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Structural Characterization of More Potent Alternatives to HAMLET, a Tumoricidal Complex of α-Lactalbumin and Oleic Acid

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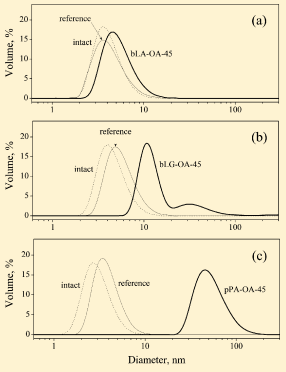
ABSTRACT: HAMLET is a complex of human α-lactalbumin (hLA) with oleic acid (OA) that kills various tumor cells and strains of *Streptococcus pneumoniae*. More potent protein−OA complexes were previously reported for bovine α-lactalbumin (bLA) and β-lactoglobulin (bLG), and pike parvalbumin (pPA), and here we explore their structural features. The concentration dependencies of the tryptophan fluorescence of hLA, bLA, and bLG complexes with OA reveal their disintegration at protein concentrations below the micromolar level. Chemical cross-linking experiments provide evidence that association with OA shifts the distribution of oligomeric forms of hLA, bLA, bLG, and pPA toward higher-order oligomers. This effect is confirmed for bLA and bLG using the dynamic light scattering method, while pPA is shown to associate with OA vesicles. Like hLA binding, OA binding increases the affinity of bLG for small unilamellar dipalmitoylphosphatidylcholine vesicles, while pPA efficiently binds to the vesicles irrespective of OA binding. The association of OA with bLG and pPA increases their α-helix and cross-β-sheet content and resistance to enzymatic proteolysis, which is indicative of OA-induced protein structuring. The lack of excess heat sorption during melting of bLG and pPA in complex with OA and the presence of a cooperative thermal transition at the level of their secondary structure suggest that the OA-bound forms of bLG and pPA lack a fixed tertiary structure but exhibit a continuous thermal transition. Overall, despite marked differences, the HAMLET-like complexes that were studied exhibit a common feature: a tendency toward protein oligomerization. Because OA-induced oligomerization has been reported for other proteins, this phenomenon is inherent to many proteins.



HAMLET (human α-lactalbumin made lethal to tumor cells) is a complex of a major milk protein, human α-lactalbumin (hLA), with oleic acid (OA, C18:1:9-*cis*) prepared from the Ca^{2+}-free protein using anion exchange chromatography on a DEAE-Trisacryl column preconditioned with OA.[1,4] Unlike intact α-lactalbumin (α-LA), HAMLET kills various tumor and immature cells, not affecting healthy, differentiated cells,[1,5] and possesses bactericidal activity against strains of *Streptococcus pneumoniae*.[6−8] The variant of HAMLET, formed from bovine α-LA (bLA), BAMLET, has similar cytotoxic properties[9] but lacks specificity toward cancer cells.[10] The therapeutic potential of HAMLET was shown in the treatment of bladder cancer[11,12] and skin papillomas[13] and in a human glioblastoma xenograft model.[14] Besides, HAMLET reduces the rate of tumor progression and mortality in mice with mutations relevant to hereditary and sporadic human colorectal tumors.[15] BAMLET was reported to inhibit tumor growth in an orthotopic rat bladder tumor model.[16] HAMLET was shown to be capable of triggering several cell death mechanisms via a set of cellular events: disintegration of the plasma membrane,[7,17,18] cellular internalization, and attack of multiple intracellular targets (for a review, see ref 19). Meanwhile, a similar spectrum of biological effects was reported for unsaturated fatty acids, including OA (reviewed in refs 20 and 21).

While OA is shown to be necessary for the formation of the cytotoxic complex with hLA,[22] the protein component is considered to be mostly an auxiliary factor, serving as a vehicle for delivery of OA.[3,20,21,23−25] The HAMLET-like cytotoxic complexes with OA can be formed from hLA and bLA following various experimental protocols (reviewed in ref 19). Similar complexes have been prepared using α-LAs from other species,[4,25,26] fragments of α-LA[23,27,28] and its derivatives,[9,27,29,30] homologues of α-LA, equine and canine lysozymes,[25,31] and some proteins structurally and functionally distinct from α-LA: bovine β-lactoglobulin (bLG),[3,32,33] pike α-parvalbumin (pPA),[3] apomyoglobin and β_2-microglobulin,[25] and others.[33] The invariance of the cytotoxicity of the resulting complexes with regard to the proteinaceous component indicates a crucial role of OA in the cytotoxic action of these complexes. An analysis of the concentration dependencies of cytotoxicity of various HAMLET-like complexes toward human epidermoid larynx carcinoma (HEp-2) cells has shown that the proteinaceous component of the complexes decreases the half-maximal effective concentration of OA by 20−49%.[3] The minor cytotoxicity of the proteinaceous component substantiates the view[3,20,21,23−25] that it mostly serves as a carrier of poorly water-soluble OA,[34,35] which is cytotoxic per se.[8,10,20,36−38] Meanwhile, the protein component of bLA−OA complexes was

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shown to induce a 4−5-fold increase in the cytotoxicity of OA against human osteosarcoma cell line U2OS.[39] Taken together, these data evidence that OA is crucial for the cytotoxicity of its protein complexes, but their proteinaceous component may slightly enhance the cytotoxicity of OA, depending on the cell line used.

The recently reported HAMLET-like complexes with OA formed from bLG and pPA (bLG−OA-45 and pPA−OA-45, respectively) possess cytotoxicities against HEp-2 and *S. pneumoniae* cells exceeding those for HAMLET.[3] The higher cytotoxic potency of these complexes was rationalized by an increased content of OA.[3] In contrast to the thoroughly characterized α-LA−OA complexes (reviewed in ref 19), the physicochemical properties of bLG−OA-45 and pPA−OA-45 complexes remain largely unexplored. Meanwhile, the initial characterization of the bLG−OA-45 complex has unexpectedly shown a stabilization of tertiary structure of bLG within this complex.[3] This behavior is opposite that of α-LA−OA complexes, because the tertiary structure of α-LA within them was shown to be destabilized.[2,24,26,38,40−42] The latter circumstance is believed to be important for the cytotoxic action of HAMLET.[43,44] A destabilization of the protein component has also been reported for the HAMLET-like complex of equine lysozyme.[31] The unusual structural properties of the bLG−OA-45 complex show that further physicochemical characterization of the recently reported HAMLET-like complexes is required to gain an insight into the structural basics of HAMLET and related protein−OA complexes.

This work is devoted to a detailed structural and physicochemical exploration of the more potent alternatives to HAMLET, bLG−OA-45, and pPA−OA-45 complexes, in comparison with HAMLET and related α-LA-OA complexes. In contrast to the classical fatty acid-binding proteins, which possess distinct site(s) specific to fatty acid(s), the proteins used for the preparation of these complexes mostly lack such sites.[28] These proteins are close to saturation with OA molecules, which seem to be kinetically trapped within a hydrophobic protein interior.[2,40] To the best of our knowledge, HAMLET-like complexes comprise the only well-described system of this kind. They are of special interest because of their well-established cytotoxic properties with regard to cancer cells and strains of *S. pneumoniae* and their ability to serve as carriers of OA (see above). It has been revealed that bLG−OA-45 and pPA−OA-45 complexes possess properties distinguishing them from other HAMLET-like complexes studied to date. Meanwhile, despite certain differences, a common feature of the HAMLET-like complexes studied to date is found: a tendency toward protein oligomerization. Because oligomeric protein species are currently considered as a major source of cytotoxicity in amyloid diseases (for a review, see ref 45), this observation may be related to a minor cytotoxicity of the proteinaceous component of HAMLET-like complexes. Furthermore, the OA-induced protein oligomerization reported for other proteins indicates that this phenomenon is inherent to many proteins, which is structurally, and likely functionally, relevant for a multitude of the proteinaceous systems interacting with OA and related fatty acids.

MATERIALS AND METHODS

Materials. Northern pike (*Esox lucius*) parvalbumin isoform α_1 was isolated from skeletal muscles as previously described.[46] Bovine β-lactoglobulin (catalog no. L3908), α-lactalbumin (catalog no. L6010), and oleic acid (catalog no. 75090) were from Sigma-Aldrich Co. Human α-lactalbumin was isolated from milk as described in refs 47 and 48. Chemistry grade 96% (v/v) ethanol was purified as described in ref 3. The pPA−OA-45, bLG−OA-45, and bLA−OA-45 complexes were prepared as previously described.[2,3] Briefly, pPA/bLG/bLA solutions were titrated with OA under Ca^{2+}-free conditions at pH 12 and 45 °C. Unbound OA was separated by adjusting the pH to 2 and centrifuging the sample at 40 °C. The water phase, free from unbound OA, was collected, and its pH was adjusted to 5.5. The solution was dialyzed against distilled water and freeze-dried. The reference protein samples were prepared using the described procedure, but in the absence of OA. HAMLET was prepared using the original procedure.[4]

pPA and bLG concentrations were determined spectrophotometrically using the following molar extinction coefficients: ε_{259} = 1810 M^{-1} cm^{-1} for pPA[49] and ε_{280} = 17460 M^{-1} cm^{-1} for bLG (calculated according to ref 50). Concentrations of α-LA samples were evaluated using the following molar extinction coefficients: ε_{280} = 25607 M^{-1} cm^{-1} for hLA (derived from ref 51) and ε_{280} = 28960 M^{-1} cm^{-1} for bLA (calculated according to ref 50). The concentrations of water stock solutions of bis-ANS and ThT were evaluated using the following molar extinction coefficients: ε_{385} = 16790 M^{-1} cm^{-1} for bis-ANS[52] and ε_{412} = 36000 M^{-1} cm^{-1} for ThT.[53]

Ultragrade H_3BO_3 and glycine were obtained from Merck. Ultragrade EGTA was obtained from Fluka. Bis-ANS (purity of >97%), ThT, ultragrade HEPES and EDTA, a grade II glutaraldehyde solution, molecular biology grade DTT, and premium grade bovine α-chymotrypsin were obtained from Sigma-Aldrich Co. Analytical grade bovine trypsin and high-purity grade PMSF were obtained from Serva and AMRESCO, respectively. Ultragrade sodium chloride, biotechnology grade β-mercaptoethanol, and molecular mass markers for SDS−PAGE were purchased from Helicon (Moscow, Russia). DPPC powder (purity of >99%) was obtained from Avanti Polar Lipids, Inc. Analytical grade formic acid and HPLC grade 2-propanol were obtained from Panreac. Water used in this work had a resistivity of 18.2 MΩ cm.

Chemical Cross-Linking of Proteins. Cross-linking of proteins with glutaraldehyde was performed in 30 mM HEPES-KOH, 150 mM NaCl, and 1 mM EDTA (pH 8.3) in the presence or absence of 2 mM β-mercaptoethanol. Glutaraldehyde (0.02%) was added to a 1 mg/mL protein solution. After incubation for 1 h at 37 °C, the reaction was terminated by an addition of an SDS sample buffer. The samples were subjected to Tricine−SDS−PAGE under reducing conditions (5% concentrating and 12% resolving gels, 4 μg of protein per lane) and staining with Coomassie Brilliant Blue R-250. The gel was scanned using a Molecular Imager PharosFX Plus System (Bio-Rad Laboratories, Inc.), and the total protein content in each band was quantitated by Quantity One.

Limited Proteolysis. An aliquot of a bovine α-chymotrypsin stock solution (0.02 or 1 mg/mL) in 20 mM H_3BO_3, 150 mM NaCl, and 1 mM EGTA (pH 8.3) (buffer A) or bovine trypsin (0.1 or 1 mg/mL) in 5 mM formic acid was added to the protein solution (0.9−1 mg/mL) in buffer A until an enzyme:substrate weight ratio of 1:200 or 1:500 was reached. The proteolysis proceeded overnight at 37 °C and was stopped by the addition of 1 mM PMSF in 2-propanol. The proteolytic mixture was analyzed via Tricine−SDS−PAGE under reducing conditions (15% resolving gel, 5 μg of protein per lane). The gel was stained with Coomassie Brilliant Blue R-

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250 and scanned using the Molecular Imager PharosFX Plus System.

Interaction of Protein with DPPC Vesicles. Interaction of protein with small unilamellar vesicles (SUVs) of DPPC was studied as previously described,[17] with minor modifications. The mean hydrodynamic diameter of SUVs of DPPC was 25 ± 10 nm, as judged by DLS measurements. The mixture of the protein sample with DPPC vesicles [lipid (1.7−2.3 mM) to protein molar ratio of ∼100] was preincubated for 15 h at 4 °C, followed by fractionation on a Sephadex G-200 column. The separation of vesicle-bound and vesicle-free protein fractions was monitored spectrophotometrically at 280 or 254 nm for bLG or pPA samples, respectively. The total amount of vesicle-free protein was estimated spectrophotometrically or by using the Bradford protein assay[54] for bLG or pPA samples, respectively.

Fluorescence Studies. Fluorescence emission spectra were recorded using a Cary Eclipse spectrofluorimeter (Varian Inc.), equipped with a Peltier-controlled cell holder, using quartz cells (10 mm × 10 mm for protein samples and a microcell for fluorescent dyes). Protein concentrations did not exceed 10 μM. The tryptophan fluorescence of proteins was excited at 280 nm. The emission band widths (Δ) were 5, 10, and 2.5−5 nm for bLG, bLA, and hLA samples, respectively. In the latter case, the position of the fluorescence spectrum maximum (λ_{max}) was determined to be independent of the Δ value. The initial 10−15 μM stock solution of the bLG−OA-45 complex or HAMLET sample was diluted with the same buffer to the desired concentration and kept at the temperature of the experiment for 15−20 min prior to measurements. Bis-ANS and ThT concentrations were 1 and 25 μM, respectively; protein concentrations in these experiments were 6−7 μM. The fluorescence of bis-ANS and ThT was excited at 385 and 450 nm, respectively; the Δ value was 5 nm. All spectra were corrected for the spectral sensitivity of the instrument and fitted to log-normal curves[55] using LogNormal (IBI RAS, Pushchino, Russia), implementing the nonlinear regression algorithm by Marquardt.[56] The maximal intensities (I_{max}) of fluorescence emission spectra and λ_{max} values were obtained from these fits.

Dynamic Light Scattering Studies. Dynamic light scattering (DLS) measurements were taken using a Zetasizer Nano ZS system (Malvern Instruments Ltd.). The back-scattered light from a 4 mW He−Ne laser (632.8 nm) was collected at an angle of 173°. The protein concentration was 1 mg/mL. The buffer conditions were pH 8.3, 10 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA. To avoid a strong scattering signal, which may arise from particulates and large OA vesicles at pH 8.3,[35] the solutions of protein−OA complexes and control protein samples were preliminarily passed through 0.1 and 0.02 μm Whatman Anotop 10 syringe filters, respectively. The sample temperature was 25.0 °C. The acquisition time for a single autocorrelation function was 70 s. The resulting autocorrelation functions are averaged values from five measurements. The volume-weighted size distributions were calculated using the following parameters: a refractive index of 1.334 [measured with an RL3 refractometer (Polskie Zaklady Optyczne)] and a viscosity value for an aqueous solution of 150 mM NaCl at 25 °C of 0.9021 cP (derived from ref 57).

Scanning Calorimetry Measurements. The scanning microcalorimetry studies were conducted on a Nano DSC microcalorimeter (TA Intruments Inc.) at a heating rate of 1 K/min and excess pressure of 4 bar (pH 9.3, 20 mM glycine-KOH, 150 mM NaCl, and 1 mM EGTA). The protein concentrations were 0.7−1.0 mg/mL. The protein specific heat capacity (C_p) was calculated as described by Privalov and Potekhin.[58] The partial molar volume and specific heat capacity of fully unfolded protein and oleic acid were estimated according to the methods of Häckel et al.[59] and Makhatadze and Privalov.[60] The specific heat capacity of the protein in complex with OA was estimated by taking the known number of OA molecules bound per protein molecule into consideration.

Circular Dichroism Studies. Far-UV CD measurements and data treatment were conducted mostly as previously described[38] using a J-810 spectropolarimeter (JASCO, Inc.) and CDPro software package with SDP48 and SMP56 reference protein sets[61] (pH 8.2, 20 mM H_3BO_3-KOH, 150 mM NaCl, and 1 mM EGTA). The protein concentration was either 0.06−0.10 or 1.0 mg/mL; quartz cells with a path length of 1 or 0.1 mm, respectively, were used. Temperature scans were performed in a stepwise manner, allowing the sample to equilibrate at each temperature. The average heating rate was 0.25−0.5 °C/min, with a bandwidth of 2 nm and an averaging time of 2−8 s.

RESULTS AND DISCUSSION

Dissolution-Induced Disintegration of Protein−OA Complexes. Because the HAMLET-like complex of equine lysozyme with OA (ELOA) was previously shown to dissociate at protein concentrations below 10 μM,[62] it is mportant to establish the protein concentration ranges that ensure the stability of the protein−OA complexes under study. To this end, the structural changes accompanying the dilution of Ca^{2+}-free HAMLET and bLG−OA-45 solutions were monitored by their tryptophan fluorescence (Figure 1) (that of the pPA−OA-45 complex was not measured, because pPA lacks Trp residues). To ensure that both OA-free and OA-bound protein forms are not thermally denatured, the measurements were performed at 8 and 20 °C for HAMLET and the bLG−OA-45 complex, respectively (see refs 3 and 40). The fluorescence spectrum maximum (λ_{max}) was monitored, because it has been

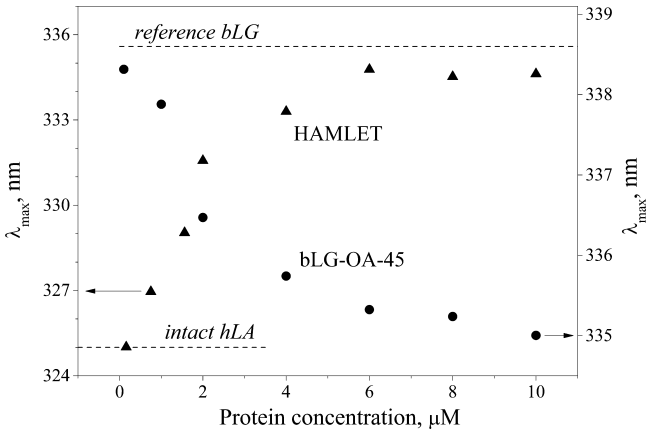

Figure 1. Concentration dependencies of the position of the fluorescence spectrum maximum (λ_{max}) for Ca^{2+}-free HAMLET (▲) and the bLG−OA-45 complex (●) at 8 and 20 °C, respectively (pH 8.3, 20 mM H_3BO_3-KOH, 150 mM NaCl, and 1 mM EGTA). Dashed lines correspond to the λ_{max} values for the intact hLA and reference bLG sample under the same conditions. The tryptophan fluorescence was excited at 280 nm.

Table 1. Distributions of Oligomeric Protein Forms at 37 °C for Various HAMLET-like Complexes and Control Samples, As Judged from Chemical Cross-Linking Experiments (0.02% glutaric aldehyde for 1 h)[a]

protein	protein form	monomer band (%)	dimer band (%)	trimer band (%)	tetramer band (%)	pentamer band (%)	higher-molecular weight fraction (%)
hLA	intact	21	51	22	7	–	–
	HAMLET	–	47	42	10	–	–
bLA	reference	29	36	21	15	–	–
	bLA–OA-45	–	24	47	30	–	–
pPA	reference	29	43	24	5	–	–
	pPA–OA-45	–	–	2	30	36	32
bLG	reference	37	43	20	–	–	–
	bLG–OA-45	–	13	34	33	–	21

[a]The protein concentration was 1 mg/mL. Buffer conditions included 30 mM HEPES-KOH, 150 mM NaCl, 1 mM EDTA, and pH 8.3. The products of cross-linking were quantitated by SDS–PAGE with Coomassie Brilliant Blue R-250 staining.

previously shown to be sensitive to binding of OA to both hLA[38] and bLG.[3]

The dilution of the HAMLET solution from 6 to 0.15 μM causes a notable gradual blue shift of the fluorescence spectrum maximum from 335 to 325 nm (Figure 1), the λ_{max} value characteristic of the apo form of intact hLA,[38] which does not reveal any spectral changes in the same protein concentration range (data not shown). The blue shift of the fluorescence spectrum evidences a decrease in the mobility and/or polarity of the local environment of emitting Trp residues, which typically reflects a decrease in their accessibility to water molecules.[63] Because the dissolution of HAMLET results in a protein state spectrally equivalent to the OA-free protein, it can be concluded that OA dissociates from HAMLET at lowered protein concentrations, as was found for the ELOA complex.[62] An alternative explanation of the observed effect is a dissolution-induced change in the oligomeric state of the protein (see below) or OA. In any case, a disintegration of HAMLET is observed at protein concentrations below 6 μM (midtransition concentration of 2 μM).

As seen for HAMLET, the decrease in the bLG–OA-45 complex concentration from 6 to 0.1 μM induces a marked but opposite shift in its fluorescence emission maximum (Figure 1) from 335.3 to 338.3 nm, the value close to the λ_{max} value of reference bLG (338.6 nm), which does not exhibit the spectral changes in this concentration range (data not shown). The observed red shift of the Trp fluorescence spectrum seems to reflect an increase in the water accessibility of Trp residue(s). Overall, the spectral properties of the bLG–OA-45 complex upon its dissolution closely approach those of the OA-free protein, which suggests disintegration of the complex at bLG concentrations below 6 μM (midtransition concentration of 2 μM).

Analogous fluorescent changes are observed for the bLA–OA-45 complex (data not shown): dilution of the complex from 1 to 0.01 μM shifts its λ_{max} from 341 to 338 nm. Because the λ_{max} value of reference bLA is 333 nm, the protein concentration of 0.01 μM is close to its midtransition concentration. This implies that bLA has OA-binding site(s) with an apparent association constant (K_{OA}) of $\sim 10^7$ M^{-1}, assuming that the observed transition reflects the dissociation of OA from the protein and considering that the bLA–OA-45 complex contains ~11 OA molecules per protein molecule.[2] A very similar affinity for OA with a K_{OA} value of $3.3–4.6 \times 10^6$ M^{-1} was previously reported for the apo form of intact bLA.[64] The analogous estimates for HAMLET and the bLG–OA-45 complex based on the known OA content of the samples[3,29] and midtransition protein concentrations (see above) evidence the presence of OA-binding site(s) with K_{OA} values of 10^5 and 3×10^4 M^{-1}, respectively. Notably, the previous estimate of the apparent OA binding constant for hLA at 17 °C under similar buffer conditions gave a similar value of 2×10^4 M^{-1}.[38] The same K_{OA} value of 10^4 M^{-1} has been reported for the apo form of intact α-LA.[40] Similarly, because the ELOA sample contains 35 OA molecules per protein molecule and its midtransition concentration for dissociation of OA is ~1.6 μM,[62] the estimate of K_{OA} gives a value of 2×10^4 M^{-1}. Overall, the comparison of literature data on the K_{OA} values with those derived from the data shown in Figure 1 confirms the validity of the method of K_{OA} estimation employed here.

In summary, HAMLET, bLG–OA-45, bLA–OA-45, and ELOA[62] complexes disintegrate at protein concentrations below 1–10 μM, which reflects the reversibility of the protein–OA interactions.

OA-Dependent Changes in the Oligomeric State of Various Proteins. Because the spectral changes accompanying dissolution of some protein–OA complexes (see Figure 1) may be at least partly due to changes in the oligomeric state of the proteins induced by dissociation of OA, we have examined this possibility using chemical cross-linking. The products of cross-linking several protein–OA complexes and respective OA-free control samples at 37 °C with 0.02% glutaric aldehyde were analyzed by SDS–PAGE (Table 1). The protein concentration used (1 mg/mL) was previously shown to be sufficient for efficient suppression of HEp-2 cell proliferation.[3] It should be emphasized that the numerical values derived from analysis of chemical cross-linking data are not absolute because of their dependence on the reaction time, concentrations of the reagents, etc. Meaningful information can be obtained only via comparative studies of the samples under identical reaction conditions. While intact hLA exists mostly as a dimer with smaller contributions of monomeric, trimeric, and tetrameric forms, HAMLET exhibits the absence of monomer, a lowered content of dimer, and increased fractions of trimeric and tetrameric species. Thus, OA binding shifts the distribution of oligomeric forms of hLA toward higher-order oligomers. A qualitatively identical effect is observed for bLA: OA association causes the disappearance of monomer and a lowered content of dimer, along with accumulation of trimeric and tetrameric protein forms. An accumulation of tetrameric and pentameric protein forms evidenced by chemical cross-linking has been also reported for OA-bound intact bLA.[24] The higher degree of protein oligomerization observed in this work is likely due to differences in the protocols of preparation of the protein–OA

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complexes and/or pH and calcium content conditions. OA-induced oligomerization of intact bLA has been also monitored by gel filtration chromatography[65] and matrix-assisted laser desorption ionization time-of-flight mass spectrometry.[66]

Even more prominent effects are observed for pPA and bLG: OA binding induces efficient population of the protein forms with a degree of oligomerization of ≥4 and the disappearance or decline in the content of monomeric and dimeric forms. It should be noted that none of the protein forms studied reveal the presence of high-molecular weight oligomers and/or aggregates, which would be unable to penetrate the 5% concentrating or 12% resolving gels. Hence, OA binding is accompanied by moderate oligomerization of the proteins studied. Furthermore, because chemical cross-linking experiments under reducing conditions (2 mM 2-mercaptoethanol) give analogous results (data not shown), the protein oligomerization cannot be ascribed to the formation of intermolecular disulfide bridges. It is of interest that a previous study of intact bLG has revealed OA-induced accumulation of disulfide-linked dimeric and trimeric forms of the protein.[32] This contradiction may be due to a difference in the methods of preparation of the bLG−OA complexes studied here and in the work by Lišková et al. (based on pH-induced and thermal denaturation, respectively).

Oligomerization of a protein should generally increase its hydrodynamic diameter, D_h. To examine this effect for the protein−OA complexes, their D_h values were estimated by dynamic light scattering at 25 °C (Figure 2). Reference bLA exhibits a size distribution very similar to that of intact bLA (Figure 2A). Meanwhile, the bLA−OA-45 complex demonstrates a prominent shift toward higher D_h values: the mean D_h value is 5.5 nm versus 4.3 nm for reference bLA. The OA-induced increase in the mean D_h value of bLA has been reported by Kehoe and Brodkorb.[42]

While the size distribution of reference bLG sample is just somewhat shifted toward higher D_h values with respect to the size distribution for intact bLG, the bLG−OA-45 complex sample exhibits a more complicated behavior (Figure 2B). In this case, two different components are observed. One of them has a higher mean D_h value (11.7 nm) compared to that of reference bLG (5.8 nm), which indicates that it corresponds to higher-molecular weight bLG oligomers. This interpretation is fully in line with the chemical cross-linking results (Table 1). The second component has a dramatically higher mean D_h value of 41 nm, a size resembling those characteristic of OA vesicles at pH 8.3.[35] Thus, this component likely corresponds to free or bLG-bound OA vesicles. The lack of this component for the bLA−OA-45 complex (Figure 2A) can be partly rationalized by a lower OA content in the sample (11 OA molecules per protein molecule vs 17 for the bLG−OA-45 complex[3]) and a higher apparent affinity of bLA for OA (see above). The previously shown ability of bLA to dissociate OA molecules[24] may exceed that of bLG, which could stabilize OA vesicles upon interaction with them.

Examination of the size distribution of the pPA−OA-45 complex did not reveal a component that resembled the intact and reference pPA samples by its D_h values (Figure 2C). Instead, a single peak was found, which has a mean D_h value of 55 nm, characteristic of OA vesicles. These facts indicate that pPA is mostly bound to the OA vesicles. This observation is in line with the previously shown ability of intact pPA to associate with DPPC vesicles.[67] The chemical cross-linking experiments

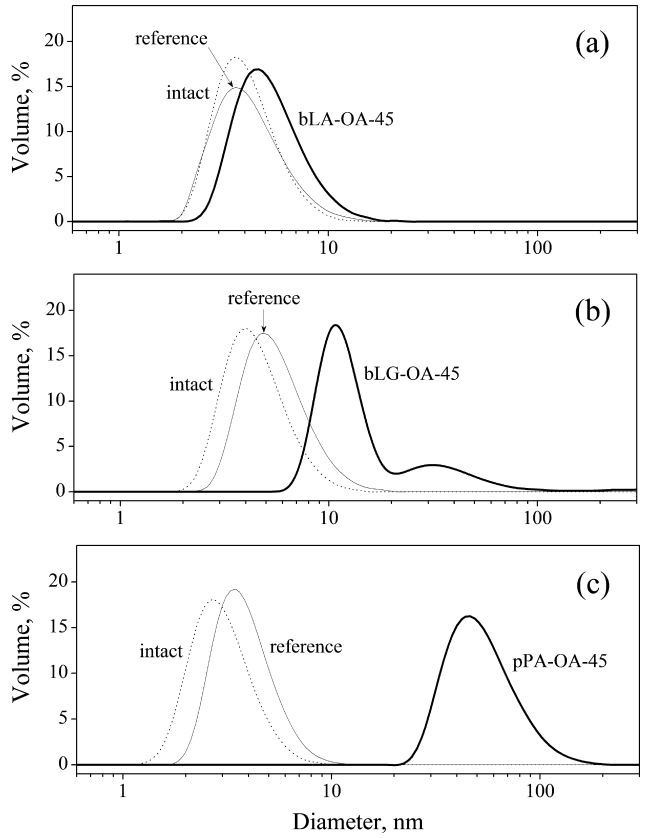

Figure 2. Volume-weighted size distributions for intact (dotted curves), reference (thin solid curves), and OA-bound (thick solid curves) forms of bLA (a), bLG (b), and pPA (c) at 25 °C, according to DLS data (pH 8.3, 10 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA). The protein concentration was 1 mg/mL.

(Table 1) evidence that pPA molecules remain in the proximity of each other, despite association with the OA vesicles.

Overall, the OA-induced protein oligomerization and concomitant increase in its hydrodynamic diameter are shown for bLA and bLG, while oligomerization of pPA is accompanied by its efficient association with OA vesicles. Equine lysozyme was previously shown to oligomerize upon OA binding.[31,68] Similarly, OA stimulates *in vitro* assembly of the filaments of the *β*-amyloid peptide and microtubule-associated protein tau.[69] Furthermore, OA associates with amyotrophic lateral sclerosis-linked superoxide dismutase 1 mutants, inducing the formation of SDS-stable oligomers.[70] Finally, the Ubxd8(Δ90−118) protein exhibits OA-induced oligomerization.[71] Thus, we can conclude that protein oligomerization is a common property of protein−OA complexes.

Association of pPA, bLG, and Their OA-Bound Forms with DPPC Vesicles. Because one of the initial cellular events triggered by HAMLET-like complexes is the disintegration of the plasma membrane,[7,17,18] it is important to compare the relative affinity of the complexes under study for lipid membranes. Though the intact forms of hLA, bLA, and pPA are known to associate with DPPC vesicles,[67] only pPA is shown to bind to OA vesicles (see above). To verify the relatively higher affinity of pPA and its complex with OA for lipid vesicles, we have studied the association of bLG, pPA, and their OA-complexed forms with small unilamellar vesicles (SUVs) of DPPC. An analogous study has been previously described for hLA and its complexes with OA.[17] The mixture of

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the protein sample with SUVs of DPPC was preincubated for 15 h at 4 °C, followed by fractionation on a Sephadex G-200 column. The calcium dependencies of the fractions of the vesicle-bound proteins (*R* values) are listed in Table 2. The *R* values for apo forms of the pPA samples exceed those for the bLG and hLA samples by at least 23% (Table 2) and 27%,[17] respectively, reaching as high as 80–92%. Hence, apo forms of intact pPA and the pPA–OA-45 complex demonstrate a higher affinity for DPPC vesicles, in accordance with the increased affinity of the pPA–OA-45 complex for OA vesicles. The high affinity of intact pPA for lipid vesicles may be physiologically important, because PA immunoreactivity was found in (or near) membranous systems, like the mitochondria and (or) the microtubuli.[72]

Table 2. Comparison of Affinities of Small Unilamellar DPPC Vesicles for bLG, pPA, and Their OA-Complexed Forms (pH 7.4, 50 mM HEPES-KOH, and 150 mM NaCl)[a]

		fraction of vesicle-bound protein (%)	
protein	protein form	1 mM EDTA	1 mM $CaCl_2$
bLG	intact	43	26
	reference	53	35
	bLG–OA-45	57	60
pPA	intact	80	23
	reference	91	33
	pPA–OA-45	92	34

[a]The mixture of the protein sample with SUVs of DPPC preincubated for 15 h at 4 °C was fractionated using a Sephadex G-200 column.

Similarly to intact hLA[17] and cod parvalbumin,[73] the addition of calcium to pPA and bLG samples is accompanied by a decrease in the *R* value (Table 2), except for that of the bLG–OA-45 complex, which exhibits a slight increase in the *R* value. In the presence of Ca^{2+}, the bLG–OA-45 complex demonstrates the highest affinity for DPPC SUVs among the studied proteins, with an *R* value of 60%. It should be noted that the calcium dependence of the *R* values observed for bLG samples may arise from its weak Ca^{2+} binding ($\sim 10^2$ M^{-1} [74]) and association of Ca^{2+} with the DPPC vesicles.[75]

The vesicle-bound fractions of reference bLG and pPA samples exceed the respective values for their intact forms by 9–11% regardless of calcium content (Table 2), which suggests notable structural differences between intact and reference forms of the proteins. The same conclusion has been drawn from intrinsic fluorescence studies of the bLG samples.[3]

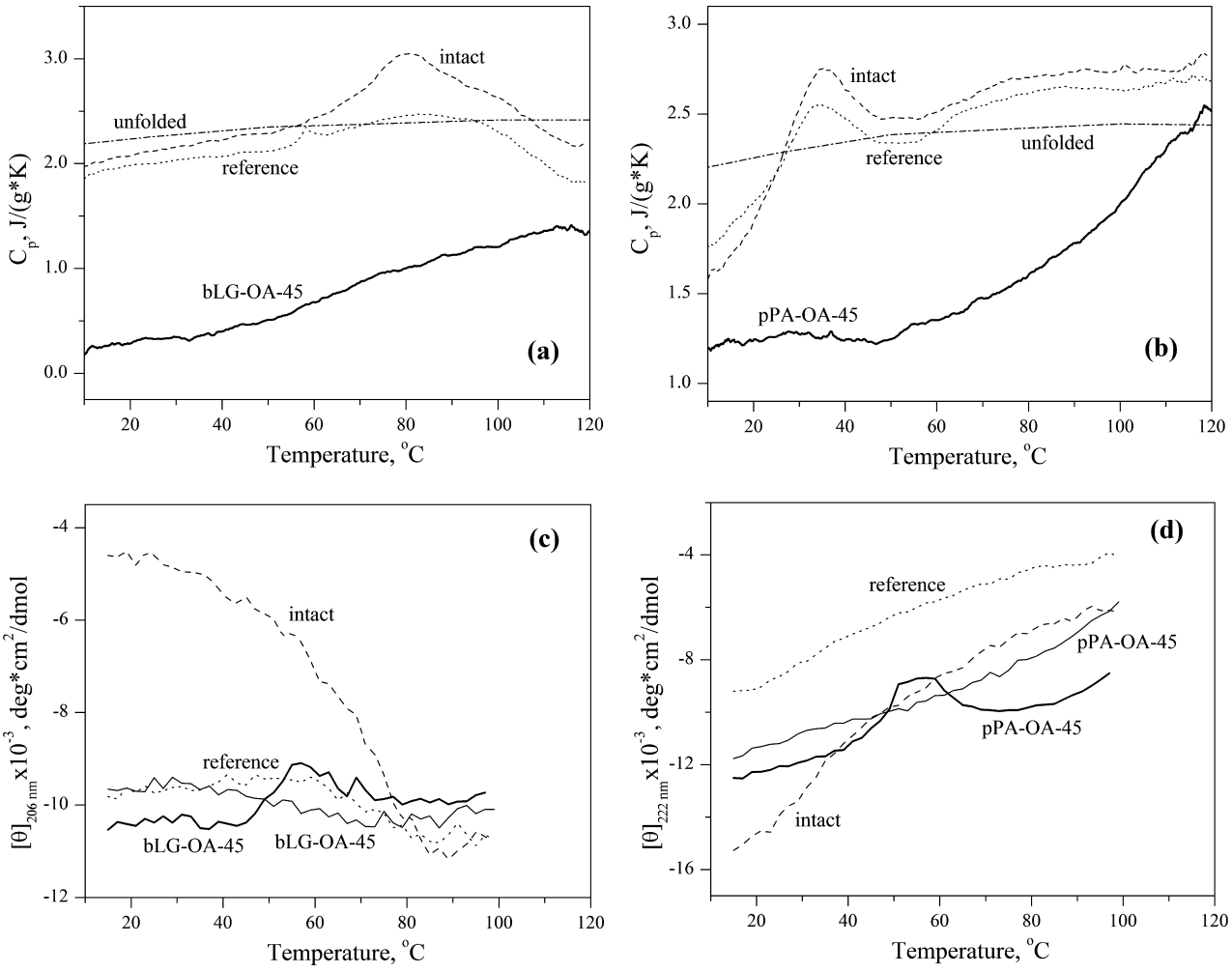

Figure 3. Thermal denaturation of intact (---), reference (···), and OA-bound (—) forms of bLG (a and c) and pPA (b and d) in the absence of calcium, monitored by DSC (a and b) and far-UV CD (c and d) methods (pH 8.2–9.3 and 1 mM EGTA). The protein concentration was 0.7–1.0 mg/mL in DSC experiments and 0.06–0.10 mg/mL (thin solid curve) or 1.0 mg/mL (thick solid curve) in CD experiments. The dashed–dotted curve corresponds to the specific heat capacity of the fully unfolded protein, estimated according to the method of Makhatadze and Privalov.[60]

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According to the DLS data (Figure 2b,c), the experimental procedures used for the preparation of reference bLG and pPA samples give rise to an increase in the hydrodynamic diameters of the proteins, which suggests partial unfolding of the reference protein forms. It is likely that the higher solvent accessibility of their hydrophobic residues promotes interaction with DPPC vesicles, as it has been previously shown for protein−membrane interactions (reviewed in ref 76). The same effect likely explains the Ca^{2+} depletion-induced increase in *R* values (Table 2 and ref 17).

The modification of the reference bLG sample by OA is accompanied by a 4% (1 mM EDTA) to 25% (1 mM $CaCl_2$) increase in the fraction of the SUV-bound protein (Table 2). This effect indicates that OA binding causes a destabilization of bLG's structure. Similarly, the binding of OA promotes association of equine lysozyme with giant unilamellar vesicles.[62] An analogous effect has been observed for hLA binding to DPPC SUVs,[17] unilamellar vesicles of lipids with varying acyl chain composition and net charge, and plasma membrane vesicles formed from intact tumor cells.[18] In contrast to that of hLA, EQL, and bLG, the binding of OA to reference pPA does not cause a noticeable change in the membrane-bound protein fraction, regardless of calcium content (Table 2). Thus, the association with OA differently affects a protein's affinity for lipid vesicles: a considerable increase is observed in the case of hLA, EQL, and bLG, but not for pPA.

Because it has been shown that the initial stages of the cytotoxic action of HAMLET-like complexes include interaction of protein with the cell membrane followed by its depolarization and rupture,[2,3,17,18,68] one may expect that the high membrane affinity observed for reference and OA-bound pPA forms (Table 2) could contribute to their cytotoxicity. Nevertheless, in contrast to the PA−OA-45 complex, reference pPA was not cytotoxic toward HEp-2 cells.[3] Thus, a high protein affinity for the lipid membrane does not ensure its cytotoxicity in this case.

Thermal Denaturation of the Protein−OA Complexes. The OA-induced changes in the affinity of bLG for lipid vesicles shown above may be related to its destabilization caused by OA binding. To explore this possibility, the thermal denaturation of bLG, pPA, and their complexes with OA was studied using differential scanning calorimetry (DSC) and circular dichroism (CD) techniques (Figure 3).

DSC measurements reveal two distinct heat sorption peaks with apparent midtransition temperatures of ∼80 and 95−100 °C for intact bLG and less defined heat capacity changes above 60 °C for the reference protein (Figure 3a). Similar but quantitatively different DSC data were previously reported for dimeric intact bLG at pH 6.8.[77] Meanwhile, the bLG−OA-45 complex lacks observable heat sorption peaks in the whole temperature range from 10 to 120 °C, which suggests that the OA-bound protein is either unfolded or hyperstabilized. The very low specific heat capacity of bLG within its complex with OA (Figure 3a) suggests a low level of entropy fluctuations in the protein.[78] The latter situation is expected for protein states with low accessibility of hydrophobic residues to water, which is achieved in compactly folded protein states (for a review, see ref 79), but in the case of the protein−OA complexes, it could be due to efficient isolation of hydrophobic residues from water because of their interaction with OA molecules. The latter possibility suggests a severe perturbation of the protein's tertiary structure, which is inconsistent with folded protein states. Both possibilities are in line with the blue-shifted fluorescence emission spectrum of the bLG−OA-45 complex (Figure 1 and ref 3). Meanwhile, only the latter possibility is consistent with the OA-induced increase in the protein's affinity for DPPC vesicles (Table 2).

The far-UV CD data for melting of the bLG−OA-45 complex at a protein concentration of 0.10 mg/mL unexpectedly reveal a cooperative thermal transition closely resembling that of the reference bLG sample (Figure 3c). Compared to the reference protein, bLG in complex with OA is ∼15 °C less thermostable (midtransition temperature of 50 °C). At the protein concentration (1.0 mg/mL) comparable to that used in the DSC experiments (Figure 3a), even more pronounced molar ellipticity changes with the same midtransition temperature are observed upon heating (Figure 3c). Because this thermal transition of the bLG−OA-45 complex is not accompanied by noticeable enthalpy changes (Figure 3a), it should be considered as a continuous (or second-order) transition, which is characterized by a gradual interconversion of protein substates without discrete states differing in enthalpy (discussed in ref 80). This unusual behavior of the OA-bound bLG is likely due to the nonspecific nature of the binding of OA to bLG, which allows the coexistence of numerous interchanging protein conformations that gradually shift toward more loose conformations upon heating, as seen from the convergence at elevated temperatures of the specific heat capacities (Figure 3a), molar ellipticities (Figure 3c), and Trp fluorescence spectrum maxima[3] of all bLG forms studied. Notably, the latter thermally induced fluorescent changes in the bLG−OA-45 complex were earlier erroneously interpreted as thermal stabilization of the OA-bound bLG.[3] The combination of data presented here suggests that bLG in the complex with OA lacks a rigid tertiary structure but has a conserved secondary structure, which experiences a continuous thermal unfolding.

Contrary to the genuine apo form of intact pPA, which lacks a rigid tertiary structure,[80] Na^+-bound intact and reference pPA forms exhibit a well-defined heat sorption peak with a midtransition temperature of 35 °C (Figure 3b). Meanwhile, the pPA−OA-45 complex shows no cooperative thermal transitions in the temperature range from 10 to 120 °C. Its heating is accompanied by a gradual growth of the specific heat capacity from the values characteristic of folded protein states up to the values inherent to the fully unfolded protein. Similarly to the bLG−OA-45 complex, far-UV CD reveals a thermal transition with a midtransition temperature of 60 °C in OA-bound pPA at a protein concentration of 0.06 mg/mL (Figure 3d). The elevated stability of the secondary structure of pPA in its complex with OA is also confirmed by guanidinium chloride unfolding experiments (data not shown). An increase in the protein concentration to 1.0 mg/mL changes its thermal behavior. In this case, two well-defined transitions are observed, with midtransition temperatures of 50 and 65 °C (Figure 3d). The difference in the thermal behavior of the protein at different concentrations of the complex reflects concentration-dependent structural rearrangements within the complex, in accord with the data described above (Figure 1). In summary, OA-bound pPA exhibits thermal behavior similar to that of the bLG−OA-45 complex, with the exception that the secondary structure of pPA in the complex with OA is stabilized. The latter effect could be due to interaction of the protein with OA vesicles (Figure 2c) or the formation of intermolecular contacts in the process of protein oligomerization (Table 1).

The unusual thermal behavior of the OA-bound forms of bLG and pPA is distinct from that reported for HAMLET, which was shown to possess a well-defined heat sorption peak shifted relative to that of the intact protein toward lower temperatures.[40] Such a principal difference between the behavior of hLA and that of bLG and pPA can be attributed to the much higher weight content of OA in the samples of bLG and pPA complexes (26 and 31%, respectively, vs 11% for HAMLET[3]), which results in a much more prominent structural disturbance. Nevertheless, such unusual behavior of bLG and pPA complexed with OA should be corroborated by alternative experimental approaches.

OA-Induced Changes in the Resistance of Various Proteins to Enzymatic Proteolysis. The decrease in protein stability is expected to promote an increase in the overall mobility of its polypeptide chain, which may lead to an increased susceptibility of the protein to enzymatic proteolysis. Therefore, limited proteolysis by bovine α-chymotrypsin (specific for large hydrophobic residues) and trypsin (specific for Lys and Arg residues) was applied for probing the stability changes accompanying the binding of OA to bLG and pPA. Because free fatty acids are known to inhibit various enzymes, including trypsin,[81,82] the reaction was performed in the presence of 150 mM NaCl, which is sufficient for suppression of the trypsin inhibition by fatty acids.[81]

As clearly seen from the results of fractionation of a proteolytic mixture of various pPA forms with α-chymotrypsin using SDS–PAGE (Figure 4b), intact and reference pPA forms are equally susceptible to proteolysis. Meanwhile, the pPA–OA-45 complex forms proteolytic fragments of higher molecular weights, which evidence its stronger resistance to enzymatic digestion. Trypsinolysis of various pPA forms gave analogous results (data not shown). The increased resistance of the pPA complexed with OA to enzymatic proteolysis despite destabilization of its tertiary structure (see above) may be due to protein oligomerization (Table 1), incorporation of pPA molecules into the OA vesicles (Figure 2c), the elevated stability of the protein secondary structure (Figure 3d), or direct protection of its hydrophobic residues via their interaction with OA molecules, as evidenced by the low specific heat capacity of the protein within the complex (Figure 3b).

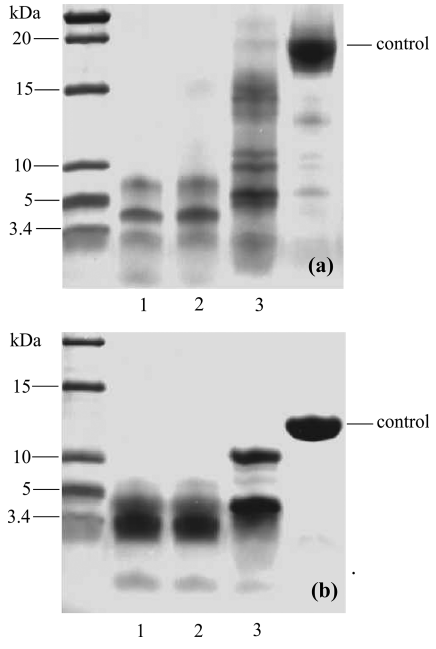

Figure 4. Limited proteolysis by bovine α-chymotrypsin for various forms of bLG (a) and pPA (b) in the absence of calcium, analyzed by SDS–PAGE (pH 8.3, 20 mM H_3BO_3, 150 mM NaCl, and 1 mM EGTA). The lines corresponding to intact, reference, and OA-bound forms of the proteins are labeled 1–3, respectively. The protein concentration was 0.8–1.0 mg/mL. The enzyme:substrate weight ratio was 1:200 for bLG and 1:500 for pPA samples. The intact protein not subjected to proteolysis was used as a control. The numbers at the left indicate the molecular masses of markers in kilodaltons.

The digestion of various forms of bLG and hLA with α-chymotrypsin was accompanied by an analogous OA binding-induced increase in the molecular weights of the proteolytic fragments (see Figure 4a for bLG). This effect was also observed for trypsinolysis of bLG samples (data not shown). In comparison with intact hLA, HAMLET was just slightly more resistant to trypsinolysis (data not shown). The OA-induced protection of bLG and hLA against enzymatic digestion despite destabilization of their tertiary structures (see above and ref 40) can be rationalized by protein oligomerization (Table 1) and by protection of their hydrophobic residues via interaction with OA molecules, in the case of bLG (see above). The inconsistency between the changes in thermal stability and resistance to proteases caused by OA binding was also observed for the Ubxd8(Δ90–118) protein.[71] Hence, fatty acid binding seems to impair the direct relationship between the stability of a protein and its resistance to proteases.

In contrast to that of hLA, bLG, and pPA, but like that of the Ubxd8(Δ90–118) protein,[71] binding of OA to EQL weakens its resistance to proteases.[62] Overall, we can conclude that the OA binding may differently affect the sensitivity of a protein to enzymatic proteolysis. Hence, limited enzymatic proteolysis does not ensure unambiguous information about the protein stability changes induced by OA binding, which raises a need in the use of alternative techniques.

Probing the Conformation of OA-Bound Protein Forms Using Hydrophobic Dye Bis-ANS. Because changes at the level of protein tertiary structure are able to affect its interaction with hydrophobic probes, conformational properties of the protein–OA complexes were studied using hydrophobic dye bis-ANS. A pronounced increase in the fluorescence quantum yield of bis-ANS, accompanied by a blue shift in its fluorescence emission spectrum, is observed upon noncovalent interaction of bis-ANS with hydrophobic surfaces of proteins (for a review, see ref 83). This feature of bis-ANS is routinely used for detection of partially folded protein states. Spectral parameters of bis-ANS fluorescence in the presence or absence of a 6–7-fold molar excess of the protein samples are listed in Table 3. The measurements were performed at temperatures ensuring folded states of HAMLET, bLA–OA-45, intact, and reference forms of the proteins (see Figure 3 and refs 2, 40, and 65).

If compared to intact protein forms, the reference forms of bLA, pPA, and bLG exhibit 2.0–5.4-fold increases in their maximal intensities of the fluorescence emission spectrum of bis-ANS (I_{max}) and 1–10 nm blue shifts in the spectrum. This observation corroborates the partial unfolding of the reference protein samples, shown by other methods (see above and refs 2 and 3).

Compared to intact hLA, HAMLET demonstrates a 3-fold higher I_{max} value, in accord with its lowered thermal stability.[40]

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Table 3. Maximal Positions (λ_{max}) and Maximal Intensities (I_{max}) of the Fluorescence Emission Spectrum or Emission Intensity at 490 nm (I_{490}) for Hydrophobic Dye Bis-ANS and Fibril-Specific Dye ThT for Various States of hLA, bLA, pPA, and bLG in the Absence of Calcium (pH 8.3, 30 mM HEPES-KOH, 150 mM NaCl, and 1 mM EDTA)[a]

			bis-ANS		ThT
protein or dye	protein form	t (°C)	λ_{max} (nm)	I_{max} (au)	I_{490} (au)
hLA	intact	10	490.5	71.7	50.0
	HAMLET		491.1	220	387
bLA	intact		490.9	52.0	50.5
	reference		486.4	281	344
	bLA–OA-45		478.1	333	241
pPA	intact		500.0	16.3	33.8
	reference		499.0	66.5	34.8
	pPA–OA-45		499.5	173	205
free dye	–		–	2.10	35.0
bLG	intact	20	500.4	95.1	54.2
	reference		490.6	186	72.7
	bLG–OA-45		496.7	92.9	375
free dye	–		–	1.90	28.1

[a]Protein concentrations were 6–7 μM. Bis-ANS and ThT concentrations were 1 and 25 μM, respectively. The fluorescence of bis-ANS and ThT was excited at 385 and 450 nm, respectively.

An analogous effect was previously reported for binding of ANS (monomeric analogue of bis-ANS) to HAMLET.[22,29] In contrast to that of ANS, binding of bis-ANS to hLA does not cause a considerable shift in its emission spectrum, which suggests an inability of larger bis-ANS molecules to incorporate into the protein interior. In comparison to that of hLA, the association of OA with bLA causes an 18% increase in I_{max} and an 8 nm blue shift of the bis-ANS emission spectrum. The latter effect seems to reflect an efficient embedding of bis-ANS molecule(s) into the protein interior. These spectral changes are in line with the lowered thermal stability of the bLA–OA-45 complex[2] and previous studies of binding of ANS to bLA.[39,66,84] Similar results were reported also for binding of ANS to ELOA.[31]

Similarly to hLA, association of pPA with OA causes a 2.6-fold increase in I_{max}, without a noticeable shift in the emission spectrum of bis-ANS (Table 3). This behavior evidences the appearance of extra hydrophobic surfaces on the protein, which is consistent with the view that binding of OA to pPA is accompanied by a loss of its fixed tertiary structure. It should be noted that fluorescence emission spectrum of bis-ANS in the presence of OA at a concentration used for the pPA–OA-45 complex is red-shifted to 538 nm. Because this effect was not observed in the presence of the pPA–OA-45 complex, in this case the bis-ANS fluorescence cannot be ascribed to an interaction of bis-ANS molecules with OA vesicles, which are present in the solution according to the DLS data (Figure 2c).

In contrast to that of the other studied proteins, the binding of OA to bLG induces a 2-fold decrease in I_{max} and a 6 nm red shift in the emission spectrum of bis-ANS. It should be mentioned that binding of ANS to thermally induced oligomers of bLG causes opposite spectral changes,[85] which confirms the specificity of the conformational rearrangements caused by OA binding. The observed spectral changes indicate a decreased water accessibility of hydrophobic residues of OA-bound bLG, which contradicts the conclusion of the lack of fixed tertiary structure in the bLG–OA-45 complex (see above). Alternatively, the binding of bis-ANS to bLG in complex with OA can be less efficient because of either OA-induced protein oligomerization (Table 1) or severe impairment of the protein's tertiary structure, which is known to prevent hydrophobic dyes from associating with proteins (reviewed in ref 83).

Overall, the use of the hydrophobic dye provides contradictory information about the protein stability changes induced by OA binding. Summarizing the experiments dedicated to this issue, one may conclude that the suggestion regarding the lack of rigid tertiary structure in bLG and pPA complexed with OA is supported by DSC and CD melting experiments (Figure 3), while the data from alternative approaches are more contradictory but can be rationalized after consideration of the available experimental material.

OA-Induced Changes in the Secondary Structure of bLG and pPA. The qualitative information about the rigidity of the tertiary structure of bLG and pPA complexed with OA was complemented by the data on their secondary structure obtained by the far-UV CD technique (Table 4). The measurements were performed in the absence of calcium at 15 and 50 °C, which correspond to the boundary points of the thermal transition of apo-pPA (Figure 3b).

Table 4. Secondary Structure Fractions Estimated from CD Data for Various Forms of bLG and pPA in the Absence of Calcium at 15 and 50 °C (pH 8.2, 20 mM H_3BO_3-KOH, 150 mM NaCl, and 1 mM EGTA)[a]

protein	protein form	t (°C)	α-helix (%)	β-sheet (%)	turn (%)	unordered structure (%)
bLG	intact	15	14.3	31.8	21.9	31.3
	reference		9.3	27.4	20.7	41.8
	bLG–OA-45		17.6	25.3	19.7	36.5
pPA	intact	15	57.6	5.9	16.9	19.6
	reference		38.7	12.5	18.1	30.3
	pPA–OA-45		53.9	5.3	15.2	26.0
pPA	intact	50	39.7	8.8	16.0	34.9
	reference		29.0	17.7	17.4	35.4
	pPA–OA-45		47.2	6.7	15.7	30.4

[a]The protein concentration was 5–6 μM.

An analysis of available NMR structures of intact bLG (PDB entries 1cj5 and 1dv9, corresponding to monomer) using the DSSP algorithm[86] gives 11–12 and 31–36% for the fractions of α-helices and β-sheets, respectively, which closely resembles the CD estimates (14 and 32%, respectively). Reference bLG exhibits an elevated content of unordered structure because of a decline in the content of α-helices and β-strands (Table 4), which confirms the structural perturbations imposed by the process of preparation of the complex. Meanwhile, OA binding is accompanied by a lowering of the level of unordered structure of bLG by 5% due to an 8% increase in α-helicity and a concomitant 2% decrease in β-sheet content. This effect is unexpected, because according to far-UV CD melting experiments OA-bound bLG exhibits a destabilized secondary structure (Figure 3c).

Like bLG, the folded state of the Ca^{2+}-free form of reference pPA (15 °C) demonstrates an increased level of unordered structure due to a decrease in α-helicity, partly compensated by an increase in β-sheet content (Table 4). On the other hand, the association with OA induces a 4% decline in the level of unordered structure in pPA, accompanied by a 15% increase in α-helicity and a 7% decrease in β-sheet content. Qualitatively

similar changes are observed for the Ca^{2+}-free form of pPA at 50 °C, which ensures its thermal denaturation. These results are in accordance with the increased thermal stability of the secondary structure of OA-bound pPA (see Figure 3d).

Evidently, the procedures used for the preparation of the protein−OA complexes distort their secondary structure. Nevertheless, despite differences in the prevailing type of secondary structure (β-sheets for bLG and α-helices for pPA), the interaction of the reference forms of the proteins with OA leads to protein structuring accompanied by an increase in α-helicity, partially compensated by a decrease in β-sheet content. The OA-induced increase in α-helicity was also observed by CD for bLA and hLA (for example, refs 2, 38, 39, 41, and 66). Meanwhile, Fourier transform infrared spectroscopy measurements did not reveal prominent OA-induced changes in the secondary structure of bLA.[42]

Because oligomerization of proteins may represent a first step toward the formation of fibril structures, possessing a specific cross-β-sheet structure (for a review, see ref 87), it is of interest to assess the presence of this structure in the oligomeric samples of our protein−OA complexes. Association of a fluorescent probe for cross-β-sheet structure, thioflavin T (ThT), with amyloid-like fibrils or aggregates causes a large increase in its fluorescence emission intensity (for a review, see ref 83). Wilhelm et al.[31] revealed the binding of ThT to oligomers of ELOA. Similarly to ELOA, an 8-fold increase in ThT fluorescence intensity with respect to the intact protein is observed for HAMLET (Table 3). The reference sample of bLA demonstrates a very similar increase in ThT fluorescence, which reflects the modification of its structure during the process of sample preparation. Unlike that of hLA, the association of OA with bLA is accompanied by a 43% decrease in ThT fluorescence. Because an inhibitory effect of OA on fibrillation of bLA at more acidic pH values has been observed,[88] it may be concluded that OA prevents the formation of amyloid-like structures of bLA. In comparison with intact and reference forms of pPA and bLG, their OA-bound forms exhibit 5−7-fold higher ThT fluorescence intensities (Table 3), which suggests an OA-induced accumulation of the cross-β-sheet structure. The ThT-positive oligomers of bLG were previously prepared via incubation of the protein at 67 °C.[85]

Overall, all protein−OA complexes studied, except for the bLA−OA-45 complex, demonstrate an increased fluorescence intensity of ThT, which suggests an elevated content of the cross-β-sheet structure in these complexes. Because the increased intensity of ThT fluorescence was reported for oligomeric forms of human α-synuclein formed in excess of docosahexaenoic acid (n-3-polyunsaturated fatty acid),[89] we can conclude that other unsaturated fatty acids are also able to promote the formation of oligomers with a cross-β-sheet structure. The latter effect is likely to affect the cytotoxic properties of protein complexes with unsaturated fatty acids.[45,87]

CONCLUSIONS

The presented detailed physicochemical study of the structural features of the complexes that are more potent than HAMLET protein−OA complexes reveals an unexpectedly complicated picture: their structural properties exhibit substantial differences, while some combinations of their properties are fairly unique and can be rationalized only by considering multiple equilibria within the protein−OA system.

HAMLET-like complexes of proteins with OA, with the exception of ELOA, are generally believed to be stable. The range of equine lysozyme concentrations ensuring the shift of the equilibrium between protein and OA toward formation of their complex has been reported previously.[62] Here we have shown that a structurally and functionally different protein, bLG, possesses a similar range of protein concentrations that ensure the stability of its complex with OA. We have shown that at protein concentrations below 1−6 μM the Trp-containing complexes studied (bLG−OA-45, HAMLET, and bLA−OA-45) disintegrate, which may be due to the dissociation of OA and oligomeric protein forms. The OA-induced protein oligomerization has been revealed for all protein−OA complexes studied using chemical cross-linking and DLS techniques. This phenomenon was observed earlier for other proteins, including equine lysozyme,[31,68] β-amyloid peptide, protein tau,[69] superoxide dismutase 1 mutants,[70] and Ubxd8(Δ90−118) protein.[71] Hence, the formation of protein oligomers in response to association with OA is a widespread phenomenon, inherent to protein−-OA interactions.

While the OA-bound forms of bLA and bLG mostly demonstrate only an increased hydrodynamic diameter, the pPA complexed with OA is shown to be bound to OA vesicles. The increased affinity of pPA for lipid vesicles has been confirmed using chromatographic separation of the protein fraction bound to SUVs of DPPC. While hLA and bLG exhibit the OA binding-induced increase in the fraction of SUV-bound protein, the binding of OA to pPA does not cause any noticeable effects. The efficient binding of intact pPA to lipid vesicles may be physiologically important, considering that PA immunoreactivity was found in (or near) membranous systems, like the mitochondria and (or) the microtubuli.[72]

The OA-induced changes in the oligomeric state and the affinity for lipid membranes of the proteins studied are a consequence of their structural rearrangements in response to OA binding. Similar to that of hLA and bLA, the interaction of the reference forms of bLG and pPA with OA leads to an increase in α-helicity. Analogous to ELOA, all the protein−OA complexes studied, except for the bLA−OA-45 complex, demonstrate an increased content of cross-β-sheet structure, as judged from the ThT fluorescence tests. The structuring effect of OA on the secondary structure of bLG and pPA is in line with their lowered sensitivity toward limited enzymatic proteolysis. Meanwhile, the characterization of OA-induced changes in the stabilities of their tertiary structures unexpectedly revealed the absence of observable heat effects accompanying thermal denaturation. Because a cooperative thermal transition at the level of secondary structure is observed for both the bLG−OA-45 and pPA−OA-45 complexes, we can conclude that the OA-bound forms of bLG and pPA lack a fixed tertiary structure but exhibit a continuous thermal transition. This drastic difference in their thermal behavior from that of HAMLET is rationalized by a considerably higher content of OA in the complexes formed by bLG and pPA.

The comparison of physicochemical properties of the protein−OA complexes studied here to those for other complexes reveals a common feature of these complexes: a tendency to protein oligomerization. Self-association of proteins that gives rise to dimers and higher-order oligomers is known to be abundant in biological systems and is supposed to provide additional functional advantages (reviewed in ref 90). In this sense, it can be expected that oligomerization of

otherwise innocuous proteins may change their functional properties, ultimately leading to their cytotoxicity. Indeed, the accumulation of oligomeric protein species is currently considered as a major source of cytotoxicity in amyloid diseases (for a review, see ref 45). In the case of bLA, IEC-6 cell death is induced by its oligomeric fraction.[91,92] Furthermore, it is probably the fact that multimeric bLA, within its complexes with OA, induces a 4−5-fold increase in the cytotoxicity of OA to U2OS cells.[39] At the same time, the degree of bLA oligomerization does not affect the cytotoxicity of bLA against Jurkat cells[24] or the cytotoxicity of bLA−OA complexes against U937 cells.[30] An analogous conclusion was reached for bLG−OA complexes.[32] Moreover, the protein component negligibly (1.3−1.7-fold) increases the cytotoxicity of the bLA−OA-45 and bLG−OA-45 complexes against HEp-2 cells.[3] Overall, the cytotoxicity of oligomeric protein seems to depend on the protein, cell line, and/or structural features of the oligomers.

Because cytotoxic oligomeric forms of α-synuclein are formed in excess of docosahexaenoic acid,[89] and arachidonic and linoleic acids associate with mutants of superoxide dismutase 1 with formation of cytotoxic oligomers,[70] we can conclude that unsaturated fatty acids different from OA are also able to promote the formation of cytotoxic oligomers. The interaction of the Nur77 ligand-binding domain with arachidonic and docosahexaenoic acids changes the oligomerization state of the receptor.[93] The proper choice of both the lipid and the proteinaceous components could result in complexes with cytotoxic activity and specificity of action exceeding those of HAMLET. As an alternative, natural proteins with a specific anticancer activity (TRAIL, apoptin, etc.[94]) along with vaccenic[22] and 2-hydroxyoleic[95] acids can be suggested.[3,20]

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ABBREVIATIONS

OA, oleic acid (C18:1:9-*cis*); intact protein, protein isolated from a natural source; α-LA, α-lactalbumin; hLA, human α-lactalbumin; bLA, bovine α-lactalbumin; HAMLET and BAMLET, complexes of human and bovine α-lactalbumin with oleic acid, respectively, prepared as described in ref 1; bLA−OA-45, complex of bovine α-lactalbumin with oleic acid, prepared as described in ref 2; bLG, bovine β-lactoglobulin; pPA, pike parvalbumin, α isoform; bLG−OA-45 and pPA−OA-45, complexes of β-lactoglobulin and pike parvalbumin with oleic acid, respectively, prepared as described in ref 3; reference bLG and pPA, samples of β-lactoglobulin and pike parvalbumin subjected to procedures described for the preparation of bLG−OA-45 and pPA−OA-45, respectively, without the addition of oleic acid; ELOA, complex of equine lysozyme with oleic acid; HEp-2, human epidermoid larynx carcinoma; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; ThT, thioflavin T; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; DLS, dynamic light scattering; CD, circular dichroism; DSC, differential scanning calorimetry; DPPC, dipalmitoylphosphatidylcholine; SUVs, small unilamellar vesicles.

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