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Electrostatic interactions play an essential role in the binding of oleic acid with α-lactalbumin in the HAMLET-like complex: A study using charge-specific chemical modifications

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ABSTRACT

Human α-lactalbumin made lethal to tumor cells (HAMLET) and its analogs are partially unfolded protein-oleic acid (OA) complexes that exhibit selective tumoricidal activity normally absent in the native protein itself. To understand the nature of the interaction between protein and OA moieties, charge-specific chemical modifications of lysine side chains involving citraconylation, acetylation, and guanidination were employed and the biophysical and biological properties were probed. Upon converting the original positively-charged lysine residues to negatively-charged citraconyl or neutral acetyl groups, the binding of OA to protein was eliminated, as were any cytotoxic activities towards osteosarcoma cells. Retention of the positive charges by converting lysine residues to homoarginine groups (guanidination); however, yielded unchanged binding of OA to protein and identical tumoricidal activity to that displayed by the wild-type α-lactalbumin-oleic acid complex. With the addition of OA, the wild-type and guanidinated α-lactalbumin proteins underwent substantial conformational changes, such as partial unfolding, loss of tertiary structure, but retention of secondary structure. In contrast, no significant conformational changes were observed in the citraconylated and acetylated α-lactalbumins, most likely because of the absence of OA binding. These results suggest that electrostatic interactions between the positively-charged basic groups on α-lactalbumin and the negatively-charged carboxylate groups on OA molecules play an essential role in the binding of OA to α-lactalbumin and that these interactions appear to be as important as hydrophobic interactions.

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Key words: HAMLET/BAMLET; protein-fatty acid complex; electrostatic interaction; charge-specific; chemical modification; lysine; protein unfolding; partially unfolded/folded; α-lactalbumin; oleic acid/oleate.

INTRODUCTION

α-Lactalbumin is a well-studied small (M_r = 14,200, 123 residues), acidic (pI 4–5), globular, Ca^{2+}-binding milk protein.[1] Upon partial unfolding and binding of oleic acid (OA), a complex named HAMLET/BAMLET (Human or Bovine Alpha-lactalbumin Made LEthal to Tumor cells) is formed that has proven to be effective against over 40 tumor cell lines while leaving healthy, differentiated cells intact.[2–7] Details of the preparation, characterization, and tumoricidal activity of HAMLET/BAMLET can be found in various comprehensive reviews.[8–11] Though this very special feature of the α-lactalbumin-oleic acid complex has attracted wide interest, the mechanisms of the interaction between α-lactalbumin and OA as well as the origin of their stoichi-

Additional Supporting Information may be found in the online version of this article.

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ometry in the complex are still poorly understood at the molecular level. It has been generally accepted that the interaction between OA and α-lactalbumin is mostly mediated by hydrophobic interactions[12,13] due to the fact that apo-α-lactalbumin, which is the form used in the original chromatographic preparation of the α-lactalbumin-oleic acid complex,[3] is more hydrophobic than the holo form.[14,15] In other words, removing Ca^{2+} ions by dialyzing against ethylenediaminetetraacetic acid (EDTA) to make the apo form, which induces a partially unfolded state and allows hydrophobic sites to be exposed, is believed to favor the interaction between α-lactalbumin and OA and hence the formation of the HAMLET-like complex. Interestingly, the classical studies of Luck and Naismith have predicted that interaction among molecules carrying similar net negative charges can take place provided that a sufficient number of positively-charged basic groups (i.e. the ε-amino group of lysine or the guanidinium group of arginine) remain to provide primary binding sites for the negatively-charged oleate anions.[16,17] Interestingly, Spolaore *et al.* have proposed that the association between α-lactalbumin and OA is likely to be mediated by both electrostatic and hydrophobic interactions,[18] however, no specific experimental evidence had been given in their work. In relation to this proposal, there have been discrepancies in the reported stoichiometries between OA and α-lactalbumin when the complex has been prepared by different methods. For example a stoichiometry of 1:5.1–1:5.4 between α-lactalbumin and OA was measured in the HAMLET complex[19] prepared by the original chromatographic method.[3,19,20] Whereas, upon mixing the two components at 17°C or 45°C, the stoichiometry reported was 1:2.9 and 1:9, respectively.[21] Spolaore *et al.* have found that the α-lactalbumin-oleic acid complex appears to be a high molecular weight complex mainly composed of 4–5 protein molecules that associate with 68–85 molecules of OA.[18] Why does the binding ratio between OA and α-lactalbumin differ so much when the α-lactalbumin-oleic acid complex is made with different methods? There has been no clear answer to date. Here, we employ charge-specific chemical modification of the lysine residues on bovine α-lactalbumin (BLA) via citraconylation, acetylation, and guanidination with the aim of elucidating whether electrostatic interactions between the positively-charged basic groups (mainly lysine and arginine side chains) on α-lactalbumin and negatively-charged carboxylate groups on OA molecules are essential for the binding. If electrostatic interactions between the positively-charged basic groups on the proteins and the negatively-charged carboxylate groups on the OA molecules are essential in forming the complex, the binding of OA to α-lactalbumin will be influenced by the experimental conditions (e.g., pH, ionic strength, temperature etc.). These conditions will influence the respective charged states of the proteins and the association/dissociation state of OA molecules in solution and thereby influence the binding of OA to α-lactalbumin.

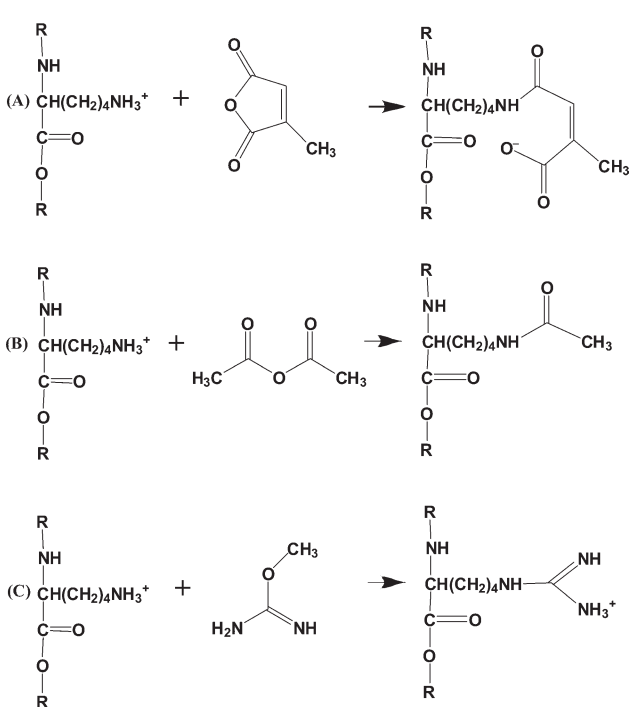

Figure 1

Chemical modification reactions applied to the lysine side-chains of BLA protein. (**A**) citraconylation with citraconic anhydride; (**B**) acetylation with acetic anhydride; (**C**) guanidination with O-methylisourea.

The chemical modification of a protein involves attaching or cleaving chemical groups in order to alter the solubility and/or other properties of the original molecule. It is commonly used to identify those residues at active sites, which contribute to substrate binding or chemical catalysis.[22] Charge-specific chemical modifications were employed in this study by using citraconic anhydride, acetic anhydride, and O-methylisourea as specific chemical modification reagents (Fig. 1). Citraconylation (the reaction product called citraconyl-BLA) introduces negatively-charged citraconyl groups in place of the positively-charged amino groups on the side chains of the lysine residues in BLA. This chemical modification is reversible under mild acidic conditions, restoring the positive charges to those of the wild type BLA.[23–25] Acetylation (the product named acetyl-BLA) converts the positively-charged lysine side chains to neutral acetyl groups, decreasing the net positive charge of the BLA molecular surface.[26,27] Guanidination (the product named guanidinated-BLA) converts lysine residues to homoarginine residues with retention of positive charges.[26,28] Following each charge-specific chemical modification of the lysine residues, the extent of OA binding was studied in detail through the use of a variety of techniques including ion exchange chromatography, ^{1}H nuclear magnetic resonance (NMR) spectroscopy, circular dichroism (CD) spectropolarimetry, intrinsic and

ANS-binding extrinsic fluorescence. In addition, the tumoricidal activity of the resulting protein or protein complexes were measured on the human osteosarcoma cell line U2OS. These experiments helped to clarify the relationship between the properties of the side chains of lysine residues of BLA and their ability to bind OA.

MATERIALS AND METHODS

Materials

Calcium depleted α-lactalbumin from bovine milk (Type III) was purchased from Sigma (Sigma-Aldrich, St. Louis, MO) and was used without further purification. Protein concentrations were evaluated either spectrophotometrically using an extinction coefficient $E^{1\%}_{1cm} = 20.1$ for bovine α-lactalbumin at 280 nm[29] or the protein assays using bicinchoninic acid.[30]

OA 200 mg (~224 μL) was dissolved in 10 mL of ethanol by vortexing, 1.0 mL of which was added to 79.0 mL 10 m*M* Tris/HCl buffer, pH 8.5 by sonication to obtain an OA stock solution (concentration of OA: ~0.885 m*M*), which was always freshly prepared prior to use, and aliquots were added to experimental samples to obtain the desired OA concentration. The final concentration of ethanol never exceeded 1.0% under all the experimental conditions.

Citraconic anhydride, acetic anhydride, O-methylisourea, OA ($C_{18:1:9cis}$), 1-anilino-8-naphthalenesulfonate (ANS), 2, 4, 6-trinitrobenzene sulfonic acid (TNBS), and urea were purchased from Sigma. Pierce® BCA™ protein assay kit was purchased from Thermo scientific. NuPAGE® Novex® Bis-Tris and Tris-Glycine Mini Gels and related sample buffer, running buffer, protein standards were purchased from Invitrogen life technologies. Dulbecco's modified eagle medium (DMEM) + GlutaMAX™-1 (Gibco-BRL cat. no. 61965-026), Dulbecco's phosphate buffered saline (DPBS) (Gibco-BRL cat. no. 14190-094) and trypsin-EDTA (Gibco-BRL cat. no. 25300-054) were purchased from Life Technologies (Karlsruhe, Germany). Alamar blue (Invitrogen cat. no. DAL1025) was purchased from Mfg (Camarillo, CA 93012). All other chemicals were of guaranteed reagent grade or better.

Citraconylation

Positively-charged lysine residues were converted to negatively-charged citraconyl groups using citraconic anhydride by following the procedure described by Dixon and Perham[23] and Habibi *et al.*[24] with some amendments. Briefly, the protein concentration was 4.0 mg/mL in 10 mL of 100 m*M* phosphate buffer (pH 8.0) and the procedure was followed, at room temperature, by the step-wise addition of 3 μL aliquots of citraconic anhydride, while maintaining the pH of the stirred solution by adding 2*M* NaOH. After ensuring a constant pH, the reaction was completed in about 1 h and the sample was removed from the solution and dialyzed against 20 m*M* phosphate buffer, pH 7.5 at 4°C. Highly citraconylated BLA was further purified by ion exchange chromatography with a Hiprep™ DEAE FF column (GE Healthcare) on an Ultimate 3000 HPLC unit (Dionex). The column was equilibrated with 10 m*M* Tris/HCl buffer, pH 8.5, and the protein was eluted with a linear gradient to 1.0*M* NaCl. If necessary, the modified protein was further purified by gel filtration chromatography with a HiPrep™ 16/60 Sephacryl™ S-200 HR column (GE healthcare). The column was equilibrated and protein eluted with 10 m*M* Tris/HCl buffer, pH 8.5, containing 0.15*M* NaCl. The purified samples were dialyzed against distilled water and then lyophilized. The number of modified lysine residues was measured using the TNBS method described below.

Decitraconylation

Regeneration of the modified amino groups was carried out by following the procedure described by Dixon and Perham[23] and Batra[25] with some amendments. Briefly, citraconyl-BLA was dialyzed against 2 L of 20 m*M* sodium phosphate buffer (pH 3.5) at room temperature for 24 h with one buffer change. Subsequently, the solution was dialyzed against 2 L of 20 m*M* phosphate buffer, pH 7.5, at 4°C with one buffer change. Regenerated BLA was further purified by ion exchange or gel filtration chromatography as described above and then dialyzed against distilled water and lyophilized. The number of modified lysine residues remaining was measured using the TNBS method.

Acetylation

Acetylation of BLA by acetic anhydride was performed by the procedure of Masuda *et al.*[26] and Yamasaki *et al.*[27] with some amendments. Briefly, the protein concentration was 4.0 mg/mL in 10 mL of half-saturated sodium acetate in 100 m*M* phosphate buffer (pH 8.0) and the modification conducted at 0–4°C by the step-wise addition of 3 μL aliquots of acetic anhydride, while maintaining the pH of the stirred solution by additions of 2*M* NaOH. The reaction was completed in about 1 h and the sample was removed from the solution and dialyzed against distilled water at 4°C. Highly acetylated BLA was further purified by ion exchange or gel filtration chromatography as described above and then dialyzed against distilled water and lyophilized. The number of modified lysine residues was measured with the TNBS method.

Guanidination

Guanidination of BLA using O-methylisourea was performed using the procedure of Masuda *et al.*[26] and Cupo *et al.*[28] with some amendments. The solution of O-methylisourea was prepared by dissolving 0.54 g in

water to make a total volume of 5 mL and the pH was adjusted to 10.6 by addition of 5*M* NaOH (final O-methylisourea solution concentration: 0.6*M*). The protein concentration was 8.0 mg/mL in 5 mL of the O-methylisourea solution. The guanidination reaction was completed by stirring at 23 ± 2°C for 4 h. The reactant was precipitated by adding 4*M* HCl (to pH 3.0–4.0). After centrifugation, the precipitate was dissolved in water (using 2*M* NaOH to adjust the pH to 7.0 if necessary) and dialyzed against distilled water at 4°C. Highly guanidinated BLA was further purified by ion exchange or gel filtration chromatography, dialyzed against distilled water, and then lyophilized. The number of modified lysine residues was measured with the TNBS method.

Lysine assay (TNBS method)

The number of free lysine groups in each protein was measured with the method of Fields[31] and Couch and Thomas[32] with some amendments. Briefly, 1.0 mL of 0.1% (w/v) TNBS in 100 m*M* $NaHCO_3$ at pH 8.5 (freshly prepared prior to use) was added to 1.0 mL of 2.5 mg/mL protein solution also in 100 m*M* $NaHCO_3$ (pH 8.5) and mixed well. The solution was incubated at 37°C for 2 h and then 1.0 mL of 10% SDS (sodium dodecyl sulfate) was added followed by 0.5 mL of 1.0*M* HCl to stop the reaction. The absorbance of the solution, diluted with 0.01*M* HCl if necessary, was read at 340 nm against a blank treated as above but without protein. The number of free amino groups was calculated from the calibration curve of the absorbance at 340 nm for protein-bound TNBS ($\varepsilon_{340\ nm}$ = 11,900 M^{-1} cm^{-1}).[33] A calibration curve C = 848.14 A – 8.7166 (R^2 = 0.9976) was generated, where C is the concentration of the free lysine residues (μM), and A is the absorbance of the measured solution at 340 nm.

^{1}H NMR spectroscopy

One-dimensional ^{1}H NMR spectra of all the samples were acquired on a Bruker Avance DRX-600 NMR spectrometer employing a cryo probehead with inverse ^{1}H detection and pulsed field gradient capabilities. Lyophilized samples (ca. 10 mg) were dissolved in 600 μL 10 m*M* sodium phosphate buffer in D_2O, pH 7.4, at a final sample concentration of 1.0 m*M*. 1,4-Dioxane was used as an internal standard reference (chemical shift: 3.75 ppm). Topspin 2.1 (Bruker Corporation) and MestReNova 5.3.2 (Mestrelab research, Spain) were used for processing and analysis of NMR data.

The signal of the olefinic protons (protons attached to carbon 9/10) of OA (chemical shift: 5.33 ppm) was used to measure the bound OA.[34] The stoichiometry of protein and OA was calculated by comparing the peak area of the aromatic region of BLA (8.5–6.5 ppm) to that of the protons on carbon 9/10 of OA[19,34].

Circular dichroism

CD measurements were performed at room temperature on a JASCO J-815 spectropolarimeter (Tokyo, Japan). Near-UV CD spectra (320–250 nm) were acquired at a protein concentration of 0.05 mg/mL (3.5 μ*M*) with or without OA using a 1.0 cm path length quartz cell. For spectral measurements in the far-UV region (250–190 nm), the protein solutions were adjusted to a concentration of 0.1 mg/mL (7.0 μ*M*) with or without OA in a cuvette with a 0.1 cm path length. Solutions containing the appropriate molar ratios of OA were used as blanks and subtracted from the data for each sample. All solutions were prepared in 10 m*M* Tris/HCl buffer, pH 8.5 or 0.01*M* HCl (pH 2.0). The CD spectra measurements were conducted at 25°C and averaged over four scans. The mean residue ellipticity $[\theta]_{MRW}$ (deg cm^2 $dmol^{-1}$) was calculated using the formula $[\theta]_{MRW} = (\theta_{obs}/10)(MRW/lc)$, where θ_{obs} is the observed ellipticity in deg, MRW is the mean residue molecular weight (116 Da for apo-BLA, 125 Da for citraconyl-BLA, and 120 Da for acetyl-BLA and guanidinated-BLA, respectively), l is the light path length in cm, and c is the protein concentration in g/mL.

Fluorescence studies

Fluorescence measurements were performed at room temperature on a JASCO FP-6200 spectrofluorometer (Tokyo, Japan). Samples were prepared in 10 m*M* Tris/HCl buffer, pH 8.5 or 0.01*M* HCl (pH 2.0). Protein solutions with or without OA had a final protein concentration of 3.5 μ*M*. Intrinsic fluorescence emission experiments were performed for each concentration of OA (excitation at 280 nm and emission scan range from 310 to 400 nm with a slit width of 5 nm). For the ANS-binding extrinsic fluorescence measurements, ANS was added to a final concentration of 10 μ*M* and the fluorescence intensity measured by exciting at 390 nm with an emission range from 410 to 600 nm (slit width 10 nm). Spectral measurements were additionally acquired for each respective OA concentration range in the absence of protein and subtracted from the spectra acquired from protein-containing samples. All fluorescence measurements were averaged over four scans.

Equilibrium unfolding transitions of wild-type and modified BLA by chemical denaturant

The equilibrium unfolding experiments of wild type BLA, guanidinated-BLA, citraconyl-BLA, and acetyl-BLA were induced by urea in 10 m*M* Tris/HCl buffer, pH 8.5, and followed by intrinsic fluorescence intensity at 350 nm (excitation wavelength: 280 nm) on a JASCO FP-6200 spectrofluorometer (Tokyo, Japan). The final protein concentration was about 5.0 μ*M*. The transition curves were analyzed by a three-state model, in which

the native, intermediate, and unfolded states are populated in the transition region.[35]

Cytotoxicity tests

The human osteosarcoma cell line U2OS was used for testing the tumoricidal activity of both wild-type and modified BLA protein-OA complexes. The cells were cultured in DMEM+ GlutaMAX^{TM}-1 with added 1% penicillin and streptomycin and 10% heat-inactivated fetal bovine serum. Adherent cells were detached with trypsin-EDTA. The cells were washed in Dulbecco's phosphate buffered saline (DPBS), harvested by centrifugation (1000 rpm, 5 min), and resuspended in DMEM. The cells were seeded into 96-well plates at a density of 5×10^3 cells/well, and incubated at 37°C in 5% CO_2 atmosphere for 24 h before being treated with protein-OA complexes. The lyophilized protein-OA complexes were dissolved at a concentration of 10 mg/mL in distilled water and added to wells at a final concentration between 0.001 and 1.0 mg/mL. The plates were then incubated at 37°C for a further 24 h treatment. Alamar blue was used to test the viability of the cells (10 µL/well). Plates in triplicate were incubated for 4 h at 37°C protected from direct light, then read at 590 nm using an excitation wavelength of 544 nm in a fluorescence plate reader (Spectra Max Gemini). Wells containing medium and distilled water-only served as blank controls, while the viability of the treated cells was taken as a percentage as compared to wells with untreated cells. The LD_{50} value of each protein-OA complex was estimated by fitting the correlation between cells viability and protein concentration or OA concentration. The OA concentration in each complex was calculated from the stoichiometry of OA obtained by the integrated ^1H area of the olefinic protons in the NMR spectra of the complexes (see above).

Schematic representation of α-lactalbumin

The solvent accessible surface areas (SASAs) and the relative surface accessibility (%) of the lysine and arginine residues in bovine apo-lactalbumin [Protein Data Bank (PDB) code 1F6R[15]] were determined with the POPS (Parameter OPtimised Surfaces; version 1.3.2) program.[36,37] The three-dimensional molecular model of bovine apo-α-lactalbumin was prepared using MOE (Molecular Operating Environment; version 2009.10; Chemical Computing Group. Montreal, Canada).

RESULTS

Characterization of charge-specific chemically modified and wild-type bovine α-lactalbumin

The role played by the positively-charged basic amino acid side chains of bovine α-lactalbumin in binding OA was investigated by their charge-specific chemical modifications (Fig. 1). Citraconylation converts the originally positively-charged lysine residues to negatively-charged citraconyl groups, resulting in a significantly different retention time than the wild-type BLA protein [Fig. 2(A,B)] using anion exchange chromatography. A significant advantage of this modification is its reversibility under mild acidic conditions to regenerate the wild-type BLA. Acetylation converts the positively-charged lysine side-chain amino groups to neutral acetyl groups, decreasing the net positive charges of the BLA molecular surface. As shown in Figure 2(D), guanidination converts

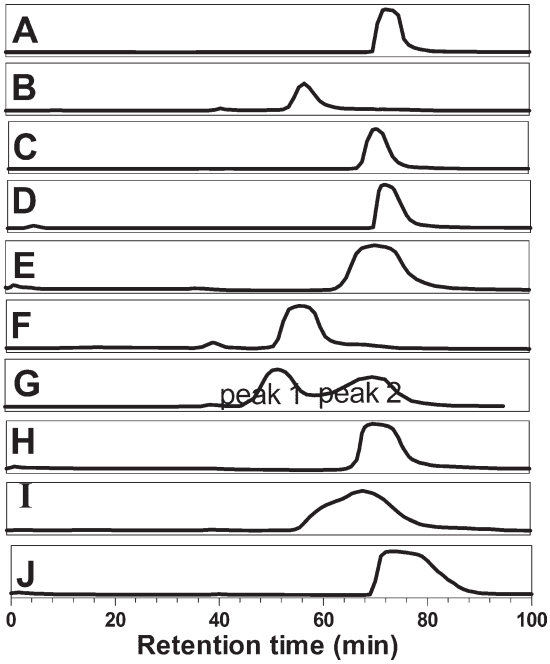

Figure 2

Anion-exchange chromatograms of BLA protein, its modified derivatives, and the corresponding protein-OA complexes. An anionic exchange column (DEAE FF column) was used and preconditioned with 200 mg OA. Thirty milligram protein was loaded thereafter. A 0–1.0*M* NaCl linear gradient was applied over 10 bed volumes between 30 and 80 min. Detection was at 280 nm. For chromatograms A, B, C, and D, 10 mg protein was loaded onto a DEAE FF column without preconditioning with OA. From top to bottom: (**A**) apo-BLA; (**B**) citraconyl-BLA; (**C**) acetyl-BLA; (**D**) guanidinated-BLA; (**E**) BLA-OA complex (BAMLET) with wild-type BLA protein was used; (**F**) citraconyl-BLA-OA complex made with citraconyl-BLA with 11.6 lysine residues blocked; (**G**) partially regenerated citraconyl-BLA was used (Citraconyl-BLA was dialyzed against 2 L of 20 m*M* sodium phosphate buffer (pH 3.5), at room temperature for 3 h, and then dialyzed against 2 L of 20 m*M* phosphate buffer, pH 7.5); (**H**) the completely regenerated BLA was used (Citraconyl-BLA was dialyzed against 2 L of 20 m*M* sodium phosphate buffer (pH 3.5), at room temperature for 24 h with buffer changed once, and then dialyzed against 2 L of 20 m*M* phosphate buffer, pH 7.5. The highly regenerated BLA was further purified by liquid chromatography. 10.9 lysine residues of the total 12 lysine were regenerated according to TNBS method); (**I**) acetyl-BLA-OA complex made with acetyl-BLA with 11.5 lysine residues blocked; (**J**) guanidinated-BLA-OA complex made with guanidinated-BLA with 10.9 lysine residues blocked.

the lysine residues to homoarginine residues, yielding an identical number of positive charges with no noticeable effect on the retention time of the guanidinated-BLA protein when compared with wild-type BLA. Care was taken with each chemical modification method to find the optimal [reagent] to [protein] ratios to ensure uniform modification of the protein. At least 11 ε-amino groups of lysine residues (without further purification) were easily modified by the three different chemical modification reagents (when [reagent]: [BLA] = 50:1). The results of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of modified and wild-type bovine α-lactalbumin proteins demonstrated that the charge-specific chemical modifications to which the proteins were subjected did not significantly alter the molecular weights observed (i.e., no crosslinking occurred). Furthermore, native polyacrylamide gel electrophoresis (native-PAGE) showed the anticipated mobility shift resulting from the altered pI (data not shown). Variation of the molar ratios of citraconic anhydride reagent to BLA produced different extents of modification. For example, at a [citraconic anhydride] to [BLA] molar ratio of 10:1, an average of 7 lysine residues were modified as assessed by the TNBS method; however, the modification was heterogeneous for different lysines (as shown in Fig. S1 of the Supporting Information), making further analysis of the protein and its OA complex impractical.

Effects of charge-specific modification of lysine residues on OA binding

The effects of citraconylation, acetylation, and guanidination of the lysine residues of BLA on OA binding were tested using complexes prepared on an OA-preconditioned DEAE-Sepharose ion exchange column with a linear NaCl gradient to elute the complexes.[3,19,20] On this type of column both ion exchange and gel filtration behavior can influence the elution time, and consequently, care must be taken in interpreting the results. When wild-type BLA protein was applied, the BLA−OA complex (named BAMLET) peak eluted at ∼0.8*M* NaCl [Fig. 2(E)] and served as the reference complex. Bound OA molecules could be detected in BAMLET based on the ^{1}H NMR spectrum [Fig. 3(F)].[19,34] In contrast, when the citraconyl-BLA protein was used with all 12 lysine residues blocked (the modified citraconyl-BLA protein was further purified by ion exchange and gel filtration chromatography and then quantified with the TNBS method before use), the citraconyl-BLA−OA complex peak eluted earlier [Fig. 2(F)], and no OA binding to the citraconyl-BLA protein was detected by ^{1}H NMR [Fig. 3(G)]. Because the citraconyl-BLA is more negatively charged than the wild type protein, it would be expected to have a longer retention time on an anion exchange column. However, the elution time was significantly shorter than that observed for the wild-type protein (cf. Fig. 2(A,B)]. A possible reason for this anomalous behavior might have been because of the introduction of the negatively-charged citraconyl groups into the protein, which might cause the protein to expand and producing a shorter retention time. When partially regenerated citraconyl-BLA protein was applied, two peaks eluted from the ion exchange chromatography [Fig. 2(G)]. Peak 1 [Fig. 2(G)], corresponding to citraconyl-BLA, eluted much earlier and exhibited no bound OA [Fig. 3(H)], while peak 2, corresponding to BLA-OA complex, appeared later and exhibited OA binding [Fig. 3(I)]. When applying the completely regenerated citraconyl-BLA protein, the resulting regenerated citraconyl-BLA-OA complex peak eluted at the same time as that of the wild-type BLA-OA complex [Fig. 2(H,E)] and regained all of its OA binding capacity [Fig. 3(J)]. When the acetyl-BLA protein was used [Fig. 2(I)], no OA binding was detected in the ^{1}H NMR spectrum [Fig. 3(K)]. In remarkable contrast, when the guanidinated-BLA [Fig. 2(J)] was used, protein-bound OA was detected [Fig. 3(L)]. For comparison purposes, wild-type BLA protein, citraconyl-BLA protein, regenerated citraconyl-BLA pro-

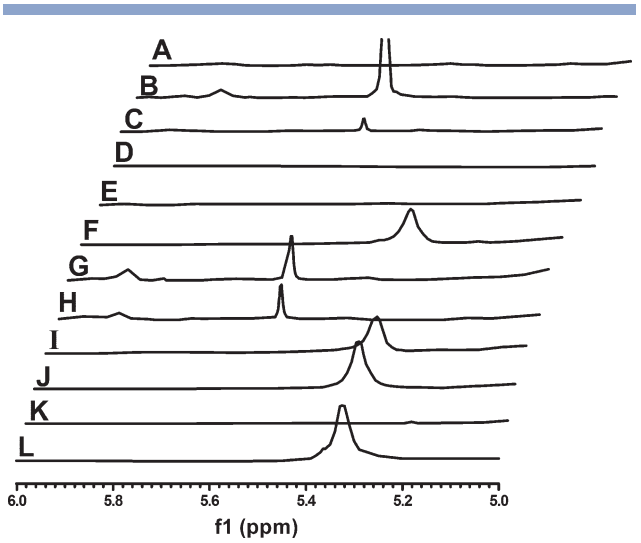

Figure 3

^{1}H NMR spectra of the olefinic proton region of oleic acid for wild-type and modified BLA protein, and protein-OA complexes (chemical shift region: 6.0–5.0 ppm). The concentrations of the proteins are approximately 1 m*M*. From top to bottom, (**A**) wild-type BLA; (**B**) citraconyl-BLA (with 11.6 lysine residues blocked); (**C**) fully regenerated citraconyl-BLA (with 1.1 lysine residues blocked); (**D**) acetyl-BLA (with 11.5 lysine residues blocked); (**E**) guanidinated-BLA (with 10.9 lysine residues blocked); (**F**) BLA-OA complex (BAMLET) made with wild-type BLA [see Fig. 2(E)]; (**G**) citraconyl-BLA-OA complex made with citraconyl-BLA (with 11.6 lysine residues blocked) [see Fig. 2(F)]; (**H**) partially regenerated citraconyl-BLA-OA complex [see peak 1 in Fig. 2(G)]; (**I**) partially regenerated citraconyl-BLA-OA complex [see peak 2 in Fig. 2(G)]; (**J**) BLA-OA complex made from completely regenerated citraconyl-BLA [see Fig. 2(**H**)]; (**K**) acetyl-BLA-OA complex [see Fig. 2(I)]; (**L**) guanidinated-BLA-OA complex [see Fig. 2(J)].

tein, acetyl-BLA protein, and guanidinated-BLA protein [Fig. 3(A–E), respectively] were included in the ^{1}H NMR spectra as well. The sharp peak at the chemical shift around 5.50 ppm [as shown in Fig. 3(B,C,G, and H), respectively] corresponds to the olefinic C_3 proton on the citraconyl groups of the citraconyl-BLA protein and can be easily separated from the OA peak at the chemical shift of 5.33 ppm. The ^{1}H NMR spectral regions, showing the aromatic peaks of the proteins, the internal reference standard and other protons attached to methyl groups are shown in Figures S2, S3, and S4, respectively, in the Supporting Information.

Effects of protein modification on OA–protein interaction studied by CD

CD spectral measurements were performed in order to study further the conformational changes of the proteins, induced by adding OA, as well as the differences in OA-binding to the wild-type and modified BLA proteins.

The far-UV CD spectra for the wild type and modified BLA proteins in the absence and presence of different concentrations of OA, illustrated in Figure 4, show the typical features of α-helical protein structure, in which the 222 nm dichroic band is predominantly associated with α-helical n–π* amide transitions, and the 208 nm dichroic band corresponding to the π–π* amide transitions.[38] When the OA concentration was increased (from absence of OA to [OA]: [protein] = 50:1), the negative mean residue ellipticity ($[\theta]_{MRW}$) values of wild-type BLA and guanidinated-BLA at 208 and 222 nm increased, indicating an increase of α-helix content in the secondary structure of the proteins. Upon further increase of the molar ratio of OA (to 65:1, 80:1, and then 100:1), no substantial changes were observed (as shown in Fig. S5 of the Supporting Information). Further quantitative calculations of α-helix and β-strand content using the program K2D3[39] confirmed the trends in the data (as shown in Fig. S6 of the Supporting Information). In the absence of OA, the contents of α-helix were calculated to be 22.22% and 25.78%, respectively for the wild-type and guanidinated-BLA. The α-helix content increased gradually with the increase of OA concentration, up to ~ 33% at [OA]: [protein] = 25:1. Persistent secondary structure was observed at all higher concentrations of OA. The spectra of both the wild-type BLA and the guanidinated-BLA proteins, when subjected to conditions inducing the acidic molten globule state (the A-state[40,41]) in 0.01*M* HCl (pH 2.0), qualitatively resembled that of the spectra of [OA]: [BLA]= 5:1 and [OA]:[guanidinated-BLA] = 25:1, respectively. In stark contrast, there was no substantial change of the negative mean residue ellipticity values at 222 and 208 nm wavelengths observed in the cases of citraconyl-BLA and acetyl-BLA, regardless of the addition of OA. Hence, the far-UV CD spectral measurement experiments demonstrated that OA has little effect on the secondary structure of citraconyl-BLA and acetyl-BLA.

The minimum intensities at 270–280 nm wavelengths range in the near-UV CD spectra characterize the asymmetry of the microenvironment around aromatic side chains: a reduction in signal intensity indicates an increase of mobility and gradual loss of rigid tertiary structure. Following the increase of OA concentration, the negative mean residue ellipticity values of wild-type BLA within the 270–280 nm wavelengths region remarkably diminished and then disappeared completely as shown in Figure 5(A), indicating the loss of protein tertiary structure. Spectral stabilization appeared to occur when [OA]: [BLA] = 50:1, and further increases in the molar ratio to 65:1, 80:1, and then 100:1 did not induce further changes in the near-UV CD spectra (spectra not shown). The wild-type BLA protein structure appeared to be partially unfolded by the addition of OA and at [OA]:[BLA] = 50:1 and resembled that of the classical molten globule induced by 0.01*M* HCl (pH 2.0). The tertiary structural changes of guanidinated-BLA [Fig. 5(B)] appeared to undergo the same trend as that of the wild-type BLA, resembling the spectrum induced by 0.01 M HCl (pH 2.0) when [OA]: [guanidinated-BLA] = 50:1. The results of near-UV CD of wild-type BLA and guanidinated-BLA indicated that the proteins gained more mobility and lost tertiary structure gradually following the increase in OA concentration. In contrast, there were no significant changes in the near-UV CD spectra of citraconyl-BLA as a function of OA concentration [Fig. 5(C)]. In fact the original citraconyl-BLA spectrum persisted throughout, suggesting little structural perturbation caused by the addition of OA. It was notable that the spectrum for citraconyl-BLA induced by 0.01*M* HCl (pH 2.0) appeared similar to the classical A-state molten globule. We were careful to ensure that the time involved in the measurement of the low-pH condition was much shorter than that for the regenerating citraconyl-BLA; therefore, any effect of low-pH to bring about chemical modifications was negligible. It was apparent that the acetyl-BLA protein itself displayed a qualitatively different near-UV CD spectrum from the spectra of the other proteins, and furthermore no changes could be observed when the OA concentration was increased or the protein placed in 0.01*M* HCl solution (pH 2.0) [Fig. 5(D)].

Protein-OA interactions studied by fluorescence

The structural perturbations of the wild-type and modified BLA proteins caused by the addition of OA in the sample solutions were monitored with intrinsic and ANS-binding extrinsic fluorescence.

When the OA concentration was increased, the intrinsic fluorescence intensity of the wild-type BLA increased accordingly but with little change in the position of the peak maxima [λ_{max} = 342–343 nm; excitation wavelength: 280 nm; Fig. 6(A)]. With a molar ratio of

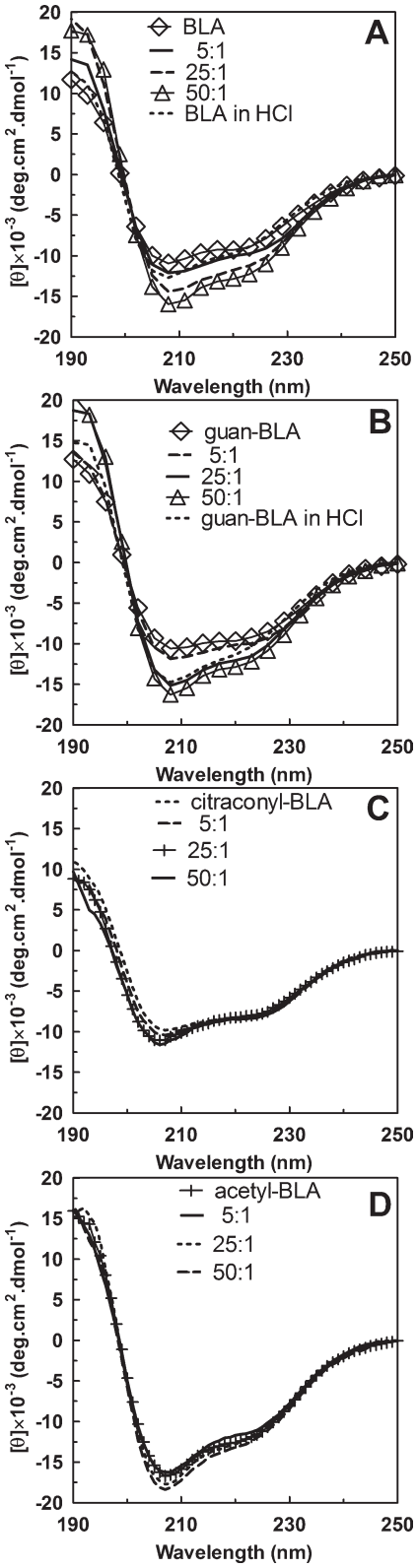

Figure 4

Far-UV CD spectra of (**A**) BLA, (**B**) guanidinated-BLA, (**C**) citraconyl-BLA, and (**D**) acetyl-BLA in the absence and presence of different [OA] to [protein] molar ratios at pH 8.5 or separately in 0.01*M* HCl (pH 2.0) with the final protein concentration at about 7.0 μ*M*.

[OA]:[BLA] = 5:1–10:1, the fluorescence spectrum became very similar to that of the protein in the molten globule state induced by 0.01*M* HCl (pH 2.0). For guanidinated-BLA [Fig. 6(B)], the spectral change of the intrinsic fluorescence appeared to be more significant than that of the wild-type BLA, with a λ_{max} red shift from 333 nm in the absence of OA to 342–343 nm in the presence of 25-fold or greater OA, accompanied with a more significant intensity increase. The fluorescence intensity of guanidinated-BLA induced by 0.01*M* HCl (pH 2.0) was very similar to that when the molar ratio of OA to guanidinated-BLA was increased, [OA]:[guanidinated-BLA] = 10:1, but with a significant longer wavelength shift (λ_{max} = 343 nm). In contrast to wild-type BLA and guanidinated-BLA, little or no perturbations were observed for citraconyl-BLA and acetyl-BLA [Fig. 6(C, D)]. The total intrinsic fluorescence intensity of citraconyl-BLA was significantly lower than those of its counterparts [Fig. 6(C)]. However, a slight spectral maxima (λ_{max}) shift from 336 nm for citraconyl-BLA in the absence of OA to 342–343 nm for [OA]:[citraconyl-BLA] =100:1 was observed. For both citraconyl-BLA and acetyl-BLA, the perturbations caused by the presence of OA in the sample solutions were much less than placing them under conditions where they adopted the acidic molten globule state (0.01*M* HCl) [Fig. 6(C,D)].

ANS fluorescence is accompanied by an increased intensity when the reporter group is exposed to a more hydrophobic environment, and this behavior is used to probe partially unfolded proteins, especially their exposed hydrophobic cores or pockets. Both wild-type BLA and guanidinated-BLA displayed prominent increases in ANS-bound fluorescence that was at its respective maxima up to [OA]:[protein] = 10:1, followed by a decrease at higher molar ratios. [Fig. 7(A,B); excitation wavelength: 390 nm]. In contrast, the ANS-bound fluorescence intensity increase for acetyl-BLA was substantially less [Fig. 7(C)]. Interestingly, the total ANS-binding extrinsic fluorescence intensity of citraconyl-BLA excited at 390 nm was nearly identical to that of the ANS-OA-only control (as shown in Fig. S7 of the Supporting Information), suggesting that citraconyl-BLA did not bind to ANS at all. The spectral intensity of the proteins in the classical molten globule state induced by 0.01*M* HCl (pH 2.0) was significantly larger such that the excitation and emission slit widths had to be reduced (result not shown). The quenching of ANS fluorescence itself as a function of increasing OA concentration in the solvent was ruled-out based on control experiments [Fig. 7(D)].

Tumoricidal activity of protein-OA complexes

The cytotoxicity of the protein-OA complexes was tested on a U2OS cell line *in vitro* after 24 h incubation and quantified with Alamar blue staining. Tumoricidal

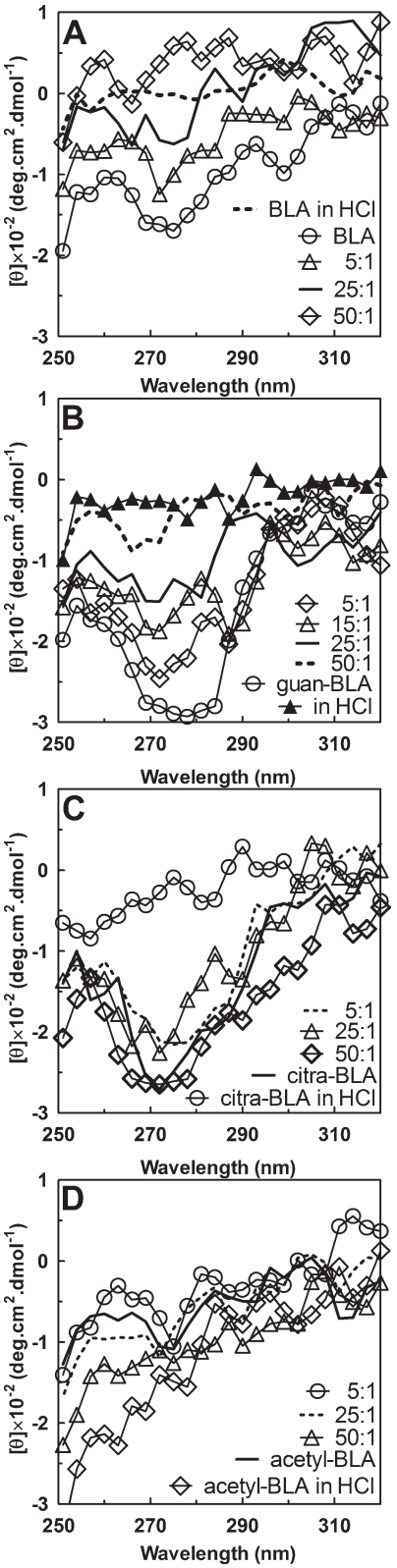

Figure 5

Near-UV CD spectra of (**A**) BLA, (**B**) guanidinated-BLA, (**C**) citraconyl-BLA, and (**D**) acetyl-BLA in the absence and presence of different [OA] to [protein] molar ratios at pH 8.5 or separately in 0.01*M* HCl (pH 2.0) with the final protein concentration at about 3.5 μ*M*.

activities of guanidinated-BLA-OA and wild-type BLA-OA complexes were found to be significantly different from those of the citraconyl-BLA-OA or acetyl-BLA-OA complexes ($P < 0.005$, one-way ANOVA). As shown in Figure 8(A), wild-type BLA-OA (named BAMLET) and guanidinated-BLA-OA complexes were found to kill tumor cells in a dose-dependent manner. About 70% of cells were killed after 24 h of exposure to either of these two complexes at the concentration of 0.1 mg/mL (7.0 μ*M*) protein. There was no significant difference in tumoricidal activity between guanidinated-BLA-OA and wild-type BLA-OA complexes ($P > 0.05$, one-way ANOVA). However, the citraconyl-BLA-OA and acetyl-BLA-OA complexes lacked tumoricidal activity, even at concentrations as high as 1.0 mg/mL protein (data not shown). The LD_{50} values calculated from fitting of the experimental data are shown in Table I. The LD_{50} values for wild-type BLA-OA and guanidinated-BLA-OA complexes were nearly identical, but citraconyl-BLA-OA and acetyl-BLA-OA did not reach a measurable LD_{50} at any of the concentrations tested.

To explore further the role of OA in killing of U2OS cells, the cytotoxicity data in Figure 8(A) were replotted versus the OA concentration within the protein-OA complexes [Fig. 8(B)]. The LD_{50} values for OA in the complexes of wild-type BLA-OA and guanidinated-BLA-OA were in the range of 25–35 μ*M*, while the LD_{50} for free OA in solution was 4–5 times greater at a concentration of about 135 μ*M* [Fig. 8(B)]. Thus, it was reasonable to conclude that the protein component of the protein-OA complexes enhances the tumoricidal effect of OA.[18,42,43] In addition, similar to the wild-type BLA-only-control, guanidinated-BLA, acetyl-BLA, and citraconyl-BLA did not reduce cell viability (data not shown).

DISCUSSION

Positively-charged basic groups are essential for oleic acid binding with α-lactalbumin

In the present study, charge-specific chemical modifications of lysine side chains were employed in order to explore the mechanisms of interaction between OA and bovine α-lactalbumin in the HAMLET-like complex. Table I summarizes the effects of charge-specific chemical modification on the OA binding ability with wild-type or modified BLA proteins. When the lysine residues on BLA are modified by citraconylation or acetylation, converting the originally positively-charged side chain amino groups to negatively-charged citraconyl groups or neutral acetyl groups, the binding interaction of OA with citraconyl-BLA or acetyl-BLA is greatly inhibited as compared to the underivatized protein. Citraconyl and acetyl modification of at least 11 lysine residues (without further purification by chromatographic methods) per BLA resulted

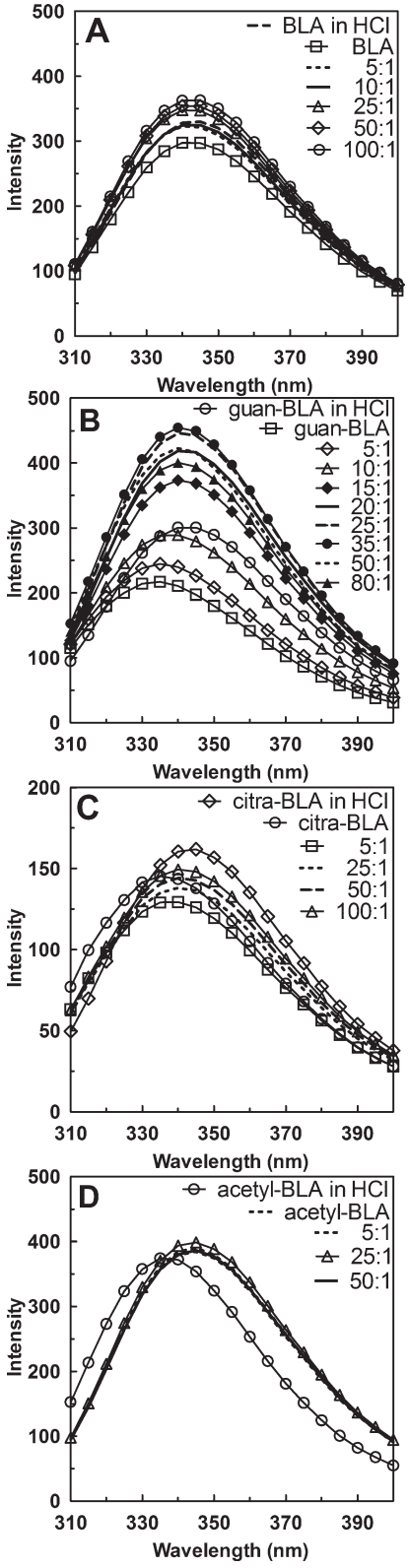

Figure 6

Intrinsic fluorescence spectra of (**A**) BLA, (**B**) guanidinated-BLA, (**C**) citraconyl-BLA, and (**D**) acetyl-BLA in the absence and presence of different [OA] to [protein] molar ratios at pH 8.5 or separately in 0.01*M* HCl (pH 2.0) with the final protein concentration at about 3.5 μ*M*. The spectra were obtained by excitation at the wavelength of 280 nm.

in no OA binding when making the complex using the original chromatograpic method.[3] Following complete regeneration of the citraconyl-BLA protein under mild acidic conditions and then loading the protein onto an OA preconditioned DEAE-Sepharose column, the protein regains its ability to bind OA as detected by ^{1}H NMR spectroscopy. In addition the guanidinated-BLA, which retains the original positive charge, also retains the ability to bind OA as well as the cytotoxic activity against the tumor cell line, U2OS.

Moreover, under the conditions used for generating the BLA-OA complex at pH 8.5,[3] BLA (pI = 4.8) will carry a net negative charge while OA (p*Ka* = 6.2–7.3)[44] will be deprotonated and also exist as a negatively-charged species. Consequently, the electrostatic repulsion between two negatively-charged species might be expected to result in little or even no binding interaction. However, the experimental results show that if sufficient positively-charged groups on the molecular surface of the protein exist to provide the initial primary binding sites for the negatively-charged oleate ions, binding can occur despite an overall negative charge on the protein. Consequently, local electrostatic attraction must play an important role in the binding of oleate anions to BLA protein.

Furthermore, as illustrated in the CD and fluorescence spectra of the wild-type BLA [Figs.4(A), 5(A), 6(A), and 7(A)] and modified guanidinated-BLA [Figs. 4(B), 5(B), 6(B), and 7(B)], these proteins undergo substantial conformational changes when the OA concentration is increased and can be characterized by the loss of rigid tertiary structure but with retention of secondary structure. However, the conformation of the citraconyl-BLA [Figs. 4(C), 5(C), 6(C), and Supporting Information Fig. S7), exhibits little change when the OA concentration is increased and also fails to bind OA. This result indicates that the binding of OA with citraconyl-BLA is inhibited due to the local electrostatic repulsion between the negatively-charged citraconyl groups and the oleate anions. In addition, the acetyl-BLA [Figs. 4(D), 5(D), 6(D), and 7(C)], displays no substantial conformational change when the OA concentration is increased and also fails to bind OA, further confirming the conclusion that the neutral acetyl groups cannot provide initial local binding sites for the negatively-charged oleate anions.

Because the lysine residues were modified globally rather than on a site-specific basis, there is presently no evidence as to which of the 12 lysine side chains are involved in the binding of OA to α-lactalbumin, in other words, our studies do not provide residue-specific modification information. However, it is clear that the positively-charged basic groups (including lysine and arginine side chains) on the α-lactalbumin protein play an essential role in the initial binding of the negatively-charged carboxylate groups of the OA molecules and that abolition of all of the positively-charged lysine charges eliminates OA binding. We note that both elimination of the

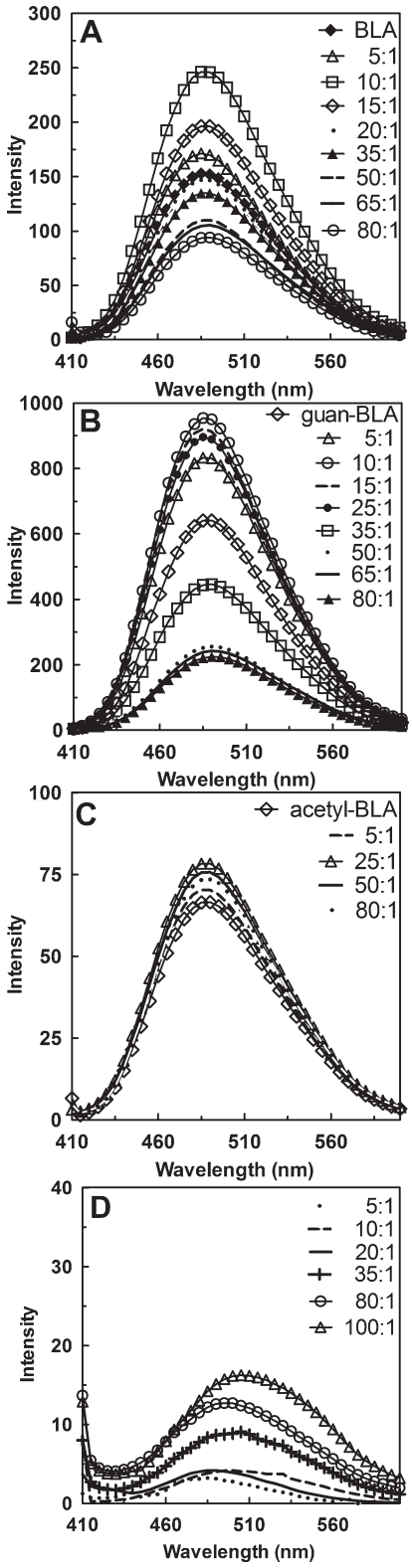

Figure 7

ANS-binding fluorescence spectra of (**A**) BLA, (**B**) guanidinated-BLA, (**C**) acetyl-BLA in the absence and presence of different [OA] to [protein] molar ratios at pH 8.5. The spectra were obtained by excitation at the wavelength of 390 nm. The final protein concentration employed was about 3.5 μ*M*, and the final ANS concentration was about 10 μ*M*. (**D**) ANS-binding fluorescence spectra of different molar ratios OA at pH 8.5.

lysine side chain charges by acetylation and their conversion to negative charges by citraconylation as well as their retention by conversion to homoarginine groups involves a relatively comparable increase in size of the side chains. Consequently, the perturbation of α-lactalbumin structure purely by the change in size of the side chains with respect to the binding properties of OA does not appear to be an issue—only the elimination of positive charges affects OA binding. Consequently, we conclude that electrostatic interactions between the positively-charged basic groups on the α-lactalbumin protein and the negatively-charged carboxylate groups on OA molecules are key in the intermolecular integrity of the α-lactalbumin-oleic acid complex under the present experimental conditions.

Both electrostatic and hydrophobic interactions are involved in OA-protein binding

Under the present experimental conditions, the nature of the interaction between OA and the BLA protein is speculated to take place through initial electrostatic associations: the negatively-charged carboxylate group of the OA molecule forms a salt bridge or a hydrogen bond with positively-charged basic amino acid residues (mainly lysine and arginine side chains) or polar amino acid side chains on the bovine α-lactalbumin proteins. These initial interactions cause the bovine α-lactalbumin protein to unfold partially and gain more conformational flexibility and molecular mobility as shown in the spectroscopic changes of the proteins observed as a function of OA concentration (Figs. 4–7), and hence the hydrophobic sites are more easily accessible. Such exposure of hydrophobic patches may in turn allow the hydrophobic long alkyl chains of OA molecules to interact with these internal hydrophobic sites of the protein after the initial electrostatic attraction.[45–47] It is important to note that, upon charge-specific chemical modification to convert the originally positively-charged lysine residues to negatively-charged citraconyl groups or neutral acetyl groups, the binding of OA to the protein is inhibited, even at high molar ratios of OA. This means that, once the initial electrostatic interactions between the positively-charged basic groups on proteins and the negatively-charged carboxylate groups on OA molecules are significantly reduced, no hydrophobic interactions can take place thereafter.

To further demonstrate the electrostatic nature of binding, a variety of screening experiments at different salt concentrations were carried out (0, 25, 100, 300, 500, 800, 1000 m*M* NaCl in 10 m*M* Tris/HCl buffer, pH 8.5). For wild-type BLA, the OA binding molar ratio to the BLA protein determined by ^{1}H NMR incrementally decreases upon the increase of salt concentrations (Supporting Information Fig. S8 and Table S1), whereas for citraconyl-BLA and acetyl-BLA, no OA molecules bound to the modified proteins under any salt concentration

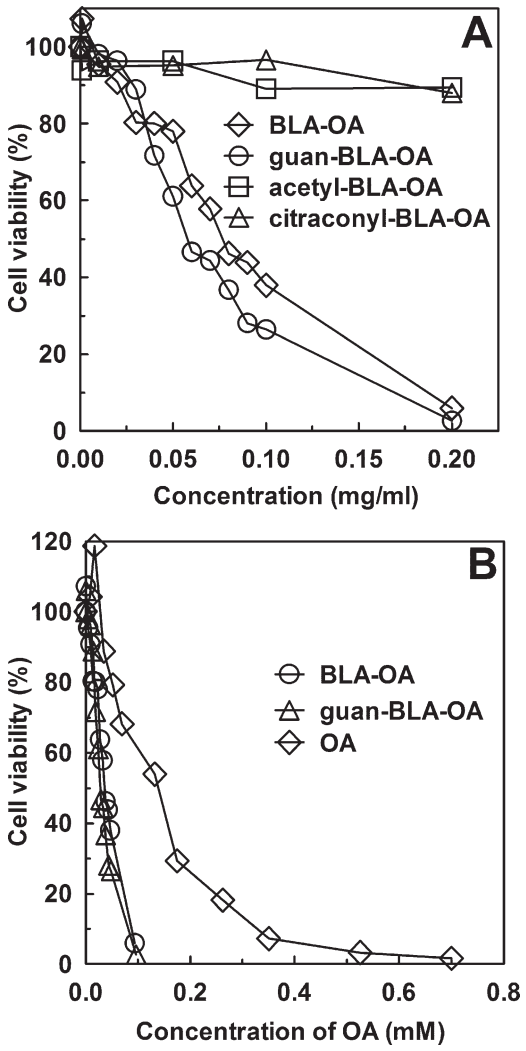

Figure 8

Viability of U2OS cells *in vitro* plotted either as a function of (**A**) protein concentration or (**B**) total concentration of OA bound to protein.

(data not shown). Consequently, the initial contact of OA with protein does appear to involve electrostatic interactions, and no Hofmeister or "specific ion" effects (usually apparent above 0.2*M* salt)[48] seem particularly prominent.

Both protein and oleic acid affect the binding ratio

The α-lactalbumin molecule is made up of two domains, a large α-helical domain and a small β-sheet domain.[1] All 12 lysine residues and one arginine residue are located on the molecular surface and are solvent accessible (Fig. 9), therefore, they can potentially contribute to the binding of OA. With regards to the individual residue SASAs [Fig. 10(A)] or relative surface accessibility (%) [Fig. 10(B)], notable differences are observed, with the relative surface accessiblities (%) of the lysine or arginine residues ranging from 12.22 to 100.0%. For example, Lys 58 is relatively less solvent accessible, while Lys 13, 16, 114, 122, and Arg 10 are more solvent accessible. When making the α-lactalbumin-oleic acid complex under different experimental conditions (such as different pH, ion strength, temperature, and initial OA/BLA molar ratio etc.),[11,12,17–19,21] the relative surface accessibilities of positively-charged basic side chains may also differ, further complicating the structural consequences in the association/dissociation of OA. We speculate that it is through these subtle differences that different binding ratios may be obtained.

Oleic acid induces the partial unfolding of α-lactalbumin in the HAMLET-like complex

The near-UV and far-UV CD and intrinsic and ANS-binding extrinsic fluorescence studies have shown that there are global conformational changes of wild-type BLA and guanidinated-BLA proteins with increasing concentration of OA.

Following an increase in the OA concentration, the far-UV CD spectra of wild-type BLA and guanidinated-BLA show that the proteins retain secondary structure [Fig. 4(A,B)] but are characterized by increased conformational mobility and gradual loss of tertiary structure as shown in the near-UV CD spectra [Fig. 5(A,B)]. When the [OA]:[BLA] and [OA]:[guanidinated-BLA] = 50:1, the resulting spectra closely resemble those of the molten globule state induced by 0.01*M* HCl [Fig. 5(A,B)], suggesting that the proteins are partially unfolded by the addition of OA. In the cases of citraconyl-BLA [Figs. 4(C) and 5(C)] and acetyl-BLA [Figs. 4(D) and 5(D)], the tertiary and secondary structures exhibit little change following an increase in the concentration of OA.

Table I

Summary of Results of Charge-Specific Chemical Modification of Lysine Side Chains for BLA

Modification	Charge type	Modified lysine	[OA]:[protein]	LD_{50} (μM)
Wild-type BLA	Positive	0	6.6	32.5
Citraconylation	Positive→Negative	11.6	No binding	No cytotoxicity
De-citraconylation	Negative→Positive	1.1	6.2	(Not tested)
Acetylation	Positive→Neutral	11.5	No binding	No cytotoxicity
Guanidination	Positive→Positive	10.9	6.9	26.7

The number of modified lysines was calculated from the free lysine residues determined by the TNBS method (ε_{340nm} = 11,900 M^{-1} cm^{-1}).[33] The [OA]:[protein] molar binding ratio was calculated by comparing the peak area of the aromatic region of the proteins (chemical shift: 8.5–6.5 ppm) to that of the protons on carbon 9/10 of OA (5.33 ppm). The LD_{50} (μ*M*) in oleic acid was calculated by fitting the curves of cell viability as a function of the concentration of protein-OA complexes as shown in Figure 8.

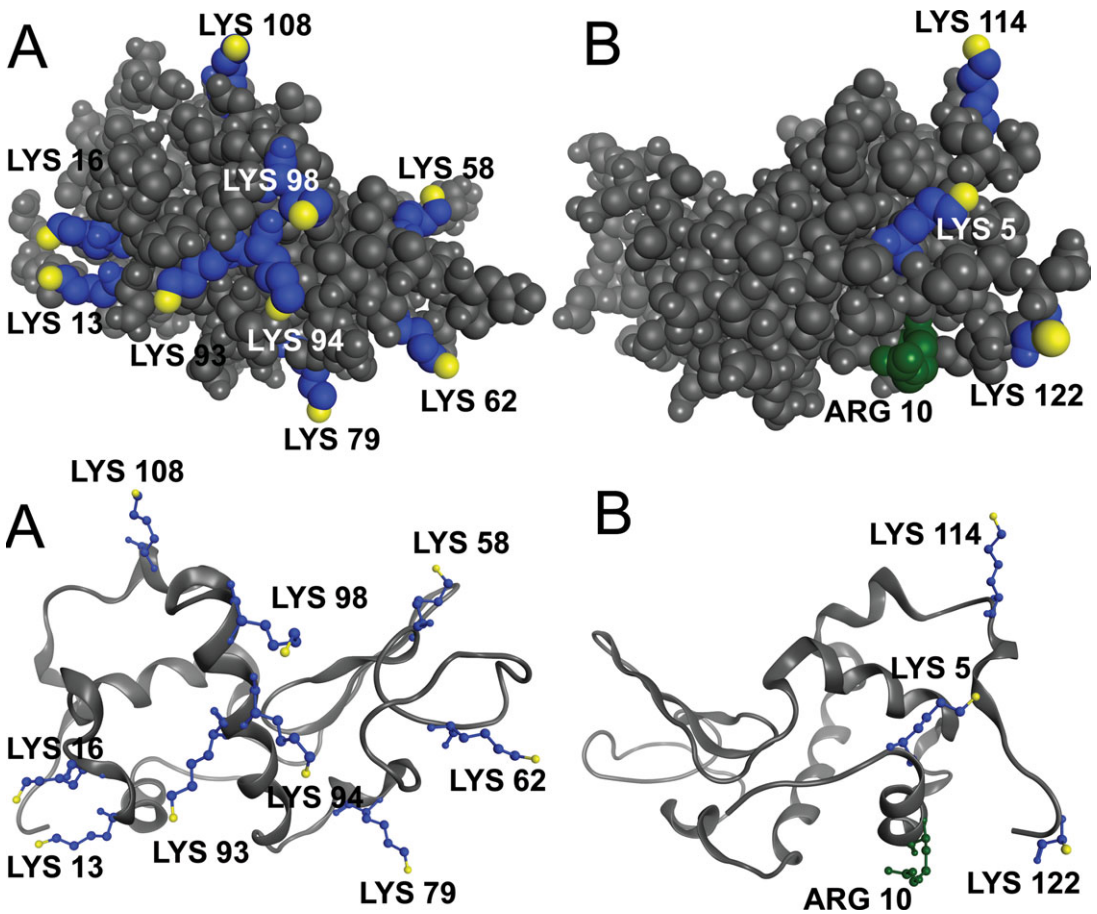

Figure 9

Representation of the three-dimensional structure of bovine apo-lactalbumin in two different orientations. (**A**) one side of α-lactalbumin; (**B**) the opposite side, rotated 180° about a vertical axis. The lysine residues are shown in blue, and the ϵ-amino groups of lysine residues are expressed in yellow. The arginine residue is indicated in green. The upper row is represented as a space-filled model, and the lower row a ribbon model. The figure was drawn using data for bovine apo-α-lactalbumin (PDB code 1F6R[15]) and the program MOE.

The intensity of intrinsic fluorescence spectral bands [Fig. 6(A,B)] increase as the molar ratio of OA over wild-type BLA or guanidinated-BLA increases up to [OA]:[protein] = 50:1. This observation indicates that the presence of OA improves the accessibility of tryptophan and tyrosine residues in wild-type BLA and guanidinated-BLA, indicative of the partial unfolding of the proteins. In addition, ANS-binding extrinsic fluorescence measurements [Fig. 7(A,B)] further confirm the conformational change of wild-type BLA and guanidinated-BLA following the addition of OA. At low ratios of OA ([OA]:[BLA] and [OA]:[guanidinated-BLA] = 5:1–10:1), the intensity of ANS-binding fluorescence increases as the molar ratio of OA over protein increases. With further increase of OA concentration up to a ratio of [OA]:[protein] = 50:1, the intensity of fluorescence decreases significantly, further implying that there may be changes in the environment surrounding the hydrophobic sites. However, the total intrinsic fluorescence intensity of both citraconyl-BLA and acetyl-BLA [Fig. 6(C,D), respectively) is much lower than that of their counterparts [Fig. 6(A,B)] and remains unchanged following an increase in the concentration of OA. The same type of observation can be made concerning the ANS-binding extrinsic fluorescence spectra of acetyl-BLA and citraconyl-BLA [Fig. 7(C) and Supporting Information Fig. S7], which further indicates very little influence of OA on the conformational changes of these two proteins.

Combining the results from the near-UV, far-UV CD, and intrinsic and ANS-binding extrinsic fluorescence, it can be concluded that wild-type BLA and guanidinated-BLA are partially unfolded by the addition of OA. In contrast, for the cases of acetyl-BLA and citraconyl-BLA, no substantial conformational changes are observed upon the increase of OA concentration. At pH 8.5, OA is known to dissociate to oleate anions (pKa = 6.2–7.3 under physiological conditions)[44] and can serve as an anionic surfactant.[49,50] Our results suggest that OA mol-

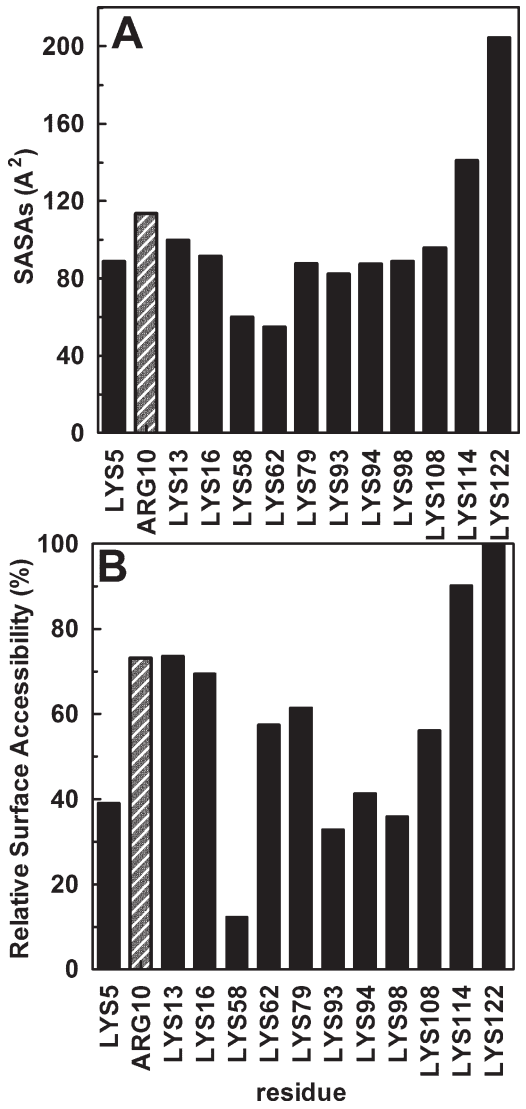

Figure 10

Average residue (**A**) solvent accessible surface areas (SASAs) and (**B**) relative surface accessibilities (%) of lysines and arginine in bovine apo-α-lactalbumin (PDB code 1F6R[15]) calculated using POPS (version 1.3.2).[36,37] Each asymmetric unit contains six chains with chains A and B possessing 12 lysines each and chains C, D, E, F possessing 11 lysines each (lacking Lys 122 due to its high flexibility and hence inability to exhibit fixed electron density in the crystal structure).

ecules may interact with BLA by modifying the conformation and/or denaturation[46,51,52] with the carboxylate groups bound to lysine or arginine side chains and the aliphatic long chains bound within hydrophobic regions.

From HAMLET to XAMLET

HAMLET was originally identified from casein fractions of human milk, and its constituting components were characterized as α-lactalbumin and OA.[2,3] Other HAMLET-like complexes made from OA and α-lactalbumin that is purified from other species such as porcine, equine, caprine, and bovine, have shown equivalent cytotoxicity to tumor cells.[53] Moreover, a variety of different complexes, including fragments of α-lactalbumin produced by limited proteolysis,[12] a recombinant variant of human α-lactalbumin with all four cysteines substituted with alanines (All-ALA form),[19] and α-lactalbumin denatured by heat treatment[13,54] can also be made into cytotoxic complexes using the OA preconditioned DEAE column technique. Even equine lysozyme, a structural homolog to α-lactalbumin can form a cytotoxic complex with OA (termed ELOA).[20] It should be noted that OA alone has been shown to exhibit cytotoxic activity[55–57]; however, as shown in this work and previous investigations, it is evident that the protein component may contribute significantly important features not found with OA alone. Our cytotoxicity tests of the wild-type BLA-OA (termed BAMLET) and guanidinated-BLA-OA complexes along with the total loss of tumoricidal activity for the citraconyl-BLA-OA and acetyl-BLA-OA complexes further demonstrate that OA binding can take place provided there exist sufficient positively-charged basic groups on the proteins.

One way of viewing the common OA-binding properties with the many variants of α-lactalbumin can be illustrated by a species sequence alignment of bovine, human, goat, pig, sheep, dog, horse, and lowland gorilla α-lactalbumin. This alignment shows that the positively-charged basic residues (including lysine and arginine side chains) are highly conserved throughout the various species, ranging from 12 to 13 lysine residues (9.76–10.57%) and 1–2 arginine residues (0.81–1.63%) per 123 residues (Table S2 of Supporting Information). Hence, while for the native protein, the lysine and arginine residues may bear important consequences either for the endogenous activity of the protein or in the integrity of its three-dimensional structure, it appears that these side chains also allow the binding of OA in the complexes.

Due to the partially-unfolded nature of the protein within the complex, it was important to rule out that neither citraconyl-BLA nor acetyl-BLA failed to bind OA because of increased (rather than decreased) thermodynamic stability. To this end, equilibrium-unfolding experiments induced by urea were performed (Table II),

Table II

Thermodynamic Parameters of Equilibrium Unfolding Transitions of Wild-Type and Modified BLA

Modification	C_M (M)	$m_{N\rightarrow I}$ (kcal/mol M)	$\Delta G^{0(N\rightarrow I)}_{H_2O}$ (kcal/mol)	$m_{I\rightarrow U}$ (kcal/mol M)	$\Delta G^{0(I\rightarrow U)}_{H_2O}$ (kcal/mol)
Wild-type	4.38	0.50	2.21	0.41	1.82
Guanidination	3.18	0.56	1.78	0.43	1.37
Citraconylation	2.47	0.50	1.23	0.47	2.03
Acetylation	3.66	0.52	1.30	0.31	1.13

The equilibrium unfolding data were fit to a three-state model[35], and the thermodynamic parameters were calculated using the equations as shown in the Supporting Information.

Table III
Proteins–Fatty Acid Interactions (Note that the following complexes do not necessarily exhibit cytotoxic activity.)

			Residue sequence composition				
Protein	pI	PDB	Length	Lys	Arg	Aromatic	References
Human α-lactalbumin	4.2–4.5	1B90[58]	123	12	1	11	2–11,19,21,59
Bovine α-lactalbumin	4.8	1F6R[15]	123	12	1	12	3,7,18,19,60
Equine lysozyme	8.4	2EQL[61]	129	15	4	14	20,62,63
Bovine β-lactoglobulin	4.8	3NPO[64]	162	15	3	10	65–69
Equine myoglobin	7.36	1MBN	153	19	4	11	70,71
Chicken lysozyme	10.5–11.0	193L[72]	129	6	11	12	73,74
Equine cytochrome C	9.5	1HRC[75]	105	19	2	9	76,77

and the results show that the conformational stabilities of the citraconyl-BLA and acetyl-BLA proteins decrease considerably whereas guanidinated-BLA exhibits a relatively less decrease when compared with that of wild-type BLA protein. These results further demonstrate that the lack of the formation of the complex (in its partially unfolded state and the presence of positive charges) is not due to a further conformational stabilization of the chemically modified variants.

Table III summarizes some recent publications that study the interactions between proteins and OA (or sodium oleate) or other fatty acids. As shown in this table, all of the proteins that were involved have sufficient positively-charged basic groups (including lysine and arginine residues), providing potential binding for the negatively-charged carboxylate groups of fatty acids via electrostatic interactions. In certain cases and under specific conditions, we speculate that the alkyl tails of fatty acids can also bind to the hydrophobic sites of the proteins (by mainly interacting with aromatic residues such as tyrosine, tryptophan, and phenylalanine) via hydrophobic interactions.

CONCLUSION

Charge-specific chemical modifications were employed to investigate the effects of the positively-charged basic groups of bovine α-lactalbumin proteins on the binding of OA. The present study demonstrates that under the present experimental conditions (10 m*M* Tris/HCl buffer, pH 8.5), electrostatic interactions between positively-charged basic groups (mainly lysine and arginine side-chains) on bovine α-lactalbumin and the negatively-charged carboxylate groups on OA play an essential role in the binding of OA to bovine α-lactalbumin in the HAMLET-like complex, and that these may be as important as the hydrophobic interactions between the alkyl tails of OA molecules and the hydrophobic sites of the proteins. Furthermore, it is speculated that the properties of both the protein and OA affect the binding ratio of OA to α-lactalbumin: first, due to the differences in the SASAs of individual positively-charged basic residues on the proteins and, second, due to the association/dissociation state of OA under different experimental conditions.

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