be applied to comparatively monitor the binding of the proteins/peptides to the membrane, a binding that is not necessarily accompanied by irreversible aggregation of the protein. Thus, as we have previously shown, the peripheral binding of BLA to membranes can be reverted by, for instance, pH changes, providing the soluble conformation of the protein,[38,39] and leakage might be a functional manifestation of the membrane disruptions observed at very low amounts of the proteins on the uncompressed monolayers (Fig. 1a and b). These disruptions might function as nucleation sites for further accumulation and aggregation of the protein in AOs, which will lead to complete and irreversible disruption of the vesicle system.

Although pores in general are often formed by helical motifs,[56] available data for other systems have indicated that AO structures are dominated by polypeptide in β-sheet conformation.[37] The dominantly helical secondary structure of Peptide C and BLA appears to be maintained upon membrane binding (Fig. 5 and Refs. 38 and 44) but might be further affected upon up-concentration and resulting aggregation. Discrete molecular dynamics have revealed the transition from helical to β-sheet conformation upon formation of annular protofibrils made of amyloid-β oligomers.[57] Further studies are thus necessary to elucidate the AOs described in this work.

AO formation and cytotoxic activity of HAMLET

While HAMLET influences the permeability of artificial membranes[30] and increases ion fluxes in tumor cells,[13,29,58] there is no evidence that AO formation occurs in intact cells exposed to HAMLET. The ability of HAMLET to form AOs in artificial lipid bilayers likely needs to be translated into a more complex molecular context in intact tumor cell membranes. In such membranes, HAMLET has recently been shown to activate specific membrane channels in conjunction with signaling pathways that determine death (C. Svanborg, unpublished results). Beyond the plasma membrane, HAMLET and HAMLET-like preparations colocalize to the membranes of both lysosomes and mitochondria in susceptible cells.[24] In lysosomes, HAMLET causes release of cathepsin, a marker for loss of lysosomal membrane integrity.[25] In mitochondria, HAMLET affects the integrity and morphology of the mitochondrial membrane.[24,59] Both lysosomal and mitochondrial membrane permeabilization are mutually reinforcing key events in cell death, either as initiating or as amplifying effects.[60] In many cases, both exogenous and endogenous killing agents such as Bax, Bak and viral proteins have been shown to form pores in both lysosomes and mitochondria.[60–62] Thus, the identification of HAMLET as an AO-forming protein complex could also be related to action on organelle membranes, and this may be a worthwhile line of investigation in exploring the full effect of this complex. Furthermore, the present study clearly suggests that the protein is critical for defining the properties of the HAMLET complex and that HAMLET does not act merely by offloading oleic acid molecules into the membrane, unlike ELOA (*e*quine *l*ysozyme multimeric complexes with *o*leic *a*cids), which is a preparation resembling HAMLET but with a large excess of fatty acid molecules.[63] The role of the oleic acids would be both to increase the membrane affinity of the HAMLET nucleation sites and to locally alter the membrane.

Materials and Methods

Protein, peptides and other materials

Peptide A [E(1)QLTKCEVFRELKDLKGY(18)] and Peptide C [I(75)SCDKFLDDDLTDDIMCVKKILDKVG(100)] were obtained from CPC Scientific Inc., San Jose, CA, and the numbers refer to positions in BLA. HAMLET complex was formed from human (and, for the experiments in Fig. S2, bovine) α-La and oleic acid, as described previously.[21] BLA (UniProt identifier: P00711 | 20-142), ANTS and DPX

were purchased from Sigma-Aldrich. PBPS and EYPC were obtained from Avanti Polar Lipids (Alabaster, AL, USA).

Deposition of mixed protein–lipid monolayers on mica surfaces

HAMLET, BLA and peptides were dissolved in a buffer containing 5 mM citrate/10 mM Na_2HPO_4, either pH 5.0 or 7.4. Depositions of lipid monolayers with and without polypeptide present in the subphase were carried out with a KSV Minitrough fitted for monolayer deposition (Helsinki, Finland) using the manufacturer's software at 37 °C. The Teflon trough (75 mm × 364 mm × 5 mm) was filled with protein- or peptide-containing buffers at the corresponding pH. The polypeptide concentration given for each AFM experiment corresponds to the amount of polypeptide added to the through subphase volume (136.5 mL). Freshly cleaved mica (Agar Scientific Ltd., England) was used as a supporting solid substrate for the film deposition (dipping method). The mica was immersed in the trough before spreading the phospholipid solution on the buffer phase. The surface was swept, and the possible impurities were removed from the air/water interface with a Pasteur pipette, prior to each experimental run. We carefully spread 20 μL of glycerophospholipid in chloroform (1 mg/mL) on the surface with a Hamilton syringe, and the chloroform was allowed to evaporate before starting the measurements. Compression of the lipid monolayers was performed at 5 mm/min while an electrobalance recorded the surface tension with a Wilhelmy plate. The film area was compressed to a surface pressure of 30 mN/m and left for approximately 20 min for stabilization. The mica sheets were detached and transferred to the AFM instrument for imaging.

AFM imaging

AFM imaging was carried out in air at room temperature using tapping mode (AC mode) on an MFP-3D-Bio™ atomic force microscope (Asylum research). Silicon cantilevers, ACL, from AppNano with a typical spring constant of 48 N/m were used. Images were captured with a size of 256 × 256 pixels, and the scan rate was adjusted for each sample to a value between 0.5 and 1 Hz.

Preparation of liposomes

The prerequisite amounts of chloroform-solved lipids were transferred using a Hamilton glass syringe to Kimble glass tubes wrapped in aluminum foil to reduce exposure to light in subsequent steps. Lipid films were produced under dry N_2 pressure, subjected to vacuum for at least 2 h and hydrated in buffer overnight. For liposome preparation, the solutions were subjected to seven freeze–thaw cycles using liquid N_2 and a warm water bath.[64] Finally, the hydrated multilamellar structures were extruded using a LIPEX extruder (Northern Lipids), assembled with two membrane filters (Whatman), pore size of 200 nm. The samples were forced through the filters 10 times using N_2 pressures of 12 bar, producing LUVs.[65,66] For the liposomes to be used in the ANTS/DPX leakage experiments, the buffer used for hydrating the lipid film contained 12.5 mM ANTS and 45 mM DPX. The unencapsulated fluorophores and quenchers were removed by gel filtration on a Sephadex G-75 exchange column.

Fluorescence-monitored leakage assays

Liposomes with ANTS/DPX encapsulation were diluted to 1 mM lipid concentration using citrate/Na_2HPO_4 at either pH 5.0 or 7.0. We added 0.5 mL volumes to a Hellma GmbH & Co. quartz cuvette with a 5-mm path length, and we measured fluorescence with a Perkin-Elmer LS5 luminescence spectrometer. Volumes of stock solutions of BLA, HAMLET or peptides were added stepwise directly to the cuvette. To be able to compare dilution and temporal effects such as spontaneous leakage of liposome contents, we used volumes of water equivalent to the volumes of the protein stocks. ANTS excitation was performed at 355 nm, and emission was measured in the range of 450–550 nm. The resulting spectra are strongly dominated by the ANTS fluorescence, with a λ_{max} at 510 nm. An increased intensity at this wavelength indicates loss of liposome integrity as the average distance between ANTS and DPX increases. After each titration, Triton X-100 was added to a final cuvette concentration of 2 mM. The resulting fluorescence at 510 nm was arbitrarily set to 100%, that is, complete liposome breakdown. Similarly, the fluorescence at 510 nm prior to addition of any stock solution or volume of water was arbitrarily set to 0%, that is, no leakage.

Circular dichroism

CD experiments were performed in the far-UV spectral range with a Jasco J-810 CD spectropolarimeter, typically using continuous scanning at 50 nm/min. Appropriate blanks were subtracted from each spectrum by using the software provided by the instrument manufacturers. We utilized 1.0-mm quartz cuvettes from Hellma. The experiments were carried out at 25 °C, and for each point, two scans were accumulated. The mean residue ellipticity (θ) was determined using the formula $\theta = \varepsilon/(10 \times C \times n \times l)$. Here, ε is the experimentally determined ellipticity in millidegrees, l is the path length of the cuvette (cm), C is the protein concentration (mol/L), 10 is a scaling factor, and n is the number of amino acids, which is 18 and 26 for Peptide A and Peptide C, respectively. This formula gives θ with units deg cm^2 $dmol^{-1}$.

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Supplementary Data

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