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# No Need To Be HAMLET or BAMLET To Interact with Histones: Binding of Monomeric α-Lactalbumin to Histones and Basic Poly-Amino Acids†

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ABSTRACT: The ability of a specific complex of human α-lactalbumin with oleic acid (HAMLET) to induce cell death with selectivity for tumor and undifferentiated cells was shown recently to be mediated by interaction of HAMLET with histone proteins irreversibly disrupting chromatin structure [Duringer, C., *et al*. (2003) *J. Biol. Chem. 278*, 42131−42135]. Here we show that monomeric α-lactalbumin (α-LA) in the absence of fatty acids is also able to bind efficiently to the primary target of HAMLET, histone HIII, regardless of $Ca^{2+}$ content. Thus, the modification of α-LA by oleic acid is not required for binding to histones. We suggest that interaction of negatively charged α-LA with the basic histone stabilizes apo-α-LA and destabilizes the $Ca^{2+}$-bound protein due to compensation for excess negative charge of α-LA's $Ca^{2+}$-binding loop by positively charged residues of the histone. Spectrofluorimetric curves of titration of α-LA by histone H3 were well approximated by a scheme of cooperative binding of four α-LA molecules per molecule of histone, with an equilibrium dissociation constant of 1.0 $\mu$M. Such a stoichiometry of binding implies that the binding process is not site-specific with respect to histone and likely is driven by just electrostatic interactions. Co-incubation of positively charged poly-amino acids (poly-Lys and poly-Arg) with α-LA resulted in effects which were similar to those caused by histone HIII, confirming the electrostatic nature of the α-LA−histone interaction. In all cases that were studied, the binding was accompanied by aggregation. The data indicate that α-lactalbumin can be used as a basis for the design of antitumor agents, acting through disorganization of chromatin structure due to interaction between α-LA and histone proteins.

α-Lactalbumin (α-LA)[1] is a classical object of numerous studies due to a unique combination of features (for a recent review, see ref *1*). This is a relatively simple protein, which is small ($M_r$ = 14.2 kDa, 123 residues) and has a well-studied globular structure. The protein possesses one strong $Ca^{2+}$/$Mg^{2+}$-binding site (*2*, *3*), a distinct strong $Zn^{2+}$-binding site, and a few weaker $Zn^{2+}$-binding sites (*4*, *5*). Despite its clear cation binding properties, the role of metal ions in α-lactalbumin functioning as a modifier protein in the lactose synthase (EC 2.4.1.22) is still poorly understood. One of the most exciting features of α-lactalbumin is its ability to form

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[1] Abbreviations: α-LA, α-lactalbumin; MAL, multimeric α-LA; HAMLET, human α-lactalbumin made lethal to tumor cells; BAMLET, bovine α-lactalbumin made lethal to tumor cells; CD, circular dichroism; UV, ultraviolet.

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the so-called molten globule state at acidic pH or at neutral pH at elevated temperatures in the absence of cations (*6*). Interest in α-lactalbumin over the past several years has grown due to several reports that α-LA itself or its fragments possess bactericidal or antitumor activity (*7*, *8*). In particular, Hakansson *et al*. (*7*) have found that a multimeric human α-LA derivative (MAL), isolated from the casein fraction of milk, is a potent $Ca^{2+}$-elevating and apoptosis-inducing agent with broad, yet selective, cytotoxic activity. In fact, MAL was shown to kill all transformed, embryonic, and lymphoid cells that were tested, but spare mature cells. It was shown that the apoptosis-inducing fraction of α-LA contains α-LA oligomers that underwent a conformational change toward a molten globule-like state (*9*). Furthermore, it has been established that direct interaction of MAL with mitochondria led to the release of cytochrome *c*, followed by activation of the caspase cascade (*10*). 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 (*11*).

Similar results were obtained with HAMLET (human α-lactalbumin made lethal to tumor cells) or BAMLET (bovine α-lactalbumin made lethal to tumor cells), which is native α-lactalbumin converted *in vitro* to the apoptosis-inducing folding variant of the protein in complex with oleic acid (*12*, *13*). HAMLET was shown to trigger apoptosis in tumor and immature cells, but healthy cells were resistant to this effect. Importantly, HAMLET passed through the cytoplasm to the nucleus and accumulated in the cell nucleus. In tumor cells *in vivo*, HAMLET colocalized with histones and perturbed the chromatin structure (*14*). HAMLET was found to bind histone HIII strongly and to lesser extent histones HIV and HIIB. The binding of the histones by HAMLET impaired their deposition on DNA. On the basis of these observations, it has been concluded that HAMLET is able to interact with histones and chromatin in tumor cell nuclei, locking the cells into the death pathway by the irreversible disruption of chromatin organization (*14*). The conformational behavior of BAMLET has recently been analyzed using limited proteolysis and CD spectroscopy, showing that BAMLET possessed structural properties of the classical molten globule (*15*). In fact, it has been established that the far- and near-UV CD spectra of BAMLET were essentially identical to those of the most studied molten globule of α-LA at pH 2.0 (so-called A state) (*15*). Proteolysis of the 123-residue chain of BAMLET by proteinase K occurs in a manner similar to that of apo-α-LA with moderate heating, or α-LA in the presence of 15% TFE, i.e., under conditions known to stabilize the molten globule state in this protein. In this study, proteinase K was shown to preferentially cleave BAMLET at the Ser34−Gly35, Gln39−Ala40, Gln43−Asn44, Phe53−Gln54, and Asn56−Asn57 peptide bonds. Interestingly, all these peptide bonds are located within the $\beta$-subdomain of the protein (chain region of residues 34−57) (*15*). Taken together, these results for BAMLET or HAMLET suggest that the interaction of α-LA with oleic acid might induce structural transformation of the protein into the molten globule-like conformation, which, in its turn, binds histone HIII and induces apoptosis.

In the work presented here, we show that modification by oleic acid is not necessary for the efficient interaction of α-LA with the primary target of HAMLET, histone HIII. Like the HAMLET state of α-LA, monomeric $Ca^{2+}$-loaded α-LA or apo-α-LA is also able to bind to the histone. Furthermore, both bovine and human α-LAs can interact with charged disordered polypeptides, resembling histones: polyl-lysine and polyarginine of various molecular masses. The data justify the electrostatic nature of the α-LA−histone interaction and allow us to consider α-lactalbumin as a basis for the design of antitumor agents, acting through disorganization of chromatin structure due to the interaction with histone proteins.

## EXPERIMENTAL PROCEDURES

*Materials.* α-Lactalbumin from bovine milk (lot no. 60K7002), histone type III-S from calf thymus (Lys-rich fraction) (lot no. 10K7435), poly-L-arginine hydrochloride, poly-L-lysine hydrobromide, and the sodium salt of poly-L-glutamic acid with an average molecular mass of 45 600 Da (lot no. 79H5918) were purchased from Sigma Chemical Co. (St. Louis, MO). Human α-lactalbumin was isolated and purified from milk as described in ref *16*. Protein concentrations were evaluated spectrophotometrically, using an extinction coefficient $E_{1\%,280}$ of 20.1 for bovine α-lactalbumin and an $E_{1\%,280}$ of 18.2 for human protein (*17*) (18.4 there). The molar extinction coefficient for bovine histone III-S was calculated using its amino acids content (*18*, *19*) according to the method described in ref *20* ($\epsilon_{280} = 4595\ M^{-1}\ cm^{-1}$). All solutions were prepared using distilled, deionized water.

*Methods*. Fluorescence studies were carried out on a computerized and automated lab-built spectrofluorimeter described previously (*21*). Protein fluorescence was excited using a 296.7 nm Hg lamp spectral line. All spectra were corrected for the spectral sensitivity of the instrument and fitted to log-normal curves (*22*) using nonlinear regression analysis (*23*). The positions of fluorescence spectrum maxima ($\lambda_{max}$) were obtained from the fits. Temperature scans were performed stepwise, allowing the sample to equilibrate at each temperature for at least 5 min. The temperature was monitored directly inside the cell. The fraction of conversion from the native to thermally denatured protein state was calculated from the plots of temperature dependence of emission intensity at a fixed wavelength as previously described (*24*, *25*). All titration experiments were performed using a lab-made automatic titrator. Estimation of parameters of theoretical models was carried out according to the method described in ref *23*. The calcium binding affinity of α-LA was measured using a spectrofluorimetric $CaCl_2$/EDTA titration method described previously (*26*).

In all fluorescence experiments, the α-lactalbumin illumination time and the UV irradiation power level were minimized to avoid the UV-induced structural rearrangements in the protein molecule (*27*, *28*).

## RESULTS AND DISCUSSION

To facilitate presentation of the results that were obtained, mostly data for bovine α-LA are depicted herein. The bovine protein was chosen mainly due to the fact that interaction of human α-LA with histone and poly-amino acids leads to extensive aggregation, precluding the protein from the quantitative analysis of experimental data. The results for the human protein will be mentioned to emphasize principal differences in the behavior of both forms of α-LA.

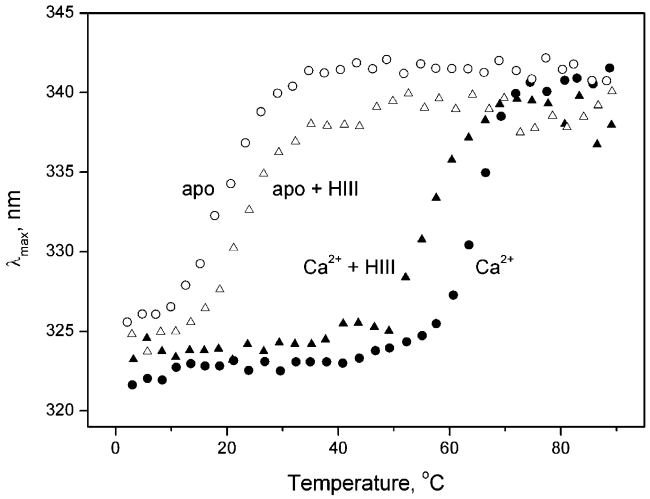


FIGURE 1: Thermal denaturation of apo [1 mM EDTA (○ and △)] and $Ca^{2+}$-saturated [1 mM $CaCl_2$ (● and ▲)] α-LA in the presence of histone HIII vs melting of native α-LA, as followed by tryptophan fluorescence of α-LA (pH 7.6 and 10 mM HEPES-KOH). The excitation wavelength was 296.7 nm. The α-LA concentration was 3−6 $\mu$M, and the histone to protein molar ratio was 5. Circles represent data for native α-LA; triangles represent data for the α-LA−HIII complex. $\lambda_{max}$ is the tryptophan fluorescence spectrum maximum position.

Table 1: Half-Transition Temperature ($t_{1/2}$) of Thermal Denaturation of Apo and $Ca^{2+}$-Saturated α-LA (3−8 $\mu$M) in the Presence or Absence of Histone HIII or Basic Poly-Amino Acids (substance to α-LA molar ratio of ∼5−9), As Measured by the Tryptophan Fluorescence of α-LA (pH 7.6 and 10 mM HEPES)

| substance | $t_{1/2}$ (°C) in 1 mM EDTA | $t_{1/2}$ (°C) in 1 mM $CaCl_2$ |
|---|---|---|
| native α-LA | 22.6 | 65.1 |
| α-LA and histone HIII | 24.0 | 58.4 |
| α-LA and poly-L-Arg (molecular mass of 10 750 Da) | 28.0 | 45.3 |
| α-LA and poly-L-Lys (molecular mass of 11 450 Da) | 34.1 | 56.4 |

*α-LA−Histone HIII Interaction.* To examine the possibility of interaction between α-lactalbumin and histone HIII, a set of thermal denaturation curves for α-LA was measured at various concentrations of the histone and $Ca^{2+}$ ions. Figure 1 depicts the thermal denaturation of α-LA at pH 7.6 as followed by its tryptophan fluorescence selectively excited using a 296.7 nm Hg lamp line. As bovine histone HIII does not contain Trp residues (*18*, *19*), only tryptophan chromophores of α-LA are excited under these conditions. It is clearly seen that addition of histone HIII to α-LA results in a notable (∼7 °C) decrease in the thermal stability of $Ca^{2+}$-loaded bovine α-LA and causes just a minor (1−2 °C) increase in the thermal stability in the case of apo-α-LA (see Table 1). Destabilization of $Ca^{2+}$-bound human α-LA induced by the histone was even more pronounced (23 °C, data not shown). These data clearly emphasize the existence of an effective interaction between α-LA and histone HIII, which occurs regardless of calcium content. The observed binding of the basic histone protein [net charge of +20 at pH 8 (*18*, *19*)] to acidic α-LA (net charge of −7 at pH 8) is accompanied by thermal stability changes, characteristic of compensation of excessive negative charge of the $Ca^{2+}$-binding loop of α-LA, as observed in ref *29*.

It should be noted that the binding of histone HIII to native or denatured apo-α-LA causes a small but reproducible blue shift in the fluorescence spectrum of 1−3 nm (Figure 1), indicating that the environment of some α-LA tryptophan residues became less polar or/and less mobile (indicative of their immersion into the protein interior). The opposite effect, a red shift in the fluorescence spectrum of 1−3 nm, was observed in the case of the histone binding to native $Ca^{2+}$-saturated α-LA. Thus, a good correlation between changes in the fluorescence spectrum position and the thermal stability of α-LA is observed, showing that a more red-shifted fluorescence spectrum corresponds to a more mobile or disordered and less stable protein state.

Examination of Figure 1 shows that at 5 and 40 °C α-LA is sufficiently far from its thermal transitions for the histone titration experiments to be carried out. Figure 2 demonstrates results of the fluorimetric titrations of apo-α-LA (panel A) and the $Ca^{2+}$-saturated protein (panel B) at 5 and 40 °C as followed by changes in emission at 340 nm. At 40 °C, the apo form of α-LA is known to represent the classical molten globule state (*6*). The binding of histone to the molten globule state of α-LA causes a slight (∼3 nm) blue shift in the fluorescence spectrum of α-LA (see Figure 1) which is accompanied by the dramatic changes in the fluorescence quantum yield, as manifested by the changes in emission intensity at 340 nm (Figure 2A). The first bend of the titration curve is achieved at a very low histone to α-LA molar ratio (∼1/30), which can be caused by aggregation of α-LA due to supersaturation of the histone with α-LA molecules. This conclusion is fully supported by the increase in intensity of the scattered Hg lamp spectral line at 365 nm (Figure 2A). The rest of the titration curve is approximated well by a simple cooperative scheme of binding of *n* α-LA molecules to a molecule of histone:

$$\text{histone} + n\alpha\text{-LA} \overset{K_{\text{eff}}}{\longleftrightarrow} \text{histone}\cdot(\alpha\text{-LA})_n \qquad (1)$$

where $K_{\text{eff}}$ is an effective equilibrium association constant. Estimation of *n* and $K_{\text{eff}}$ values using the nonlinear least-squares method gave an *n* of 3.9 (Table 2) and a $K_{\text{eff}}$ of $1.0 \times 10^6$ $M^{-1}$ (dissociation constant of 1.0 $\mu$M). Analogous analysis of human α-LA gave similar values, although experiments were accompanied with severe aggregation and precipitation.

At the same time, the binding of histone HIII to native apo-α-lactalbumin at 5 °C was characterized by the negligible (∼1 nm) blue shift in the fluorescence spectrum of α-LA (see Figure 1) and by the much less complicated changes in the fluorescence quantum yield (Figure 2A). In fact, the titration curve in this case lacked the first sharp phase with a stoichiometry of binding around 1/30 and demonstrated a single peak in the region of relative histone to α-LA concentrations around 1/4.2. This peak is likely to imply that the formation of the histone−α-LA complex is followed by its partial dissociation due to the redistribution of α-LA molecules over the increasing number of histone molecules. Thus, interaction between α-LA and histone in this case cannot be approximated by the simple cooperative binding scheme (eq 1).

Fluorescent titration of native $Ca^{2+}$-saturated α-lactalbumin with histone HIII at 40 °C did not reveal the evident changes

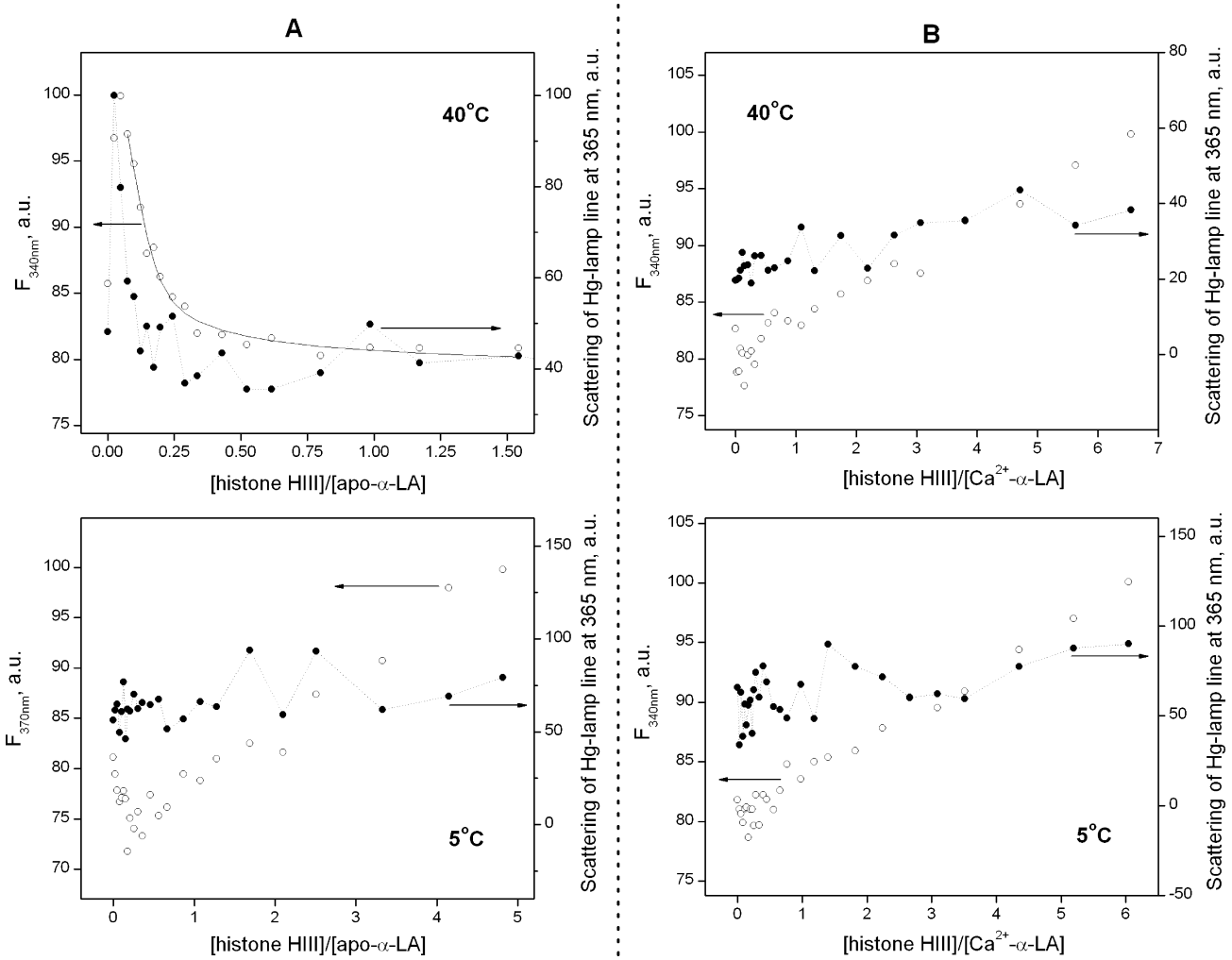


FIGURE 2: Spectrofluorimetric titration of apo (1 mM EDTA, A) and $Ca^{2+}$-saturated (1 mM $CaCl_2$, B) α-LA by histone HIII at 5 and 40 °C (pH 7.6 and 10 mM HEPES-KOH). The excitation wavelength was 296.7 nm. The α-LA concentration was 3−5 μM. Empty circles represent the fluorescence intensity at 340 nm in arbitrary units. Filled circles represent the intensity of the scattered Hg lamp spectral line at 365 nm in arbitrary units. The solid curve fitted to the experimental points is calculated from a simple cooperative scheme of the binding of $n$ α-LA molecules to a molecule of histone [histone + $n$α-LA ↔ histone·$(α\text{-LA})_n$].

Table 2: Change in the Stoichiometry of Binding[a] of Histone HIII or Basic Poly-Amino Acids to Apo-α-LA (1 mM EDTA) upon Conversion of α-LA to the Molten Globule State (pH 7.6 and 10 mM HEPES)

| substance | $n$ at 5 °C | $n$ at 40 °C |
|---|---|---|
| histone HIII | 4.2 | 3.9 |
| poly-L-Arg (molecular mass of 10 750 Da) | 2.6 | 5.4 |
| poly-L-Lys (molecular mass of 11 450 Da) | 3.3 | 4.4 |

[a] The stoichiometry of binding ($n$) represents the number of α-LA molecules bound per molecule of substance. The stoichiometry values were evaluated from the spectrofluorimetric titrations.

at low histone concentrations, i.e., when histone to α-LA molar ratios were less than 1 (Figure 2B), but demonstrated a slow increase (>20%) in the fluorescence quantum yield and a red shift (>2 nm) in the α-LA fluorescence spectrum at higher histone to α-LA molar ratios (see Figure 2B). These changes were accompanied by a gradual increase in sample scattering (Figure 2B), indicative of the activation of aggregation processes. A similar situation was observed at 5 °C (Figure 2B). Thus, the fluorescence titrations did not reveal high-affinity binding of histone HIII to $Ca^{2+}$-loaded α-lactalbumin. Nevertheless, the observed prominent shift in thermal stability of $Ca^{2+}$-bound α-LA upon addition of histone (see Table 1) implies that the high-affinity binding is likely to be masked due to the decreased sensitivity of fluorescence to the binding of histone. A similar situation is observed for native apo-α-LA at 5 °C, where strong binding of the histone is accompanied by an only ∼8% change in the fluorescence intensity (Figure 2A).

*Interaction between α-Lactalbumin and Charged Poly-Amino Acids.* We assume that the observed specific binding of approximately four molecules of apo-α-LA per histone HIII molecule indicates that the binding process is likely to be not site-specific with respect to histone, but is driven by electrostatic attraction of negative α-LA molecules (net charge of −7) to positively charged histone molecules (net charge of +20).

It has been established previously that naturally folded and intrinsically unstructured (or natively unfolded) proteins occupy nonoverlapping regions in the charge−hydrophobicity plots (*30*). This means that folded and natively unfolded proteins could be distinguished on the basis of their amino acid composition (*30*−*32*). Analysis of the amino acid sequences of bovine histone HIII showed that this protein, as well as the majority of other core histones, is characterized by the amino acid composition typical for the natively

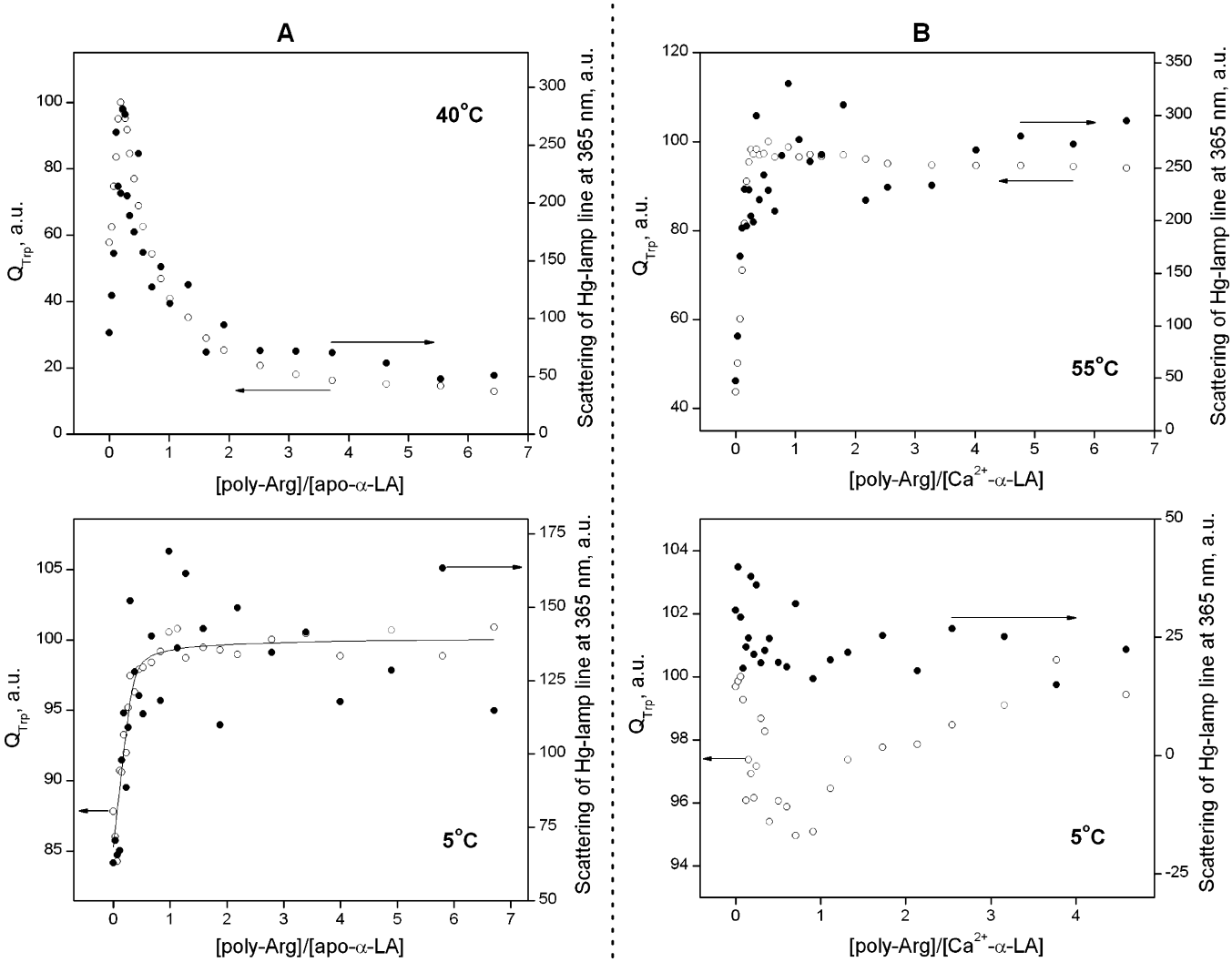


FIGURE 3: Spectrofluorimetric titration of apo (1 mM EDTA, A) and $Ca^{2+}$-saturated (1 mM $CaCl_2$, B) α-LA by poly-Arg (molecular mass of 10 750 Da) at 5, 40, or 55 °C (pH 7.6 and 10 mM HEPES-KOH). The excitation wavelength was 296.7 nm. The α-LA concentration was 4−6 $\mu$M. Empty circles represent the area under the fluorescence spectrum (arbitrary units), which is proportional to fluorescence quantum yield, $Q_{Trp}$. Filled circles represent the intensity of the scattered Hg lamp spectral line at 365 nm in arbitrary units. The solid curve fitted to the experimental points is calculated from a simple cooperative scheme of the binding of $n$ α-LA molecules to a molecule of histone [histone + $n$α-LA ↔ histone·$(α\text{-LA})_n$].

unfolded proteins. This predicts that bovine histone HIII should have a low content of ordered structure (*33*). Furthermore, using a variety of biophysical techniques, it has been recently established that all bovine core histones (HIIA, HIIB, HIII, and HIV) possess little or no ordered structure under physiological conditions, thus demonstrating clear signs of natively unstructured proteins (*33*). This is in good agreement with earlier studies, where it has been shown that pure histones dissolved in water with no added salt are in an "extended loose form" (*34*−*38*); however, in the presence of salt, they adopt a folded conformation (*34*−*38*).

To test the possibility of the electrostatic nature of the interactions between α-LA and histone HIII, we have performed a set of titrations of α-LA with charged poly-amino acids possessing molecular masses similar to that of histone HIII. Poly-Arg and poly-Lys molecules are positively charged at neutral pH and are known to resemble random coil structures at low ionic strengths (*39*−*41*), which makes them a simple model of a highly positively charged histone molecule.

Changes in the thermal stability of α-LA, caused by interaction with poly-L-Arg (molecular mass of 10 750 Da) at pH 7.6, were analogous to those observed for histone HIII (Table 1), but their magnitude was larger. The interaction stabilizes the apo form of α-LA by 5−6 °C, while the $Ca^{2+}$-saturated protein becomes less stable by 20 °C. Similarly, changes in the fluorescence spectrum maximum position caused by the binding of α-LA to poly-Arg resemble those induced by the histone binding, being, however, even more pronounced. In fact, the poly-Arg-induced blue shift in the fluorescence spectrum for the molten globule state of apo-α-LA was ∼5 nm versus an ∼3 nm shift induced by histone HIII binding. The more pronounced changes in the structural properties and thermal stability of α-LA correlate well with a higher net charge of the poly-Arg molecule: +55 versus +20 for histone HIII.

Interaction of the α-lactalbumin molten globule state (apo form at 40 °C) with poly-Arg (molecular mass of 10 750 Da), as followed by the tryptophan fluorescence of α-LA, exhibited a single maximum of fluorescence intensity at an α-LA to poly-Arg molar ratio of ∼5.4 (Figure 3A). This indicates that the binding of five to six molecules of α-LA to a molecule of poly-Arg was not cooperative. Formation of the α-LA−poly-Arg complex was followed by its partial

dissociation after an increase in the poly-Arg concentration due to the redistribution of α-LA molecules over the poly-Arg molecules.

The analysis of the spectrofluorimetric titration of native apo-α-LA with poly-Arg (molecular mass of 10 750 Da) at a low temperature (5 °C) (Figure 3A) using the cooperative scheme (eq 1) gave a stoichiometry of binding of 2.5 molecules of α-LA per poly-Arg molecule and an effective equilibrium association constant of $2.6 \times 10^6$ $M^{-1}$ (dissociation constant of 0.38 $\mu$M). The light scattering changes of the sample during the titration experiment indicated that formation of the α-LA−poly-Arg complex was accompanied by the irreversible aggregation, as the decrease in scattering at elevated concentrations of poly-Arg was not observed in this case. The irreversibility of aggregation probably results in the disappearance of the phase of partial dissociation of the α-LA−poly-Arg complex.

Interestingly, according to the data presented in Table 1, the binding of $Ca^{2+}$-saturated α-LA to poly-Arg at 55 °C converted native α-LA to an unfolded state (Figure 3B). As expected for α-LA unfolding, the binding of poly-Arg causes a pronounced (∼12 nm) red shift in the protein fluorescence spectrum and a significant (>2-fold) increase in the fluorescence quantum yield. The stoichiometry of binding in this case equals 3.8 molecules of α-LA per poly-Arg molecule. Analogous experiments with native $Ca^{2+}$-saturated α-lactalbumin at 5 °C did not reveal noticeable aggregation effects and demonstrated both formation and dissociation of the α-LA−poly-Arg complex (Figure 3B), with ∼2.3 molecules of α-LA being bound per poly-Arg molecule.

Interaction of α-lactalbumin with poly-L-Lys (molecular mass of 11 450 Da) shares many common features with that for histone HIII and poly-L-Arg (molecular mass of 10 750 Da) but shows some differences. For example, the binding of poly-Lys caused similar changes in the thermal stability of α-LA, but the magnitude of these changes was quite surprising. Despite the similarity in structures and the identical average degree of polymerization (55−56), the destabilization of $Ca^{2+}$-saturated α-LA by poly-Lys was twice as weak as that of poly-Arg (∼9 °C vs ∼20 °C for poly-Arg), resembling the effect of histone HIII (∼7 °C; see Table 1). At the same time, stabilization of apo-α-LA upon the binding of poly-Lys (∼11−12 °C) was twice as strong as that induced by the poly-Arg binding (∼5−6 °C). Furthermore, the fluorescence spectrum maximum position of α-LA turned out to be sensitive to the binding of poly-Lys only in the case of the apo form, demonstrating an ∼1−3 nm blue shift.

The titration of the molten globule of α-LA (apo form at 40 °C) with poly-Lys resulted in spectral changes indistinguishable from those induced by poly-Arg, demonstrating a slightly smaller stoichiometry of binding (4.4 vs 5.4 for poly-Arg). This difference may result from the shorter hydrodynamic radius of the poly-Lys chain due to the shortened and less bulky side chain of lysine, compared to that of the arginine residue. On the contrary, the binding of poly-Lys to native apo-α-LA at 5 °C demonstrated a higher stoichiometry of binding: 3.3 versus 2.6 for poly-Arg. This contradiction could be explained by the distortion of the poly-Arg titration curve due to the irreversible aggregation of the α-LA−poly-amino acid complex that is evident from consideration of scattering changes, accompanying titration, which was absent in the case of titration of α-LA with poly-Lys. Nevertheless, the same relation between the stoichiometries (4.0 for poly-Lys vs 2.3 for poly-Arg) remained in the case of the binding to native $Ca^{2+}$-saturated α-LA at 5 °C, despite the absence of any accompanying aggregation.

An interesting feature common to all titrations of α-LA with the positively charged poly-amino acids is the increase in the stoichiometry of binding upon thermal denaturation of α-LA (see Table 2). Thus, the loss of the rigid tertiary structure of α-LA promotes the binding of more molecules to poly-amino acids. Surprisingly, this phenomenon was not observed for the binding of histone HIII, demonstrating virtually the same stoichiometry of binding regardless of the α-LA state.

As expected, the analysis of the interaction of α-LA with poly-L-Arg and poly-L-Lys with different molecular masses has shown that changes in the α-LA thermal stability accompanying the binding process were virtually independent of the molecular mass, whereas the binding stoichiometries were nearly proportional to the charged molecule surface area, estimated as an area of a sphere with a radius equal to the hydrodynamic radius of the random coil structure (data not shown).

Spectrofluorimetric experiments on binding of negatively charged poly-L-Glu (molecular mass of 45 600 Da) to α-LA did not reveal any spectral or thermal stability changes in α-LA (data not shown), indicating the lack of direct interactions between the homopolymer and α-LA in this case. Taking into account the fact that like poly-Arg and poly-Lys, the poly-L-Glu molecule at neutral pH is a random coil (*39*), we find that the absence of binding of poly-L-Glu to α-LA confirms that the interaction between charged poly-amino acids and α-LA is driven by electrostatic interactions between negatively charged α-LA and positively charged side chains of poly-amino acids, and excludes the possibility that this interaction is primarily due to formation of hydrogen bonds between the α-LA molecule and the backbone of poly-amino acids.

Since formation of the complex between α-LA and histone HIII shares many common features with the analogous interaction of a protein with poly-Arg and poly-Lys (stoichiometry of binding, interaction-induced spectral and thermal stability changes of α-LA, and the tendency of the complexes to aggregate), it can be concluded that the nature of the interaction in both cases is the same. The electrostatic nature of the interaction between α-LA and histone HIII was also corroborated by titration experiments performed under high-ionic strength conditions. Addition of 100 mM KCl results in essential reduction of all the spectral effects upon titration of α-LA with histone HIII (data not shown).

The fact that several molecules of α-LA bind to a single histone HIII, poly-Arg, or poly-Lys molecule and not vice versa suggests that the binding of α-LA occurs via a single negatively charged site. Most likely, this site consists of carboxylic groups of the $Ca^{2+}$-coordinating loop of the strong α-LA calcium-binding site (D82, D83, D84, D87, and D88) along with some other nearby negative groups (for example, E7, D37, and D78) (*42*). This idea is supported by the character of α-LA thermal stability changes caused by histone HIII and poly-Arg and poly-Lys binding (see Table 1). The stabilization of the α-LA apo form is likely to result

from a compensation of excessive negative charge in the $Ca^{2+}$-binding loop of the protein, which destabilizes apo-α-LA (*43*). The destabilization of $Ca^{2+}$-saturated α-LA upon the binding of histone HIII and poly-Arg and poly-Lys seems to reflect a decrease in the affinity of α-LA for calcium ions caused by a competition between the positively charged compounds and $Ca^{2+}$ ions for the same site. Indeed, an addition of a 0.6-fold molar excess of poly-Arg over α-LA results in a 2.5 order of magnitude decrease in the apparent $Ca^{2+}$ binding equilibrium association constant ($7 \times 10^4$ $M^{-1}$ vs $2 \times 10^7$ $M^{-1}$ without poly-Arg, 40 °C), as estimated by spectrofluorimetric titrations according to the method described in ref *26*. Identical stability changes were observed upon replacement of Asp87, coordinating the calcium ion in the $Ca^{2+}$ site of α-LA, with Asn (*29*).

Importantly, this conclusion is further supported by the results of earlier studies on the effect of different polyamines (spermine, spermidine, and putrescine) on the spectral properties and thermal stability of several α-LAs (*44*). It has been shown that the addition of spermine to the apo form of α-LAs resulted in spectral changes similar to those induced by $Ca^{2+}$ binding and gave rise to the noticeable increase in thermal stability. Furthermore, a direct competition of spermine with $Mn^{2+}$ for the binding to the $Ca^{2+}$ site of bovine α-LA has been observed (*44*). A binding model based on the atomic coordinates of α-LA and spermine was constructed by computer modeling. To explain the results of binding experiments, this model used the cluster of negatively charged glutamate residues present in the N-terminal sequence of bovine α-LA, sterically close to the $Ca^{2+}$ site. It has been shown that the spermine molecule could be nicely fitted in a space delineated by the $Ca^{2+}$ site, on one hand, and Glu1, Glu7, and Glu11, on the other (*44*).

It is important to emphasize that the low-ionic strength conditions inside cell nucleus, which facilitate interaction between histone proteins and the DNA molecule, will also promote electrostatic interaction between α-LA and histones. This shows that α-LA can be used as a histone-arresting agent in an effort to design tumor-suppressing medicinal preparations. The results of Svanborg's group on modification of α-lactalbumin by oleic acid imparting marked antitumor properties to α-LA via this mechanism (*14*) are very encouraging from this point of view.

Interestingly, bovine α-LA itself also has antiproliferative effects in human colon adenocarcinoma cell lines (*45*). It has been shown that low α-LA concentrations (10−25 μg/mL) stimulated cell growth during the first 3−4 days. After the cells had grown for 4 days, the proliferation ceased and the number of viable cell decreased dramatically in the α-LA-treated cultures, suggesting a delayed initiation of apoptosis (*45*). Thus, the modification of α-LA with a fatty acid is not an absolute prerequisite for the antiproliferative effect with respect to some tumor cells. The observed delay for several days in α-LA action is possibly due to the passive trafficking of the protein to nucleus, where it effectively blocks histones. The passive transport of α-LA to the nucleus is potentially feasible, taking into account the ability of this protein to interact with lipid membranes (*46*−*48*). Thus, the ability of α-LA to bind histones and the ability of the protein to bind to lipid membranes are possibly sufficient to impart antitumor properties to α-LA.

Overall, the data indicate that α-LA can be used as a basis for design of antitumor agents, acting through disorganization of chromatin structure due to electrostatic interaction between α-LA and histone proteins. Oleic acid modification of α-LA is probably just a first successful step to creation of medicinal preparations of this kind.

## REFERENCES


1. Permyakov, E. A., and Berliner, L. J. (2000) α-Lactalbumin: structure and function, *FEBS Lett. 473*, 269−274.
2. Hiraoka, Y., Segawa, T., Kuwajima, K., Sugai, S., and Murai, N. (1980) α-Lactalbumin: A calcium metalloprotein, *Biochem. Biophys. Res. Commun. 95*, 1098−1104.
3. Permyakov, E. A., Kalinichenko, L. P., Morozova, L. A., Yarmolenko, V. V., and Burstein, E. A. (1981) α-Lactalbumin binds magnesium ions: Study by means of intrinsic fluorescence technique, *Biochem. Biophys. Res. Commun. 102*, 1−7.
4. Murakami, K., and Berliner, L. J. (1983) A distinct zinc-binding site in the α-lactalbumins regulates calcium-binding: Is there a physiological role for this control, *Biochemistry 22*, 3370−3374.
5. Permyakov, E. A., Shnyrov, V. L., Kalinichenko, L. P., Kuchar, A., Reyzer, I. L., and Berliner, L. J. (1991) Binding of Zn(II) ions to α-lactalbumin, *J. Protein Chem. 10*, 577−584.
6. Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. V., Bychkova, V. E., Semisotnov, G. V., Venyaminov, S. Y. U., and Ptitsyn, O. B. (1981) α-Lactalbumin: compact state with fluctuating tertiary structure? *FEBS Lett. 136*, 311−315.
7. Hakansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H., and Svanborg, C. (1995) Apoptosis Induced by a Human-Milk Protein, *Proc. Natl. Acad. Sci. U.S.A. 92*, 8064−8068.
8. Pellegrini, A., Thomas, U., Bramaz, N., Hunziker, P., and von Fellenberg, R. (1999) Isolation and identification of three bactericidal domains in the bovine α-lactalbumin molecule, *Biochim. Biophys. Acta 1426*, 439−448.
9. Svensson, M., Sabharwal, H., Hakansson, A., Mossberg, A. K., Lipniunas, P., Leffler, H., Svanborg, C., and Linse, S. (1999) Molecular characterization of α-lactalbumin folding variants that induce apoptosis in tumor cells, *J. Biol. Chem. 274*, 6388−6396.
10. Kohler, C., Hakansson, A., Svanborg, C., Orrenius, S., and Zhivotovsky, B. (1999) Protease activation in apoptosis induced by MAL, *Exp. Cell Res. 249*, 260−268.
11. Hakansson, A., Andreasson, J., Zhivotovsky, B., Karpman, D., Orrenius, S., and Svanborg, C. (1999) Multimeric α-lactalbumin from human milk induces apoptosis through a direct effect on cell nuclei, *Exp. Cell Res. 246*, 451−460.
12. Svensson, M., Hakansson, A., Mossberg, A. K., Linse, S., and Svanborg, C. (2000) Conversion of α-lactalbumin to a protein inducing apoptosis, *Proc. Natl. Acad. Sci. U.S.A. 97*, 4221−4226.
13. Svensson, M., Fast, J., Mossberg, A. K., Duringer, C., Gustafsson, L., Hallgren, O., Brooks, C. L., Berliner, L., Linse, S., and Svanborg, C. (2003) α-Lactalbumin unfolding is not sufficient to cause apoptosis, but is required for the conversion to HAMLET (human α-lactalbumin made lethal to tumor cells), *Protein Sci. 12*, 2794−2804.
14. Duringer, C., Hamiche, A., Gustafsson, L., Kimura, H., and Svanborg, C. (2003) HAMLET interacts with histones and chromatin in tumor cell nuclei, *J. Biol. Chem. 278*, 42131−42135.
15. de Laureto, P. P., Frare, E., Gottardo, R., and Fontana, A. (2002) Molten globule of bovine α-lactalbumin at neutral pH induced by heat, trifluoroethanol, and oleic acid: A comparative analysis by circular dichroism spectroscopy and limited proteolysis, *Proteins: Struct., Funct., Genet. 49*, 385−397.
16. Kaplanas, R. I., and Antanavichyus, A. I. (1975) Isolation and purification of α-lactalbumin, a component of lactose synthetase, *Biochemistry (Moscow) 40*, 493−495.
17. Kronman, M. J., Andreotti, R. E., and Vitols, R. (1964) Inter- and intramolecular interactions of α-lactalbumin. II. Aggregation reactions at acid pH, *Biochemistry 3*, 1152−1160.
18. Marzluff, W. F., Jr., Sanders, L. A., Miller, D. M., and McCarty, K. S. (1972) Two chemically and metabolically distinct forms of calf thymus histone F3, *J. Biol. Chem. 247*, 2026−2033.
19. Patthy, L., and Smith, E. L. (1975) Histone III. VI. Two forms of calf thymus histone III, *J. Biol. Chem. 250*, 1919−1920.

20. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein, *Protein Sci. 4*, 2411−2423.
21. Permyakov, E. A., Burstein, E. A., Sawada, Y., and Yamazaki, I. (1977) Luminescence of phenylalanine residues in duperoxide-dismutase from green pea, *Biochim. Biophys. Acta 491*, 149−154.
22. Burstein, E. A., and Emelyanenko, V. I. (1996) Log-normal description of fluorescence spectra of organic fluorophores, *Photochem. Photobiol. 64*, 316−320.
23. Marquardt, D. W. (1963) An algorithm for least squares estimation of nonlinear parameters, *J. Soc. Ind. Appl. Math. 11*, 431−441.
24. Permyakov, E. A., and Burstein, E. A. (1984) Some aspects of studies of thermal transitions in proteins by means of their intrinsic fluorescence, *Biophys. Chem. 19*, 265−271.
25. Permyakov, E. A. (1993) *Luminescent Spectroscopy of Proteins*, CRC Press, Inc., Boca Raton, FL.
26. Permyakov, E. A., Yarmolenko, V. V., Kalinichenko, L. P., Morozova, L. A., and Burstein, E. A. (1981) Calcium-binding to α-lactalbumin: Structural rearrangement and association constant evaluation by means of intrinsic protein fluorescence changes, *Biochem. Biophys. Res. Commun. 100*, 191−197.
27. Vanhooren, A., Devreese, B., Vanhee, K., Van Beeumen, J., and Hanssens, I. (2002) Photoexcitation of tryptophan groups induces reduction of two disulfide bonds in goat α-lactalbumin, *Biochemistry 41*, 11035−11043.
28. Permyakov, E. A., Permyakov, S. E., Deikus, G. Y., Morozova-Roche, L. A., Grishchenko, V. M., Kalinichenko, L. P., and Uversky, V. N. (2003) Ultraviolet illumination-induced reduction of α-lactalbumin disulfide bridges, *Proteins: Struct., Funct., Genet. 51*, 498−503.
29. Permyakov, S. E., Uversky, V. N., Veprintsev, D. B., Cherskaya, A. M., Brooks, C. L., Permyakov, E. A., and Berliner, L. J. (2001) Mutating aspartate in the calcium-binding site of α-lactalbumin: effects on the protein stability and cation binding, *Protein Eng. 14*, 785−789.
30. Uversky, V. N., Gillespie, J. R., and Fink, A. L. (2000) Why are "natively unfolded" proteins unstructured under physiologic conditions? *Proteins 41*, 415−427.
31. Uversky, V. N. (2002) Natively unfolded proteins: a point where biology waits for physics, *Protein Sci. 11*, 739−756.
32. Uversky, V. N. (2002) What does it mean to be natively unfolded? *Eur. J. Biochem. 269*, 2−12.
33. Munishkina, L. A., Fink, A. L., and Uversky, V. N. (2004) Formation of amyloid fibrils from the core histones *in vitro*, *J. Mol. Biol.* (in press).
34. Bradbury, E. M., Crane-Robinson, C., Phillips, D. M. P., Johns, E. W., and Murray, K. (1965) Conformational investigations on histones, *Nature 205*, 1315−1316.
35. Boublik, M., Bradbury, E. M., and Crane-Robinson, C. (1970) An investigation of the conformational changes in histones F1 and F2a1 by proton magnetic resonance spectroscopy, *Eur. J. Biochem. 14*, 486−497.
36. Li, H. J., Wickett, R., Craig, A. M., and Isenberg, I. (1972) Conformational changes in histone IV, *Biopolymers 11*, 375−397.
37. Wickett, R. R., Li, H. J., and Isenberg, I. (1972) Salt effects on histone IV conformation, *Biochemistry 11*, 2952−2957.
38. Isenberg, I. (1979) Histones, *Annu. Rev. Biochem. 48*, 159−191.
39. Holzwarth, G., and Doty, P. (1965) The ultraviolet circular dichroism of polypeptides, *J. Am. Chem. Soc. 87*, 218−228.
40. Rialdi, G., and Hermans, J., Jr. (1966) Calorimetric heat of the helix-coil transition of poly-L-glutamic acid, *J. Am. Chem. Soc. 88*, 5719−5720.
41. Tiffany, M. L., and Krimm, S. (1968) New chain conformations of poly(glutamic acid) and polylysine, *Biopolymers 6*, 1379−1382.
42. Acharya, K. R., Ren, J. S., Stuart, D. I., Phillips, D. C., and Fenna, R. E. (1991) Crystal structure of human α-lactalbumin at 1.7 Å resolution, *J. Mol. Biol. 221*, 571−581.
43. Griko, Y. V., and Remeta, D. P. (1999) Energetics of solvent and ligand-induced conformational changes in α-lactalbumin, *Protein Sci. 8*, 554−561.
44. Morozova, L., Desmet, J., and Joniau, M. (1993) Complexes of the polyamines spermine, spermidine and putrescine with α-lactalbumins, *Eur. J. Biochem. 218*, 303−309.
45. Sternhagen, L. G., and Allen, J. C. (2001) Growth rates of a human colon adenocarcinoma cell line are regulated by the milk protein α-lactalbumin, *Adv. Exp. Med. Biol. 501*, 115−120.
46. Grishchenko, V. M., Kalinichenko, L. P., Deikus, G. Y., Veprintsev, D. B., Cawthern, K. M., Berliner, L. J., and Permyakov, E. A. (1996) Interactions of α-lactalbumins with lipid vesicles studied by tryptophan fluorescence, *Biochem. Mol. Biol. Int. 38*, 453−466.
47. Cawthern, K. M., Permyakov, E., and Berliner, L. J. (1996) Membrane-bound states of α-lactalbumin: implications for the protein stability and conformation, *Protein Sci. 5*, 1394−1405.
48. Hanssens, I., Houthuys, C., and Cauwelaert, F. H. (1979) Interaction between DMPC vesicles and the peripheral protein α-lactalbumin, *Arch. Int. Physiol. Biochim. 87*, 1017−1018.

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