# Who Is Mr. HAMLET? Interaction of Human α-Lactalbumin with Monomeric Oleic Acid[†]

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ABSTRACT: A specific state of the human milk $Ca^{2+}$ binding protein α-lactalbumin (hLA) complexed with oleic acid (OA) prepared using an OA-pretreated ion-exchange column (HAMLET) triggers several cell death pathways in various tumor cells. The possibility of preparing a hLA−OA complex with structural and cytotoxic properties similar to those of the HAMLET but under solution conditions has been explored. The complex was formed by titration of hLA by OA at pH 8.3 up to OA critical micelle concentration. We have shown that complex formation strongly depends on calcium, ionic strength, and temperature; the optimal conditions were established. The spectrofluorimetrically estimated number of OA molecules irreversibly bound per hLA molecule (after dialysis of the OA-loaded preparation against water followed by lyophilization) depends upon temperature: 2.9 at 17 °C (native apo-hLA; resulting complex referred to as LA-OA-17 state) and 9 at 45 °C (thermally unfolded apo-hLA; LA-OA-45). Intrinsic tryptophan fluorescence measurements revealed substantially decreased thermal stability of $Ca^{2+}$-free forms of HAMLET, LA-OA-45, and OA-saturated protein. The irreversibly bound OA does not affect the $Ca^{2+}$ association constant of the protein. Phase plot analysis of fluorimetric and CD data indicates that the OA binding process involves several hLA intermediates. The effective pseudoequilibrium OA association constants for $Ca^{2+}$-free hLA were estimated. The far-UV CD spectra of $Ca^{2+}$-free hLA show that all OA-bound forms of the protein are characterized by elevated content of α-helical structure. The various hLA−OA complexes possess similar cytotoxic activities against human epidermoid larynx carcinoma cells. Overall, the LA-OA-45 complex possesses physicochemical, structural, and cytotoxic properties closely resembling those of HAMLET. The fact that the HAMLET-like complex can be formed in aqueous solution makes the process of its preparation more transparent and controllable, opening up opportunities for formation of active complexes with specific properties.

α-Lactalbumin (α-LA) is a well-studied small (molecular mass of 14 kDa, 123 residues) acidic (p*I* 4−5) globular calcium metalloprotein (*1*) from milk. It is one of the nutrients of milk which is believed to play a neoprotective role (for review, see ref *2*). In the lactating mammary gland, α-LA functions as a noncatalytic regulatory subunit of the lactose synthase enzyme complex (E.C. 2.4.1.22) (*3*). The protein has a single strong calcium binding site (apparent binding constant $3 \times 10^8$ $M^{-1}$ at 20 °C (*4*)) and is able to bind other physiologically significant cations such as $Mg^{2+}$, $Na^+$, $K^+$, and $Mn^{2+}$ (*5*). The binding of the metal ions causes pronounced changes in the thermodynamic properties of α-LA (*6*). It is highly destabilized in the apo state (half-

transition temperature 10−30 °C (*7*)), while calcium association increases its unfolding transition temperature by more than 40 °C. The calcium- and temperature-induced structural transitions in α-LA are adequately described on the basis of a four-state scheme (*8*).

Native α-lactalbumin consists of two domains: a large α-helical domain (composed of three major α-helices and two $3_{10}$ helices) and a small β-sheet domain (contains a small three-stranded antiparallel β-pleated sheet and a $3_{10}$ helix) (*9*). The two domains are linked by a $Ca^{2+}$ binding loop which contains two residues less than the classical EF-hand domain; moreover, the loop is cycled by characteristic Cys73−Cys91 disulfide bond. The significance of calcium ions for the functioning of α-LA remains to be elucidated; nevertheless, calcium binding is required for proper disulfide bond formation during the protein folding (*10*). The cation binding to α-LA modulates its interaction with both fatty acids (*11*) and model lipid membranes (*12−15*), which may be of physiological significance.

Although α-LA has been studied for at least 3 decades, a new wave of interest in this protein appeared in 1995 after the report on its selective antitumor activity. The group of C. Svanborg found that a unique multimeric form of α-LA,

isolated from the casein fraction of milk, induces apoptosis of transformed, embryonic, and lymphoid cells but spares mature and healthy cells (*16*). This form of α-LA, named MAL,[1] was shown to possess molten globule-like properties (*17*). MAL crosses the plasma membrane and cytosol and enters the cell nucleus, where it induces DNA fragmentation through a direct effect at the nuclear level (*18*). Moreover, MAL interacts with mitochondria, which induces the release of cytochrome *c* and activation of the caspase cascade (*19*). Later on, it was reported that a complex of oleic acid ($C_{18:1}$) with human or bovine α-LA (called HAMLET (*20*) and BAMLET (*21*), respectively) demonstrates similar cytotoxic activity. The $Ca^{2+}$-free protein was complexed with oleic acid by means of ion-exchange chromatography on a DEAE-Trisacryl M column preconditioned with oleic acid and was subsequently eluted at high salt concentration (0.8 M NaCl). Neither saturated $C_{18}$ fatty acids nor unsaturated $C_{18:1}$ *trans* conformers or fatty acids with shorter or longer carbon chains can form complexes with apo-α-LA (*22*). Remarkably, unsaturated *cis* fatty acids other than $C_{18:1:9cis}$ are able to form stable complexes with apo-α-LA, but they are not active in the apoptosis assay (*22*). HAMLET (human alpha-lactalbumin made lethal to tumor cells) triggers several cell death pathways in various tumor cell lines and undifferentiated cells while healthy and mature cells are spared (for review, see ref *23*). First, it causes mitochondrial damage followed by the release of cytochrome *c* (*24*). One more cell death pathway is the reduction of proteasome activity (*25*). Moreover, HAMLET induces macroautophagy in tumor cells, which also might contribute to cell death (*23*). Interestingly, the HAMLET complex passes through the cytoplasm to the nucleus where it accumulates and interacts with histones disrupting chromatin assembly and interfering with intact chromatin, thus preventing the cell from transcription, replication, and recombination (*26*). It was shown that this interaction has electrostatic nature and the modification of α-LA by oleic acid actually is not necessary for effective binding to histones (*27*). The molecular details of the above-mentioned cell events caused by HAMLET remain mostly unclear.

Molecular characterization of the HAMLET complex showed that it represents mostly monomeric protein with a single bound oleic acid molecule and possesses decreased thermal stability but native-like calcium affinity. Gel-filtration and magnetic resonance dispersion measurements showed the presence of maximum 5% of dimers or higher order oligomers in the HAMLET preparations (*28*), in accord with the report by Casbara et al. (*29*). At the same time, much higher population of multimeric forms of HAMLET was detected using gel-filtration chromatography; prolonged incubation of the complex at elevated temperatures caused conversion of the mostly monomeric protein into mostly multimeric forms (*30*). According to gas chromatography

and mass spectrometry data, the average number of oleic acid molecules bound per HAMLET complex is 0.9, although the real values depend upon the particular batch (0.6−1.3) (*22*). The thermal stability of apo-HAMLET is decreased (*ca.* 15 °C) compared to the intact apoprotein (*31*). The effective $Ca^{2+}$ binding constant (*K*) for HAMLET measured at 25 °C ($5.9 \times 10^8 M^{-1}$) is within experimental error of that for intact α-LA ($K = 1.8 \times 10^9 M^{-1}$) (*21*). The near-UV CD spectrum of HAMLET at 25 °C (pH 7.5) is similar to that of the classical molten globule of α-LA (*22*), i.e., a state with lost rigid tertiary but native-like secondary structure. Since apo-α-LA at 25 °C represents a mixture of the native and thermally denatured forms, adequate interpretation of the data obtained is complicated.

Distinct structural differences between HAMLET and intact protein were detected (*29*). H/D exchange experiments showed that HAMLET incorporates a greater number of deuterium atoms than does intact apoprotein. HAMLET and the intact apoprotein are both accessible to proteases in the β-domain but have substantial differences in accessibility to proteases at specific sites. Nuclear Overhauser enhancement spectroscopy showed that the two ends of the oleic acid molecule in HAMLET are in close proximity to each other and close to the double bound, which demonstrates that OA is bound in a compact conformation (*28*).

Notably, the complex between α-LA and oleic acid can be formed by simply adding OA to the protein solution (without the conventional use of ion-exchange chromatography). The $^1H$ NMR investigation of calcium-independent OA binding to 1 mM hLA at pH 7.0 and 37 °C gave an apparent dissociation constant ($K_d$) of 0.1 mM (*31*). Since oleic acid has a very low CMC under these conditions, the reported value of $K_d$ seems to be affected by the process of micelle formation. According to partition equilibria and fluorescent spectroscopy data, bovine α-lactalbumin has one binding site for oleic acid with a binding constant of $(4.6−3.3) \times 10^6 M^{-1}$ (*32*). The addition of 7.5 equiv of oleic acid to bovine apo-α-lactalbumin (pH 8.3, 5 mM EDTA) at 20 °C induces CD changes resembling those observed in the formation of the molten globule state of α-LA (*30*). According to gel-filtration data, the resulting complex is fully monomeric, in contrast to HAMLET (*30*). At the same time, despite the effective association of OA with the protein under solution conditions, the resulting α-LA−OA complex is believed to possess lower antitumor activity than HAMLET at similar protein and lipid concentrations (*20*).

It should be noted that complete systematic studies of the process of the α-LA−OA complex formation under solution conditions have not been carried out to date. In this work we present optimal temperature, calcium, and monovalent salt conditions for formation of complexes showing maximal OA binding capacity. The numbers of oleic acid molecules irreversibly bound per one α-lactalbumin molecule in these complexes were estimated. Characterization of physicochemical, conformational, and cytotoxic properties of the resulting α-LA−OA complexes formed in aqueous buffers showed that they share many common features with HAMLET. Although the protein states reported here exhibit certain differences from HAMLET, their cytotoxic activities against tumor cells are close to that of HAMLET, which makes them well suited for further cytotoxic characterization. The fact that hLA−OA complexes having well-defined cytotoxic

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[1] Abbreviations: OA, oleic acid ($C_{18:1:9cis}$); CMC, critical micelle concentration; hLA or intact hLA, α-lactalbumin isolated from human milk; OA-saturated hLA, human α-lactalbumin titrated by oleic acid up to its critical micelle concentration; MAL, multimeric form of human α-LA extracted from casein milk fraction as described in ref *16*; HAMLET (BAMLET), specific complex of human (bovine) α-lactalbumin with oleic acid as described in ref *20*; LA-OA-17 and LA-OA-45, complexes of human α-lactalbumin with oleic acid, prepared at 17 and 45 °C, respectively, as described in Materials and Methods; CD, circular dichroism; HEp-2, human epidermoid larynx carcinoma cells.

activities can be formed in aqueous solution makes the process of preparation of the active complex more transparent and controllable, which opens up the additional opportunities for fine-tuning the active complexes for specific properties.

## MATERIALS AND METHODS

*Materials.* Human epidermoid larynx carcinoma (HEp-2) cells were obtained from the Russian Cell Culture Collection (Institute of Cytology, Russian Academy of Sciences, St. Petersburg). Fetal bovine serum was from HyClone; culture medium DMEM, crystal violet, and other chemicals for cytotoxicity tests were purchased from Sigma Chemical Co.

Human α-lactalbumin (hLA) was isolated and purified from human milk as described in ref *33*. The protein concentrations were evaluated spectrophotometrically using an extinction coefficient $A_{1cm,280}^{1\%} = 18.2$ (*34*). Oleic acid ($C_{18:1,9cis}$) was bought from Aldrich. Chemistry grade 96% (v/v) ethanol was treated with $K_2MnO_4$ and activated coal, followed by double distillation; the absence of organic impurities was confirmed spectrophotometrically. Stock solution of oleic acid in ethanol was prepared by means of sonication of OA suspension for 3 min, and the solution was stored at room temperature. Ultragrade Tris and $H_3BO_3$ were bought from Merck; ultragrade HEPES and DEAE-Trisacryl M were from Sigma Chemical Co. Calcium chloride and EDTA standard solutions were from Fluka and Fisher Scientific, respectively. Other reagents were of analytical grade or better. Dialysis tubing was from Pierce. All solutions were prepared using nanopure water (conductivity 18.2 MΩ·cm).

*Methods. Preparation of HAMLET.* HAMLET was prepared at room temperature (20−22 °C) as described in ref *35*. Briefly, a 14 × 1.6 cm column was packed with 10 mL of DEAE-Trisacryl M in 10 mM Tris-HCl and 100 mM NaCl, pH 8.5 (buffer A). Ten milligrams of oleic acid were dissolved in 0.5 mL of ethanol by sonication. After addition of 9.5 mL of buffer A, the lipid solution was applied to the column and dispersed throughout the matrix using NaCl gradient: 40 mL of linear gradient (100−85% buffer A, 0−15% buffer B), 20 mL of A/B (85%/15%), 10 mL of A/B (20%/80%), and 20 mL of A (100%); buffer B was 10 mM Tris-HCl and 1 M NaCl, pH 8.5. Elution rate was 0.37 mL/min. Eight milligrams of $Ca^{2+}$-free hLA in 10 mL of buffer A with 5 mM EDTA was applied to the oleic acid conditioned column and eluted as described above. The protein fractions collected at about 0.82 M NaCl were dialyzed against distilled water (four times, 100-fold volume excess), lyophilized, and stored at −18 °C.

*The Choice of pH and Temperature Conditions.* In all studies of hLA states at fixed temperature, a temperature-corrected pH value of 8.3 was used, similar to that used in HAMLET preparation. All titration experiments were performed in the temperature range from 17 °C (above the freezing point of oleic acid, 16 °C, and below the thermal transition of the apoprotein) to 45 °C (above the thermal transition of the apoprotein).

*Preparation of LA-OA-17 and LA-OA-45 States.* A diluted solution of $Ca^{2+}$-free hLA (*ca.* 10 μM; pH 8.3, 20 mM $H_3BO_3$−KOH, 150 mM NaCl, 1 mM EDTA; 25 mL) was titrated by small aliquots (3−8 μL) of the solution of oleic acid in 96% ethanol (100 mM) under continuous stirring.

The resulting solutions contained no interfering fatty acid film on the surface. About 10 additions were required to reach the effective OA CMC value at 17 °C (for LA-OA-17) or 45 °C (LA-OA-45 state). The LA−OA complexes were dialyzed against distilled water (four times, 100-fold volume excess), lyophilized, and stored at −18 °C.

*Calcium Depletion from α-Lactalbumin Samples.* Purification of protein samples from $Ca^{2+}$ ions was performed according to Blum et al. (*36*) using Sephadex G-25 media (1.2 × 20 cm column; 20 mM HEPES−KOH, pH 8.2, buffer). The residual calcium content was less than 2−3 μM, as estimated from spectrofluorimetric calcium and EDTA titrations of α-lactalbumin (*37*).

*Estimation of the Effective Critical Micelle Concentration (CMC) of Oleic Acid.* The CMC values of OA were measured using a Cary 100 Bio spectrophotometer (Varian Inc.), equipped with the cell holder, thermostated by a NESLAB RTE 7 (Thermo Electron) water bath. Sample temperature was measured inside the sample cell.

Buffer solution (3 mL) was titrated by small aliquots (2−6 μL) of stock solutions of oleic acid in 96% ethanol (1−100 mM). Mixing of oleic acid was achieved by manual pipetting of the solution. No interfering fatty acid film was found on the surface of the solution. Maximal ethanol concentration was below 2% (v/v). The appearance of OA micelles in the solution was monitored by increase in light scattering (absorbance) at 310 nm. The CMC value of free OA, $CMC_0$, was estimated from the spectrophotometric titration curves, as an oleic acid concentration corresponding to the point of intersection of the two linearly approximated parts of the spectrophotometric titration curve. The effective CMC values of OA in the presence of hLA, CMC, were measured in identical manner.

An apparent CMC value of OA in the presence of protein at concentration [P] is linearly related to the OA binding capacity of hLA, $n_{max}$:

$$CMC = CMC_0 + n_{max}[P] \qquad (1)$$

Since OA binding to hLA may promote protein aggregation (accompanied by an increase in light scattering), a corresponding decrease of the CMC value may be observed. Hence, the slope of the dependence of CMC upon protein concentration can be used as the lowest estimate of the real OA binding capacity of hLA.

*Fluorescence Studies.* Fluorescence spectra were measured using a Cary Eclipse spectrofluorimeter (Varian Inc.), equipped with a Peltier-controlled cell holder, using a 10 × 10 mm quartz cell. Fluorescence of α-LA was excited at 280 nm. The excitation and emission band widths were 5 and 2.5 nm, respectively. All spectra were corrected for the spectral sensitivity of the instrument and fitted to log-normal curves (*38*) using LogNormal software (IBI RAS, Pushchino) written in Delphi 2005 (Borland Software Corp.), implementing the nonlinear regression algorithm by Marquardt (*39*). The positions of the fluorescence spectra maxima ($\lambda_{max}$) were obtained from these fits. The values of fluorescence emission intensities were normalized for 100.

Spectrofluorimetric temperature scans were performed stepwise, allowing the sample to equilibrate at each temperature. Temperature was monitored inside the sample cell. The average heating rate was 0.5 °C/min. At temperatures below room temperature the cell compartment was purged

Table 1: Dependence of Critical Micelle Concentration of OA (CMC, $\mu$M) at pH 8.3 (20 mM $H_3BO_3$–KOH) upon Calcium and Monovalent Salt Concentrations and Temperature Conditions Estimated from Spectrophotometric Titrations

| temp (°C) | 1 mM $CaCl_2$ | | 1 mM EDTA | | |
|---|---|---|---|---|---|
| | CMC, no salts | CMC, 150 mM NaCl | CMC, no salts | CMC, 150 mM NaCl | CMC, 150 mM KCl |
| 17 | 4 ± 1 | 3 ± 1 | 20 ± 4 | 22 ± 6 | 25 ± 8 |
| 45 | 4 ± 1 | 3 ± 1 | 69 ± 6 | 35 ± 5 | 48 ± 6 |

with dry nitrogen (dew point of −40 °C) to avoid condensation of water vapor on the cell surface.

In the course of a spectrofluorimetric titration of hLA by oleic acid, small aliquots (2−3 $\mu$L) of OA stock solution in 96% (v/v) ethanol (5−10 mM) were added to the protein solution under hand stirring. The total OA concentration was kept below its effective CMC value.

*Circular Dichroism.* Circular dichroism (CD) measurements were carried out with a JASCO J-810 spectropolarimeter (JASCO Inc., Japan), equipped with a Peltier-controlled cell holder, using quartz cuvettes with path lengths of 10 and 1.00 mm for near- and far-UV regions, respectively. The instrument was calibrated with an aqueous solution of *d*-10-camphorsulfonic acid (JASCO Inc.) according to the manufacturer's instruction. The cell compartment was purged with dry nitrogen (dew point of −40 °C) to avoid condensation of water vapors on the cuvette surface at lowered temperatures. Protein concentrations were 40 and 4 $\mu$M for measurements in near- and far-UV regions, respectively. The small contribution of buffer (20 mM $H_3BO_3$–KOH, 150 mM NaCl, 1 mM EDTA, pH 8.3) was subtracted from the experimental spectra. Band width was 2 nm, averaging time 1−2 s, and accumulation 3.

Quantitative estimations of the secondary structure contents were made using the CDPro software package, which includes the programs SELCON3, CDSSTR, and CONTIN (http://lamar.colostate.edu/~sreeram/CDPro) (*40*). The experimental data in the 200−240 nm range were treated by all three programs, using the SDP48 reference protein set. The final secondary structure fractions reported represent averaged values.

*Measurements of Protein Calcium Affinity.* Calcium affinity of protein was measured by spectrofluorimetric titration of $Ca^{2+}$-depleted protein with a $CaCl_2$ standard solution at pH 8.2 and 45 °C. Calculation of the equilibrium calcium association constant $K$ of protein (P) from the experimental data was based on the cooperative binding scheme:

$$P + nCa^{2+} \overset{K}{\leftrightarrow} P\cdot Ca^{2+}_n \qquad (2)$$
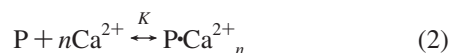
where $n$ is a cooperativity of calcium binding (Hill coefficient). The experimental data were globally fitted using FluoTitr v.1.2 software (IBI RAS, Pushchino) written in Delphi 2005 (Borland Software Corp.), implementing the nonlinear regression algorithm by Marquardt (*39*). The fit was achieved by variation of $n$ and $K$ parameters. The error in experimental estimation of the $K$ value was about ±0.25 of the order of its magnitude.

*Measurements of Protein Affinity to Oleic Acid.* Estimation of OA affinity of hLA was performed similarly to the method described for measurement of $Ca^{2+}$ affinity. A solution of α-lactalbumin (5−8 $\mu$M for spectrofluorimetric titrations; 40 $\mu$M for CD titrations, near-UV region) in 20 mM $H_3BO_3$–KOH, 150 mM NaCl, and 1 mM EDTA, pH 8.3,

was titrated with small aliquots (2 $\mu$L) of OA stock solution in 96% (v/v) ethanol (1−100 mM) up to the CMC of oleic acid, under hand stirring. The experimental spectrofluorimetric and CD titration data were fitted according to the cooperative binding scheme:

$$P + nOA \overset{K}{\leftrightarrow} P\cdot OA_n \qquad (3)$$

*Cytotoxicity Assay.* HEp-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (w/v) fetal bovine serum, 40 mg/L gentamycin, 35 mM sodium bicarbonate, and 20 mM HEPES at 37 °C in an atmosphere of 5% $CO_2$.

For the cytotoxicity assay, cells were seeded in 96-well microplates or culture dishes (Corning, USA) at a concentration of $5 \times 10^4$ cells/mL ($5 \times 10^3$ cells in 100 $\mu$L/well). Freshly prepared solutions of hLA and complexes of α-lactalbumin with oleic acid in growth medium as well as OA (1 M stock solution in ethanol) were added to culture medium 24 h after seeding the cells. Cytotoxicity was evaluated using the crystal violet assay from the ratio of optical densities at 560 nm in treated and untreated (control) cultures at 48 h after adding the toxic agents (*41*). The optical density value was in direct proportion to the number of viable cells. Each experiment was performed at least three times. All of the values represent the means ± SEM. The statistical significance of the results was analyzed using Student's *t* test.

## RESULTS

*Estimation of the Critical Micelle Concentration of Oleic Acid.* The preparation of the hLA−OA complex without the use of ion-exchange media was carried out at pH 8.3 (used in the preparation of HAMLET). Since oleate at pH 8.3 may exist also in lamellar and crystalline phases (*42, 43*), the term *critical micelle concentration* used here strictly speaking represents a boundary concentration of OA above which accumulation of extensive phases, contributing to the light scattering, is observed (see Materials and Methods section).

Since the p$K_a$ of monomeric oleic acid in aqueous solution is close to that for short-chain carboxylic acids (4.8) (*42, 43*), the carboxylate of OA at pH 8.3 is fully deprotonated. This deprotonated carboxylate can bind calcium ions, which in turn may affect the critical micelle concentration of OA (CMC). Indeed, the CMC value for $Ca^{2+}$-free buffer (1 mM EDTA) is more than 5 times higher than that measured in excess of calcium (1 mM $CaCl_2$) (Table 1). Thus, calcium ions promote aggregation of oleic acid molecules, thereby narrowing the region of OA concentrations suitable for studies of interaction between the protein and monomeric OA. Therefore, in most of our measurements calcium ions were avoided to ensure the most favorable conditions for the OA−protein interaction. The addition of NaCl or KCl causes much smaller effects on the CMC value (Table 1).
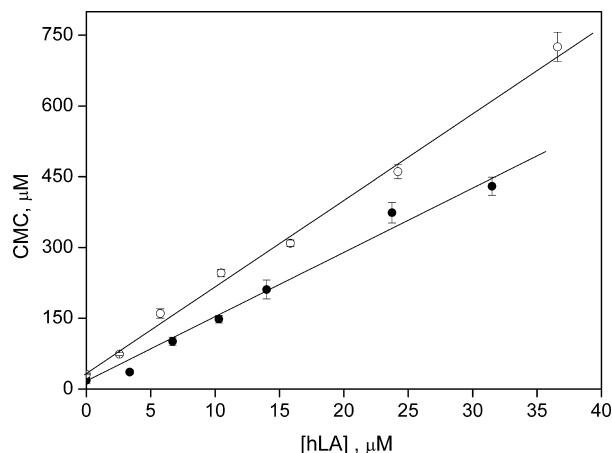
FIGURE 1: Dependence of the effective CMC of OA upon hLA concentration at 17 °C (filled circles) and 45 °C (open circles) (20 mM $H_3BO_3-KOH$, 150 mM NaCl, 1 mM EDTA, pH 8.3). The values of critical micelle concentration were estimated from spectrophotometric titrations of the protein by OA, monitored by absorbance at 310 nm. The solid lines represent linear fits of experimental data.

Table 2: OA Binding Capacity of Various States of hLA ($n_{max}$) at 17 and 45 °C (20 mM $H_3BO_3-KOH$, pH 8.3, 1 mM $CaCl_2$ or 1 mM EDTA, in the Presence or Absence of 150 mM NaCl or KCl), Estimated from the Dependence of Effective CMC of OA upon Protein Concentration[a]

| | 1 mM $CaCl_2$ | 1 mM EDTA | | |
|---|---|---|---|---|
| temp (°C) | $n_{max}$ | $n_{max}$, no salts | $n_{max}$, 150 mM NaCl | $n_{max}$, 150 mM KCl |
| 17 | 0 | 3.7 | 13.5 | 12.7 |
| 45 | 0 | 2.2 | 18.5 | 18.0 |

[a] See Figure 1 and eq 1.

*Oleic Acid Binding Capacity of* α-*Lactalbumin.* In order to estimate the OA binding capacity of hLA ($n_{max}$), the dependence of the effective CMC of OA upon protein concentration was measured at 17 and 45 °C (Figure 1). The CMC value linearly increases with protein concentration, and the slope of the straight line gives the $n_{max}$ value (see eq 1). Apparently, $n_{max}$ for the $Ca^{2+}$-free protein in the presence of salts is notably (*ca.* 40%) higher at the elevated temperature. The $n_{max}$ value does not depend on the salt type (KCl or NaCl). The importance of ionic strength conditions for efficient OA binding is corroborated by the fact that the absence of salts results in a 3−8-fold decrease in $n_{max}$. Spectrofluorimetric KCl/NaCl titrations of OA-saturated $Ca^{2+}$-free hLA at 45 °C demonstrate that the salt-induced fluorimetric changes reach a plateau at 150 mM of either salt (data not shown). Meanwhile, calcium conditions are crucial for OA association, since $Ca^{2+}$-saturated (1 mM $CaCl_2$) protein exhibits a nonmeasurable $n_{max}$ value, demonstrating inability of the $Ca^{2+}$-hLA to bind OA.

Overall, an examination of Tables 1 and 2 shows that the most favorable conditions for formation of the complex between hLA and monomeric oleic acid ensuring maximal OA binding capacity of the protein are achieved in the absence of $Ca^{2+}$ at 45 °C in the presence of 150 mM NaCl or KCl. Since the apparent $Na^+$ association constant for α-lactalbumin is $10^2$ $M^{-1}$ at 20 °C (6), the application of 150 mM sodium ions in the absence of calcium ions (1 mM EDTA) results in partial loading of the strong $Ca^{2+}$ binding site of hLA with sodium ions. Importantly, HAMLET is
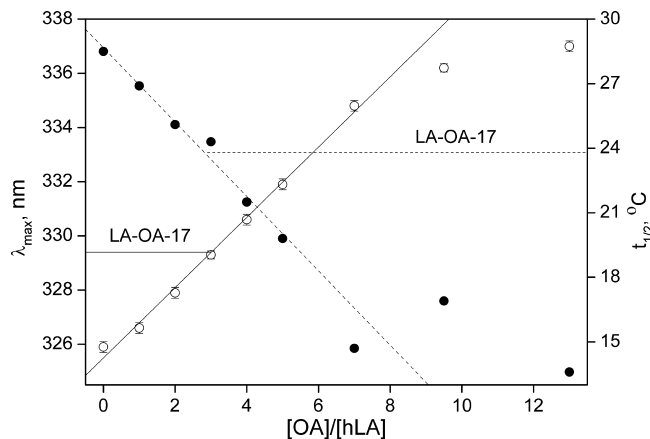


FIGURE 2: Dependence of the spectral and thermodynamic parameters of $Ca^{2+}$-free hLA on OA to protein molar ratio (20 mM $H_3BO_3-KOH$, 150 mM NaCl, 1 mM EDTA, pH 8.3). Filled circles represent the effective midtransition temperature ($t_{1/2}$) for hLA titrated at 17 °C by oleic acid up to given OA to protein ratio, estimated from spectrofluorimetric data as described in the text. Open circles represent the tryptophan fluorescence spectrum maximum position ($\lambda_{max}$) at 10 °C for the OA-titrated protein. The hLA concentration was 6−7 $\mu$M. The solid and dash lines represent linear fits of experimental data. The horizontal lines correspond to the values of respective parameters estimated for LA-OA-17. The fluorescence was excited at 280 nm.

formed in the presence of sodium ions (above 150 mM NaCl), which makes sodium chloride the most attractive choice for the formation of the HAMLET-like hLA state. We will refer to the sodium-loaded form of α-lactalbumin as "$Ca^{2+}$-free" hLA.

It should be noted that in the presence of calcium ions (1 mM $CaCl_2$) all OA-bound forms of hLA studied (HAMLET, LA-OA-17, and LA-OA-45) demonstrate an increased level of light scattering (protein concentration below 10 $\mu$M), as evidenced by spectrophotometric measurements. This phenomenon may be both due to the formation of extented oligomeric forms of the protein or due to the $Ca^{2+}$-induced liberation of OA molecules bound followed by formation of OA micelles. These effects were not observed in the absence of calcium ions; therefore, these conditions were chosen for further studies.

*Number of Oleic Acid Molecules Irreversibly Bound per* α-*Lactalbumin Molecule in LA−OA Complexes.* For estimation of the number of oleic acid molecules irreversibly bound per α-lactalbumin molecule in the LA-OA-17 state ($n_{irr}$) (after dialysis of the OA-saturated preparation against water followed by its lyophilization) some of its physicochemical parameters were compared with those of the hLA−OA complex formed at 17 °C by addition of oleic acid aliquots up to the fixed OA to protein molar ratio. The results of experiments on thermal denaturation of the resulting hLA−OA complexes monitored by intrinsic tryptophan fluorescence are shown in Figure 2. Oleic acid association results in a pronounced decrease in hLA thermal stability, as clearly seen from spectrofluorimetric measurements for various states of $Ca^{2+}$-free hLA, shown in Figure 3a. The thermally induced unfolding of hLA is reflected in a tryptophan fluorescence red shift due to transfer of some tryptophan residue(s) from the interior of the protein to the surface in contact with water molecules. These changes can be used for evaluation of thermal stabilities of various protein states. The effective

Table 3: Equilibrium $Ca^{2+}$ Association Constant ($K$) and Hill Coefficient ($n$) at 45 °C for Various States of hLA, Estimated from Spectrofluorimetric $Ca^{2+}$ Titrations of the $Ca^{2+}$-Depleted Protein (see Figure 3c), According to the Cooperative Metal Binding Scheme (eq 2)

| protein state | $K$ ($M^{-1}$) | $n$ |
|---|---|---|
| intact hLA | $1.3 \times 10^6$ | 0.95 |
| HAMLET | $8 \times 10^5$ | 0.98 |
| LA-OA-17 | $7 \times 10^5$ | 0.87 |
| LA-OA-45 | $7 \times 10^5$ | 0.86 |
| OA saturated at 17 °C | $2.7 \times 10^5$ [a] | 1.8 |
| OA saturated at 45 °C | $1.5 \times 10^5$ | 2.3 |

[a] An additional $Ca^{2+}$ binding site with $K$ value about $10^6-10^7$ $M^{-1}$ is observed. The values in the table were estimated based upon the sequential binding scheme, in which binding of a single calcium ion is followed by the cooperative binding of $n$ $Ca^{2+}$ ions.

cence spectrum maximum position ($\lambda_{max}$) by a sigmoidal curve. The initial parts of the dependencies of $t_{1/2}$ and $\lambda_{max}$ at 10 °C vs OA to protein ratio can be successfully approximated by a straight line which allowed estimation of $n_{irr}$: $2.9 \pm 0.1$. Analogous measurements for the hLA−OA complex incubated at 17 °C for 96 h under continuous stirring gave $n_{irr} = 3.3-4.3$ (data not shown). Further increase of OA to protein ratio is accompanied with saturation of the effects observed due to the approach to the $n_{max}$ value (Table 2). The estimation of the $n_{irr}$ value for the LA-OA-45 state performed in similar manner gave $n_{irr} \approx 9$. In this case the dependence of $\lambda_{max}$ upon OA to protein molar ratio lost linearity above about 10:1, which hinders the accuracy of the $n_{irr}$ value. The value of $\lambda_{max}$ for HAMLET is very close to that for LA-OA-45 in the absence of calcium ions. Nevertheless, the $n_{irr}$ value for HAMLET cannot be determined in this way due to specific conditions of the complex formation.

*Effect of Oleic Acid Binding upon Thermal Stability and Calcium Affinity of α-Lactalbumin.* Oleic acid binding results in a pronounced decrease of hLA thermal stability, as seen from spectrofluorimetric measurements of thermally induced unfolding of various states of $Ca^{2+}$-free hLA (Figure 3a). While the LA-OA-17 complex is characterized by a slight decrease (about 5 °C; see Figure 2) in thermal stability with respect to intact hLA, protein saturation with oleic acid up to its CMC at 17 °C causes more pronounced thermal stability changes (*ca.* 12 °C), comparable to those observed for HAMLET (see also ref *31*) and LA-OA-45 states. It is noteworthy, that the ethanol concentration during OA titrations was below 2% (v/v), which does not effect thermal denaturation of $Ca^{2+}$-free hLA (data not shown). The OA-induced decrease in hLA thermal stability is likely to reflect the more efficient binding of oleic acid at temperatures above the midtransition temperature for the $Ca^{2+}$-free protein (Table 2). The more pronounced (versus LA-OA-17) thermal stability changes observed for both LA-OA-45 and OA-saturated protein correlate with the higher number of OA molecules bound per α-LA molecule. Moreover, the slight red shift of the tryptophan fluorescence spectrum maximum at temperatures above the thermal denaturation transition of LA-OA-17 seems to reflect the gradual dissociation of OA with temperature, which is nearly complete at 90 °C. On the contrary, HAMLET, LA-OA-45, and OA-saturated hLA demonstrate here a slightly (2 nm) blue-shifted tryptophan fluorescence spectrum, suggesting that they remain OA-bound at these temperatures.
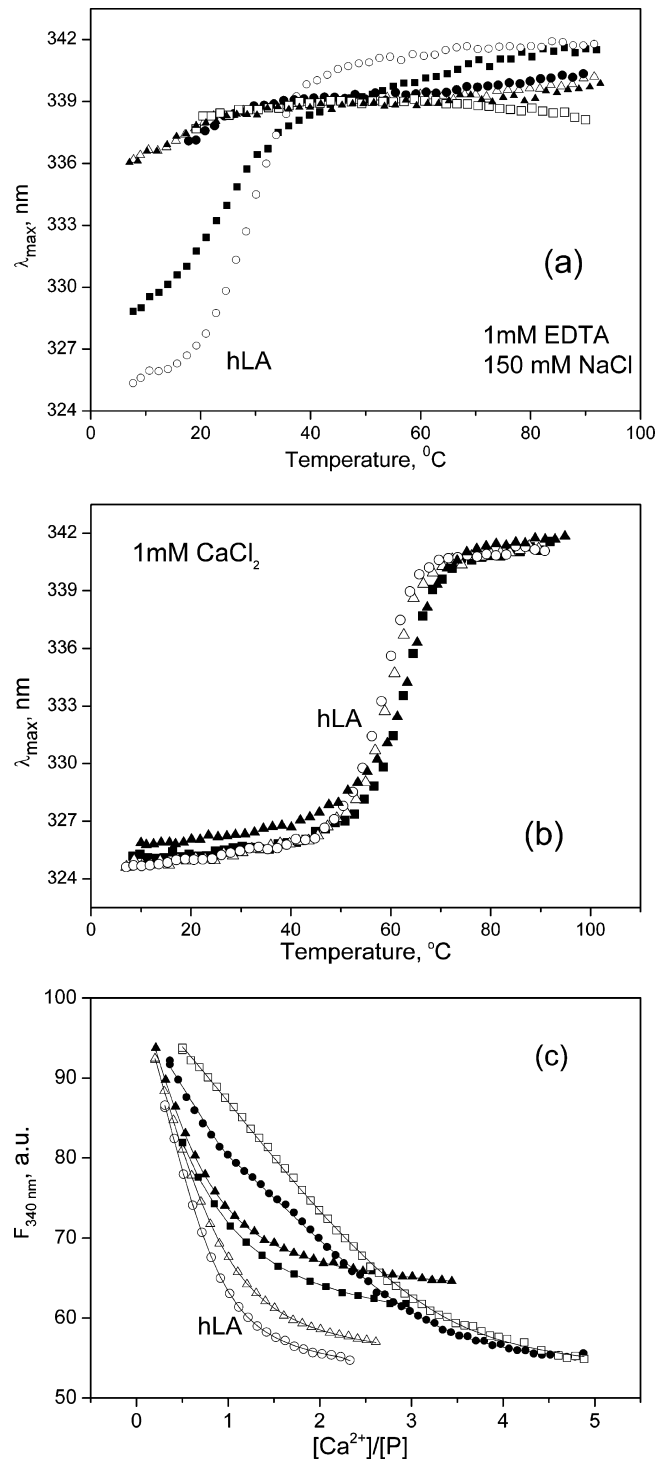
FIGURE 3: Spectrofluorimetric thermal stability (a, b) and calcium affinity (c) measurements for various states of hLA: intact hLA (open circles), HAMLET (open triangles), LA-OA-17 complex (filled squares), LA-OA-45 (filled triangles), and hLA titrated by oleic acid up to CMC at 17 °C (filled circles) or 45 °C (open squares). Fluorescence was excited at 280 nm. Protein concentration was 4−11 $\mu$M. (a, b) Thermal denaturation of hLA monitored by fluorescence spectrum maximum position, $\lambda_{max}$. Buffer conditions: 20 mM $H_3BO_3$−KOH, pH 8.3, 150 mM NaCl, and 1 mM EDTA (a) or 1 mM $CaCl_2$ (b). (c) Fluorimetric titration of calcium-depleted hLA by $CaCl_2$ at 45 °C, traced by emission at 340 nm. Buffer conditions: 20 mM HEPES−KOH, pH 8.2. Solid lines represent theoretical curves fitted to the experimental data according to the cooperative binding scheme (eq 2) or the sequential binding scheme (see Table 3).

midtransition temperature ($t_{1/2}$) was estimated from the fitting of the experimental temperature dependence of the fluores-

In contrast to the apoprotein, calcium-saturated forms (1 mM $CaCl_2$) of HAMLET, LA-OA-17, and LA-OA-45 exhibit a slight (less than 4 °C) increase of thermal stability, with respect to intact hLA (Figure 3b). Nevertheless, the difference between thermal denaturation transitions of different hLA−OA complexes suggests an effective irreversibility of the process of OA binding, since reversible processes would result in relatively rapid dissociation of OA (see Table 2).

The effects of the OA association on calcium affinity of hLA were studied by means of a spectrofluorimetric $Ca^{2+}$ titration of the calcium-depleted protein at 45 °C (Figure 3c). The apparent equilibrium $Ca^{2+}$ association constant $K$ and Hill coefficient $n$, estimated according to the cooperative binding scheme (eq 2). for various protein states, are collected in Table 3. Most of the OA-bound forms of α-LA have slightly lower $Ca^{2+}$ affinity compared with the intact protein. Interestingly, hLA titrated by OA up to its CMC at 17 or 45 °C exhibits binding cooperativity exceeding 1, and 0.5−1 order of magnitude lower $K$ values. Moreover, an additional $Ca^{2+}$ binding site with a $K$ value about $10^6–10^7$ $M^{-1}$ is observed in the case of hLA titrated by OA up to CMC at 17 °C.

*Mechanism of Oleic Acid Binding by α-Lactalbumin.* The changes in fluorescence emission intensities at 370 and 310 (320) nm accompanying oleic acid binding to $Ca^{2+}$-free hLA at 17 and 45 °C (Figure 4a,b) are absolutely different, as well as changes in fluorescence quantum yield (60% increase at 17 °C and 10% decrease at 45 °C) and spectrum maximum position (9 nm red shift at 17 °C and 2 nm blue shift at 45 °C), which emphasizes the fact that temperatures of 17 and 45 °C correspond to different thermodynamic states of the apoprotein (see Figure 3a). At 17 °C OA binding causes partial unfolding of the protein, which is accompanied by a substantial increase in fluorescence quantum yield and a spectral red shift. On the contrary, at 45 °C the thermal transition of $Ca^{2+}$-free hLA is complete, and OA-induced changes in intrinsic fluorescence intensity reflect structural changes in the local environment of tryptophan residues of the protein caused by oleic acid binding. The association of OA slightly decreases the mobility and polarity of the environment of emitting tryptophan residues, which seems to reflect their lower accessibility to the solvent.

Notably, replacement of NaCl by KCl in the experiments in Figure 4 results in qualitatively the same results (data not shown). This fact suggests that the oleic acid binding process is insensitive to the specific binding of monovalent cations to hLA.

In order to unravel the details of the mechanism of oleic acid binding by hLA, we constructed a fluorescence phase plot, which shows the dependence of the fluorescence emission intensity at fixed wavelength upon the fluorescent intensity at another fixed wavelength. The method was first introduced by Burstein (*44, 45*) for protein fluorescence data analysis but can be easily extended on any other parameter, linearly related with populations of the system's states (*37, 46*). Each straight line in the phase plot corresponds to a transition between two protein conformations. Thus, experimental conditions at which discrete protein states are formed and the transitions between them are clearly revealed. Results of the application of this approach to the spectrofluorimetric titrations of $Ca^{2+}$-free hLA by oleic acid up to CMC at 17
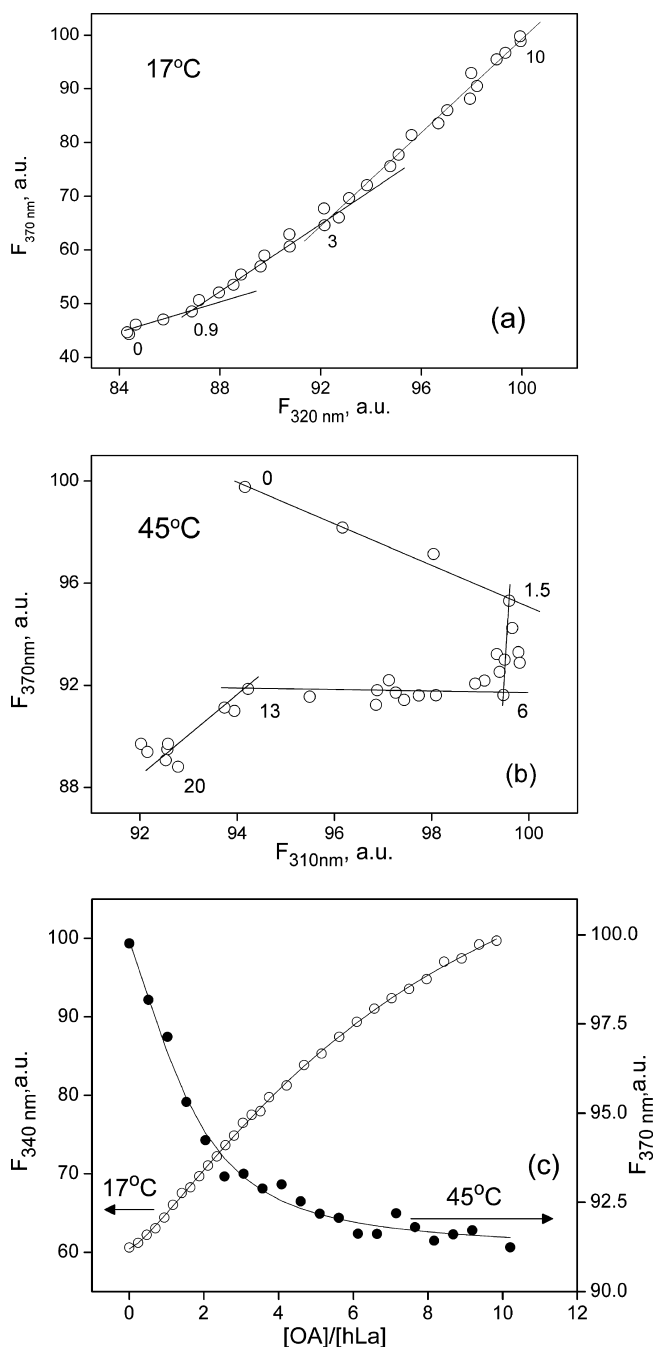


FIGURE 4: Phase plot analysis of spectrofluorimetric titrations of $Ca^{2+}$-free hLA by OA at 17 °C (a) and 45 °C (b) and their theoretical description (c) (20 mM $H_3BO_3$−KOH, 150 mM NaCl, 1 mM EDTA, pH 8.3). The protein concentration was 5−8 μM. Each straight line on the phase plots represents a transition between two conformations of the protein. The values of OA to protein molar ratios corresponding to the key points of the phase plots are indicated. (c) Spectrofluorimetric titrations of hLA by OA at 17 °C (open circles) and 45 °C (filled circles), traced by emission at 340 and 370 nm, respectively. Solid lines represent theoretical curves fitted to the experimental data according to the cooperative OA binding scheme (eq 3). The fluorescence was excited at 280 nm.

or 45 °C are shown in Figure 4. The fluorescence phase plot (emission intensity, $F$, at 370 nm versus $F$ at 310 (320) nm) reveals that the process of oleic acid binding by hLA at 17 °C passes through at least two intermediates, most populated around the OA to protein molar ratios 0.9 and 3 (Figure 4a). The phase plot for spectrofluorimetric titration at 45 °C exhibits even more complicated behavior, indicating the

presence of at least three intermediates most populated at OA to protein molar ratios about 1.5, 6, and 13 (Figure 4b). Analogous phase plots constructed for OA titration monitored by near-UV circular dichroism reveal similar behavior at both temperatures (data not shown).

Despite the fact that OA binding by $Ca^{2+}$-free hLA is a complex multistate irreversible process, the apparent pseudoequilibrium oleic acid binding constant $K$ can be estimated using the cooperative scheme (eq 3) of association of $n$ oleic acid molecules per one protein molecule. The resultant theoretical curves for fluorimetric OA titrations at 17 and 45 °C fitted to the experimental points are shown in Figure 4c. Estimation of $K$ and $n$ values gave $K = 2 \times 10^4$ M$^{-1}$ and $n = 1.3$ at 17 °C. At 45 °C about an order of magnitude increase in apparent affinity of hLA to OA occurs. Similar $K$ and $n$ values were obtained in the absence of monovalent salts at both temperatures (data not shown). These observations are fully in line with the OA-induced decrease in protein thermal stability (Figure 3a), reflecting higher OA affinity of $Ca^{2+}$-free hLA at temperatures above its thermal transition.

*Structural Changes in α-Lactalbumin Induced by Oleic Acid Binding.* Oleic acid binding induces pronounced changes in both the thermal stability of $Ca^{2+}$-free hLA and the environment of its tryptophan residues (Figures 3a,b and 4). Far-UV CD measurements for various states of the protein at 5 and 45 °C show that all of the OA-bound forms of apo-hLA exhibit an increased negative molar ellipticity in the 210−225 nm region (Figure 5). The estimation of secondary structure for different forms of the $Ca^{2+}$-free hLA using the CDPro software package (*40*) reveals that OA-induced changes at 5 °C are mostly due to an increase in α-helical structure content (6.9%); the effect increases in the order LA-OA-17 < HAMLET < LA-OA-45. Similar changes of helical content are observed at 45 °C (6.5%). The helical content of hLA OA-saturated at 17 and 45 °C is hardly distinguishable from those for HAMLET and LA-OA-45 states. At both temperatures the most pronounced changes of helicity are observed for LA-OA-45 and HAMLET states. Nevertheless, these forms are characterized by the most destabilized tertiary structure along with the most mobile and polar environment of emitting tryptophan residues (Figure 3a). OA titration at 17 °C monitored by near-UV CD displays spectral changes indicative for OA-induced disappearance of rigid tertiary structure of the apoprotein (data not shown). This behavior is in line with Figure 3a and the previously described observations for bovine apo-α-lactalbumin at 20 °C (*30*).

*Comparison of Cytotoxicities of HAMLET and LA-OA Complexes.* The concentration dependence of the cytotoxic effects of HAMLET and LA-OA complexes on human larynx carcinoma HEp-2 cells *in vitro* is shown in Figure 6. Intact hLA at concentrations up to 1 mM was shown to be nontoxic. Oleic acid causes a toxic effect on HEp-2 cells only at concentrations exceeding 300 $\mu$M. The $IC_{50}$ value for HAMLET is about 40 $\mu$M. The toxicities of LA-OA complexes resemble that for HAMLET. The $IC_{50}$ values for these complexes (about 50 $\mu$M) and HAMLET differ insignificantly ($p > 0.05$). Meanwhile, the cytotoxic action of HAMLET becomes visible at somewhat lower concentration, about 20−30 $\mu$M.
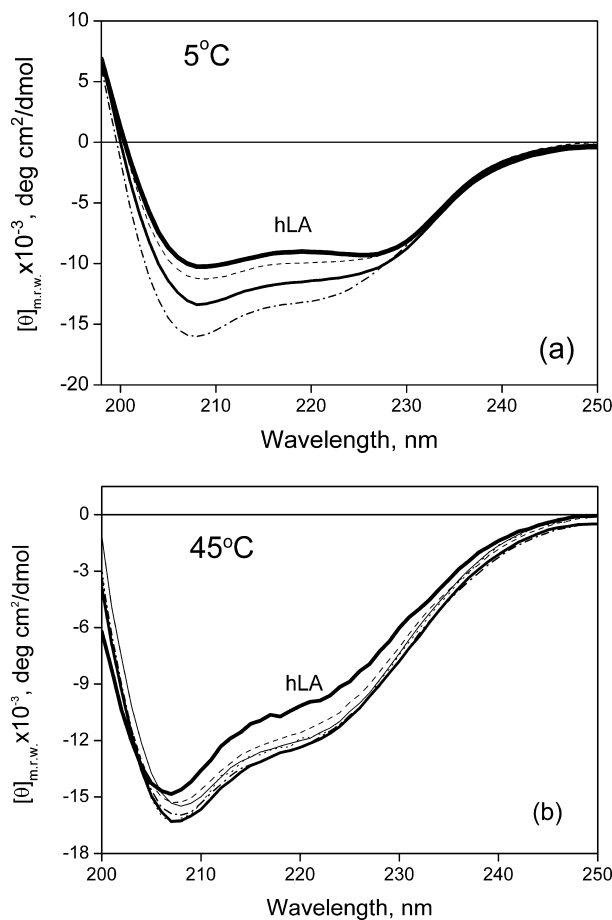


FIGURE 5: Far-UV CD spectra at 5 °C (a) or 45 °C (b) for $Ca^{2+}$-free hLA (thick solid curves), HAMLET (solid curves), LA-OA-17 (dashed curves) and LA-OA-45 (dashed dotted curves) states, and hLA titrated by oleic acid up to CMC at 45 °C (dotted curves) and 17 °C (thin solid curves). Buffer conditions: 20 mM $H_3BO_3$−KOH, 150 mM NaCl, 1 mM EDTA, pH 8.3. Protein concentration was 4 $\mu$M.
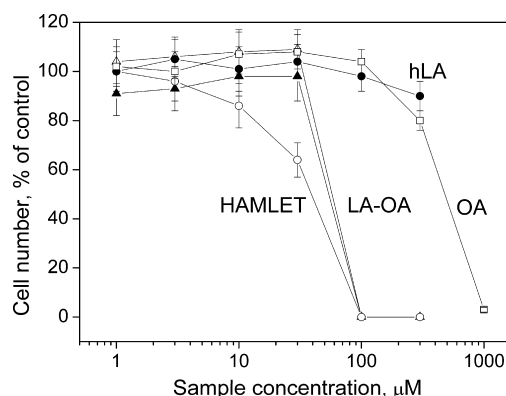


FIGURE 6: Concentration dependence of cytotoxicity against human larynx carcinoma cells, evaluated using the crystal violet assay for hLA−OA complexes and controls: HAMLET (open circles), LA-OA-17 (filled triangles), LA-OA-45 (open triangles), OA (open squares), and intact hLA (filled circles).

## DISCUSSION

The spectrophotometric measurements of CMC of oleic acid (Table 1) have shown that the addition of 1 mM $CaCl_2$ results in about 5-fold narrowing of the region of OA concentrations suitable for studies of interaction between α-lactalbumin and monomeric oleic acid. Moreover, the OA binding capacity of calcium-saturated hLA ($n_{max}$) is negligible

(Table 2), which implies the inability of the $Ca^{2+}$-bound protein to bind OA. This conclusion is supported by the spectrofluorimetric and far-UV CD OA titration experiments, which did not reveal any spectral changes for $Ca^{2+}$-saturated hLA (data not shown). Our data are in line with the earlier report that shows no HAMLET formation with the calcium-bound protein ([20]). At the same time, the NMR data by Fast et al. ([31]) suggest that interaction between the $Ca^{2+}$ form of hLA and OA may occur at pH 7.0 and 37 °C, at OA concentrations above its CMC.

Estimations of CMC of oleic acid (Table 1) and OA binding capacity of hLA (Table 2) show that the most favorable conditions for formation of the complex between hLA and monomeric oleic acid are achieved in the absence of $Ca^{2+}$ at 45 °C in the presence of 150 mM NaCl or KCl. The decrease of temperature down to 17 °C is accompanied with about 40% decrease in OA binding capacity of hLA. Moreover, it results in about a 3-fold decrease in the number of irreversibly bound OA molecules per protein molecule: 9 versus 2.9. The elevation of temperature up to 45 °C causes about an order of magnitude increase in apparent affinity of hLA to OA ($K = 2 \times 10^5$ M$^{-1}$ and $n = 1.3$) in comparison with that at 17 °C ($K = 2 \times 10^4$ M$^{-1}$ and $n = 1.3$). Overall, thermal denaturation of apo-α-lactalbumin results in partial exposure of its hydrophobic residues to the solvent, which seems to promote the binding of hydrophobic molecules of oleic acid. This conclusion is corroborated by the OA-induced decrease in thermal stability of $Ca^{2+}$-free forms of the hLA−OA complexes (Figure 3a). Notably, HAMLET exhibits analogous thermal stability change upon OA association, which similarly suggests that protein unfolding facilitates formation of the complex. The necessity of hLA unfolding for HAMLET formation was shown previously ([20]).

The average number of oleic acid molecules irreversibly bound per HAMLET molecule determined with the aid of gas chromatography and mass spectrometry (from 0.6 to 1.3) ([22]) is much less than that estimated for LA-OA complexes (from 2.9 to 9). The discrepancy between the estimates of $n_{irr}$ for HAMLET and LA-OA could be rationalized by the differences between techniques employed for its determination. Strictly speaking, the measured value of $n_{irr}$ may be overestimated since it includes both the actual number of irreversibly bound OA molecules per protein molecule and some fraction of free OA molecules in solution. Meanwhile, the correct comparison of these two components of $n_{irr}$ value is hardly possible due to the irreversibility of the OA binding process.

Despite markedly decreased thermal stabilities of all apo-hLA−oleic acid complexes, except for LA-OA-17 (Figure 3a), their apparent affinities to calcium ions are just about 2 times lower, which is within the accuracy of the measurements (Table 3). The only exception is the hLA titrated by OA up to its CMC at 17 or 45 °C, exhibiting 0.5−1 order of magnitude lower $K$ values. These data are consistent with the data of Svensson et al. ([21]), who reported that HAMLET at 25 °C is characterized by about 3-fold decreased $Ca^{2+}$ affinity (in the absence of monovalent salts). The more pronounced calcium affinity changes observed in the cases of the hLA saturated by OA are likely due to the competition between protein and oleic acid for $Ca^{2+}$ ions.

For all the hLA−oleic acid complexes studied here, the binding of oleic acid at temperatures above the unfolding transition for the apoprotein causes a slight decrease of mobility and polarity of the environment of emitting tryptophan residues of the $Ca^{2+}$-free hLA, which is consistent with their lower accessibility to the solvent (Figure 3a). This observation is supported by far-UV CD data demonstrating the OA-induced increase in α-helical structure at 45 °C (Figure 5b). It is noteworthy that the stabilization of helical structure of hLA upon OA association is also observed below the unfolding transition temperature for the $Ca^{2+}$-free protein (Figure 5a) despite the fact that fluorescence spectra of the resulting complexes are red shifted (Figure 3a). Similar OA-induced changes of protein secondary structure were previously reported by Polverino de Laureto et al. for bovine α-lactalbumin ([30]). Thus, oleic acid association induces in the $Ca^{2+}$-free hLA the increase in α-helical structure independent of temperature conditions and at the same time makes the polar environment of emitting tryptophan residues of the protein more mobile depending upon the folding state of hLA.

According to the phase plot analysis of spectrofluorimetric and CD data, the processes of oleic acid binding by hLA at 17 and 45 °C pass through at least two and three protein intermediates, respectively (Figure 4a,b). The presence of the intermediates in the course of oleic acid−hLA interaction is in full accordance with our estimates of $n_{max}$ and $n_{irr}$ values. Overall, the process of OA binding by hLA represents a multistage process, involving a number of irreversible steps.

The comparison of physicochemical and structural properties of different hLA−oleic acid complexes presented here shows that the LA-OA-45 state is the most analogous to HAMLET. It has the same thermal stability (Figure 3a,b) and effective calcium affinity (Table 3), demonstrates a similar environment of tryptophan residues, and highly increased, with respect to the intact protein, α-helical structure (Figure 5). The evident difference between LA-OA-45 and HAMLET states is the increased α-helical structure for LA-OA-45, which seems to be related with its higher number of irreversibly bound molecules of OA per protein molecule (9 versus 1).

The intriguing observation is that despite the different number of OA molecules bound per α-lactalbumin molecule, HAMLET, LA-OA-17, and LA-OA-45 complexes possess similar cytotoxic activities against tumor cells (Figure 6).

Taken together, the available experimental data suggest that monomeric oleic acid is able to effectively bind to human α-lactalbumin in aqueous buffer under optimized solvent conditions, giving rise to the LA-OA-45 state, containing multiple irreversibly bound OA molecules per protein molecule. This state resembles HAMLET by combination of physicochemical and cytotoxic properties. Although the LA-OA-45 state exhibits certain structural differences from HAMLET, the similarity of its cytotoxic activity to that of HAMLET makes it suited for further cytotoxic and physicochemical characterization. The data obtained provide better understanding of who Mr. HAMLET is.

It was previously speculated that HAMLET-like molecules can promote lowering the incidence of cancer in breast-fed children by purging of tumor cells from the gut of the neonate ([20]). Indeed, the low pH conditions in the stomach of the breast-fed child favor the release of calcium from α-lactal-

bumin, while the acid lipase hydrolyses triglycerides and fatty acids are released. Thus, a HAMLET-like state of α-lactalbumin could be formed, having altered biological activity. The ability of α-lactalbumin to associate with oleic acid at decreased pH values has been confirmed previously (47). The findings presented here suggest that simple mixing of α-lactalbumin and oleic acid may result in formation of the complex possessing cytotoxic activity with respect to tumor cells, which further supports this idea.

Overall, the conventional specific protocol used for preparation of HAMLET is not an absolute requirement for formation of the active form of hLA−OA complex. The finding that α-lactalbumin−oleic acid complexes possessing HAMLET-like cytotoxic activities may be formed in aqueous solution makes the process of preparation of the active complex more transparent, controllable, thereby opening up the wide opportunities for fine-tuning the presented active complexes for achievement of specific properties. Modification of this procedure by introduction of auxiliary solution components or use of other fatty acids or their derivatives instead of or in addition to oleic acid could be of help in preparing the active LA complexes possessing improved selectivity with respect to particular tumor cell lines or other precious properties.

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