

Short communication

A differential scanning calorimetric study of the influence of copper and dodecyl trimethyl ammonium bromide on the stability of bovine α -lactalbumin

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Abstract

Bovine α -lactalbumin (α -LA) has been studied by differential scanning calorimetry (DSC), fluorescence spectroscopy and viscometry with various concentrations of Cu^{2+} and DTAB to elucidate the effect of these ligands on its thermal properties. The DSC profile of dialyzed form of α -lactalbumin (m- α -LA) contrary to the undialyzed form (holo-form, h- α -LA) shows two temperature induced heat absorption peaks. The m- α -LA is not a new form of α -LA. It contains mixture of the apo (a- α -LA) and holo (h- α -LA) forms of α -LA at low and high temperatures, respectively. Therefore, these two states of α -LA (apo and holo) are equilibrating with together after dialyze experiment. The Cu^{2+} as a metal ion and DTAB as a non metal ion alter the two heat-absorption peaks, in such a manner that, the addition of Cu^{2+} to the m- α -LA increases partial molar heat capacity and enthalpy change values of the h- α -LA form at high temperature because the molecular population of the a- α -LA form changes into the h-like- α -LA. On the contrary, the interaction between the DTAB and the m- α -LA increases these thermodynamic values for the a- α -LA at low temperature. However, DTAB bound to m- α -LA prevents from Ca^{2+} binding to protein, because there are positive charges repulsion between them. The high temperature peak occurs at the same temperature as the unfolding of the h- α -LA, while the low temperature peak lies within the temperature range associated with the unfolding of the a- α -LA. The R_s values of m- α -LA, h- α -LA and a- α -LA forms confirmed the folding and unfolding of the m- α -LA during the addition of Cu^{2+} and DTAB at different concentration, respectively.

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1. Introduction

The α -lactalbumin (α -LA) is a 14300 Da acidic milk protein, which is the specifier component of lactose synthase in the lactating mammary gland [1,2]. Its physical characteristics and folding properties are significantly affected by specific interactions with Ca^{2+} . It is a metal-binding protein,

which binds Ca^{2+} and Na^+ ions competitively to one specific site, giving rise to a large conformational change of the protein. The removal of bound calcium greatly decreases the thermal stability of α -LA but the protein retains essentially the same folded conformation [3,4]. One of the most interesting properties of α -LA is its transition to the molten globule state [5–7]. At elevated temperatures above the thermal denaturation transition (ca. $T = 323$ K for apo-protein), at intermediate denaturant concentrations (3–4 M urea), or when Ca^{2+} dissociates from α -LA at acidic pH (< 3), the protein adopts the molten globule conformation, which has been described as a compact state containing a significant degree of the secondary structure present in the native pro-

Abbreviations: a- α -LA, apo form of α -LA; DSC, differential scanning calorimetry; DTAB, dodecyl trimethyl ammonium bromide; h- α -LA, holo (undialyzed) form of α -LA; α -LA, α -lactalbumin; m- α -LA, dialyzed form of α -LA (mixture of apo-like and holo-like forms); R_s , Stoke radius

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tein, but with fluctuating tertiary structure [8]. At neutral pH and in the presence of calcium, α -LA unfolds cooperatively at high temperatures, with significant increase of enthalpy and heat capacity [9]. At lower pH, α -LA unfolds in two stages [9,10]. The first stage is highly cooperative and proceeds with significant and sharp heat absorption, but at this stage the heat capacity does not reach the value expected for the fully unfolded polypeptide chain [8]. This value is reached at the second stage, which proceeds without a significant heat absorption peak. It is therefore unclear whether this second stage represents a cooperative process with a small enthalpy, or is a gradual process [10]. Analysis of the change of optical properties associated with these two stages, of their dependence on the presence of calcium and comparison with the unfolding of the structurally related equine lysozyme, permits the assignment of the first cooperative stage to the unfolding of the β -domain of α -LA and the second stage to the unfolding of the α -domain [9,10]. The presence of domains that have temperature-dependent unfolding was also verified by fluorescence studies [11]. However, it was unclear as to how independent are these domains, i.e., whether one can fold and be stable without the other. This point needs to be clarified in order to understand the nature of the intermediate state of α -LA, which is usually regarded as a liquid-like “molten globule” state [12,13], rather than a partly unfolded state of a two-domain protein with one retained domain.

Yutani et al. [14] have studied calorimetrically the calcium-free a- α -LA, which is supposed to be in the molten globule state in low ionic strength solutions: they did not observe any excess heat effect upon heating. It was therefore assumed that this state of α -LA is close to the unfolded state [7]. However, Relkin et al. [15] studied α -LA at low concentrations of calcium using a Perkin-Elmer scanning calorimeter: they observed two heat absorption peaks upon heating the solution. The first peak was attributed to the thermal denaturation of the a- α -LA form and the second one to the h- α -LA form.

This partly unfolded state occurs because removal of Ca^{2+} results in the appearance of strong repulsive forces between uncompensated negative charges at the calcium-binding site. In the presence of monovalent salts a- α -LA has a native like structure that unfolds cooperatively upon heating with significant heat absorption, although at much lower temperatures than the h- α -LA [3,9]. The Ca^{2+} binding is necessary for the native folding of α -LA, and the structure of the metal ion-depleted form is a typical molten globule [16–19]. Although the structure of a- α -LA has been frequently investigated, the structure of Zn^{2+} -h- α -LA in solution is not well known. Berliner and co-workers suggested that Zn^{2+} binding to Ca^{2+} -bound α -LA (h- α -LA) shifts the structure towards a new apo-like conformer [20–25]. The intrinsic fluorescence spectrum of h- α -LA is shifted to that of a- α -LA by the second ion binding. The binding constant of the hydrophobic fluorescent probe for h- α -LA also increased when Zn^{2+} was bound to h- α -LA. On the other hand, results from X-ray crystallography revealed that the crystal structure of Zn^{2+} -bound

h- α -LA is not significantly different from that of h- α -LA [26].

In this paper we demonstrate, using sensitive scanning microcalorimetry techniques, that a solution of α -LA at pH 8.0 in dialyzed condition represents a mixture of apo- α -LA (a- α -LA) and holo- α -LA (h- α -LA) forms that do not readily interconvert, and therefore unfold at different temperatures. The interaction between α -LA and ions (Cu^{2+} and DTAB) show that these ions change the partial heat capacity and enthalpy change values of a- α -LA and h- α -LA forms. These calorimetric data permit a reliable determination of the molecular population partition for a- α -LA and h- α -LA forms interaction with Cu^{2+} and DTAB, a quantity difficult to obtain by other methods.

2. Materials and methods

2.1. Materials

Bovine α -LA was obtained from Merck Chemical Co. Purity of the protein was monitored by PAGE under native and denatured conditions. The concentration of protein solution was measured spectrophotometrically using an extinction coefficient of $E^{1\%1\text{cm}}(280\text{ nm}) = 20.9$ [27] with correction for light-scattering effects. A- α -LA was prepared by previously described procedures [28]. The concentrations of a- α -LA and m- α -LA were measured by Bradford assay [29] and Stoschek method's [30]. Dodecyl trimethyl ammonium bromide (DTAB) and copper sulfate (Cu^{2+}) were obtained from Sigma and Merck, respectively. Visking membrane dialysis tubing (molar mass cut-off 10,000–14,000 g mol^{-1}) was obtained from SIC, Eastleigh, Hampshire, UK. All other materials and reagents were of analytical grade, and solutions were made in double-distilled water. Tris solution of concentration 10 mmol dm^{-3} , pH 8.0 was used as a buffer.

Visking tubing, as the semipermeable membrane, was boiled three times, each time for 15 min in ethylenediaminetetraacetic acid (EDTA) and sodium bicarbonate and then washed several times with distilled water and stored in (0.2 $\text{C}_2\text{H}_5\text{OH} + 0.8 \text{H}_2\text{O}$). Bovine α -LA solution was dialyzed against buffer (Tris, 10 mM, pH 8.0). The buffer had been changed with new buffer every 8 h, m- α -LA was ready after 24 h.

2.2. Methods

2.2.1. Fluorescence measurements

Fluorescence measurements were made on Jasco SP-6200 spectrofluorometer at an excitation wavelength of 280 nm (while identical spectral line shapes were observed over the excitation wavelength range 280–295 nm, it was experimentally preferable to use a 280 nm excitation to reduce any light scattering problems in the emission spectra on this instruments). Trp fluorescence for apo- α -LA (a- α -LA) and holo- α -LA (h- α -LA) forms was followed at 335 and 325 nm,

respectively [31]. The Cu^{2+} and DTAB significantly affect the fluorescence of free tryptophan under the experimental conditions used. The temperature of the cell compartments was kept constant at $T = 293$ K by water circulations.

2.2.2. Measurements of viscosity and Stokes radius

The viscosity was measured using a Haake D8 (W. Germany) microviscometer. The intrinsic viscosities, $[\eta]$, and Stokes radii, R_s , of the m- α -LA, a- α -LA and h- α -LA forms and Cu^{2+} , DTAB- α -LA complexes at different concentrations of ligands were determined using the equation [32,33]:

$$\frac{\eta_{\text{sp}}}{c} \cong [\eta] = \lim_{c \rightarrow 0} \left[\frac{\eta/\eta_0 - 1}{c} \right] = \frac{2.5N_A}{M} \left(\frac{4}{3}\pi R_s^3 \right) \quad (1)$$

where η_{sp} is the specific viscosity, c the protein concentration in g cm^{-3} , N_A the Avogadro's number, M the molar mass of the protein and π is equal to 3.14

2.2.3. Differential scanning calorimetric (DSC) measurements

DSC experiments were performed on a Scal differential scanning microcalorimeter (Institute for Biological Instrumentation, Pushchino, Russia) with cell volumes of 0.48 cm^3 at a scanning rate of 1 K min^{-1} (was kept constant in all experiments), interfaced with a personal computer (IBM compatible). Prior to DSC experiments, the protein solutions were dialyzed for 24 h at $T = 277$ K against three changes of a large volume of 10 mM Tris at pH 8.0, Cu^{2+} and DTAB at different concentrations. Before the measurements, samples were degassed by stirring in an evacuated chamber at room temperature and then immediately loaded into the calorimeter cell; the final dialysis buffer (also degassed) was loaded into the reference cell. A pressure of 152 kPa (1.5 atm) of dry nitrogen was always kept over the liquids in the cells through out the scans to prevent any degassing during heating. The reversibility of the thermal transitions was checked by a second heating of the cool sample immediately following the first scan. The calorimetric traces were corrected for the instrumental background by subtracting a scan with buffer in both cells. The reactive errors of the values of molar enthalpy changes are in the range of 3% and the absolute errors of given transition temperatures T_m are 0.3°C . The fittings were done based on Privalov and Potekhin theory [34], which was installed as DOS programme in the software package (named Scal-2) and supplied by Scal (Russia). The Scal-2 programme, which is installed in the DSC instrument, allows the determination of the native and denatured lines based on fitting error. The best fitting error is selected as a best curve. The baseline preparation was done by Tris (10 mM) including Cu^{2+} and DTAB at different concentrations in both sample and reference cells. The molar heat capacity was calculated for bovine α -LA according [34] using a molar mass of 14300 Da and a partial molar specific volume $v = 0.709 \text{ cm}^3 \text{ g}^{-1}$. The concentration of protein used in the calorimetric experiments was 1 mg cm^{-3} . The temperature dependencies of the heat capacity of α -LA in the unfolded state were calculated at different temperatures according to [35].

ity of α -LA in the unfolded state were calculated at different temperatures according to [35].

3. Results and discussion

3.1. Differential scanning calorimetry

Fig. 1 shows the DSC profiles for h- α -LA, m- α -LA and a- α -LA forms in 10 mM Tris (pH 8.0). The native state of h- α -LA showed a sharp heat absorption peak with a $271 \pm 3 \text{ kJ mol}^{-1}$ calorimetric enthalpy change value representing the cooperative melting of the a- α -LA exhibited a small and broad curve with a $126 \pm 3 \text{ kJ mol}^{-1}$ calorimetric enthalpy change value. The shapes of the DSC profiles and the calorimetric enthalpy change values of the h- α -LA and a- α -LA forms closely resemble those reported by Hendrix et al. [36], thus implying a close structural similarity. The transition points (T_m) of h- α -LA and a- α -LA forms were $T = 337.3$ and 303.2 K, respectively (see Fig. 1). The temperature dependencies of partial molar heat capacities of m- α -LA in 10 mM Tris (pH 8.0) with the same condition has been shown as (3) curve. The DSC profile of the m- α -LA showed two peaks, a sharp heat absorption peak and a small, broad curve with the transition temperature of 337.3 and 302.2 K, respectively. The low-temperature peak can be assigned to the a- α -LA and the high-temperature curve can be related to the h- α -LA. The transition points (T_{ms}) of the a- α -LA and h- α -LA in DSC profile of α -LA after dialyze experiment are similar to the a- α -LA and h- α -LA forms, respectively. The DSC scan was

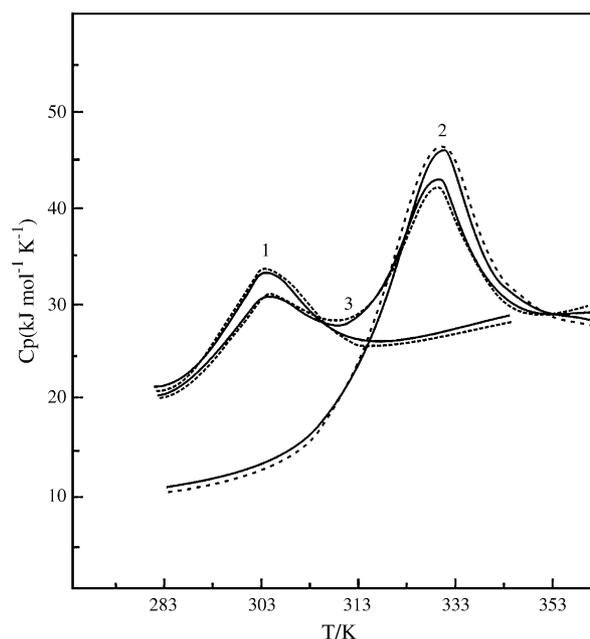


Fig. 1. Plot of measured heat capacity against temperature for the various conformational states of α -LA. (1) a- α -LA form in 10 mM Tris (pH 8.0); (2) h- α -LA form in 10 mM Tris (pH 8.0); (3) the m- α -LA form in 10 mM Tris (pH 8.0). Dashed curves show the repeated scan of a- α -LA, h- α -LA and the m- α -LA in 10 mM Tris (pH 8.0) that are keeping the same sample solution of them in the cell in each experiments. Protein concentration was $70 \mu\text{M}$.

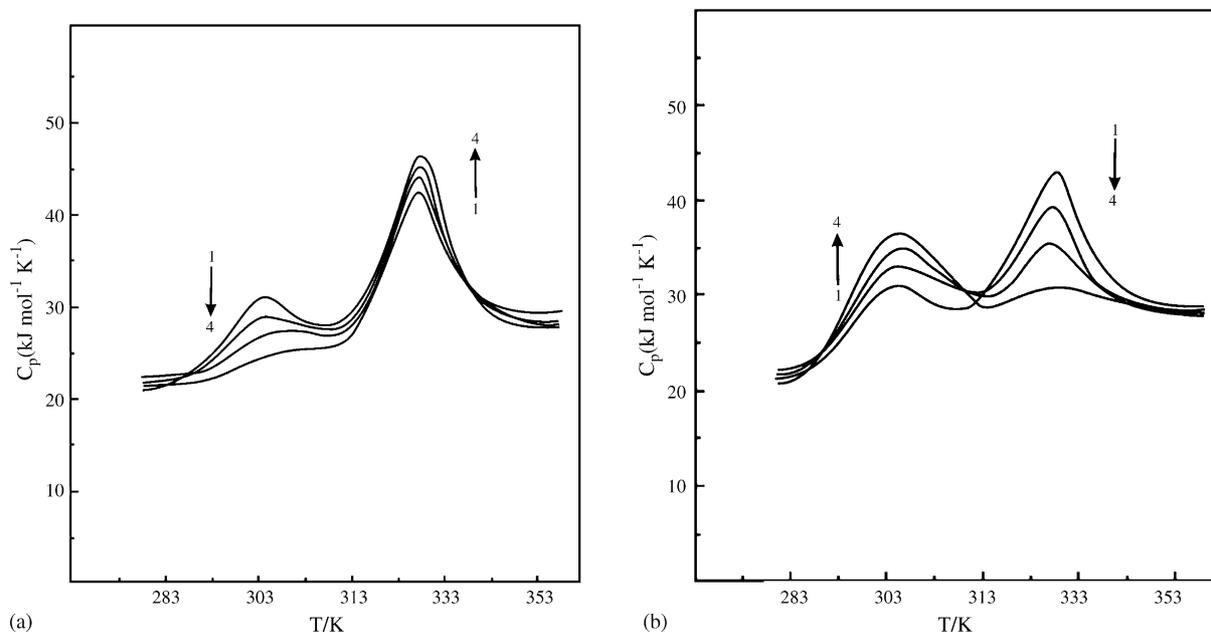


Fig. 2. (a) Plot of measured heat capacity against temperature of the m- α -LA in 10 mM Tris (pH 8.0) and various concentrations of Cu^{2+} . (1) 0 mM Cu^{2+} (m- α -LA); (2) 0.1 mM Cu^{2+} ; (3) 1 mM Cu^{2+} ; (4) 10 mM Cu^{2+} . (b) DSC thermograms of m- α -LA in 10 mM Tris (pH 8.0) and various concentrations of DTAB. (1) 0 mM DTAB (m- α -LA); (2) 0.5 mM DTAB; (3) 1 mM DTAB; (4) 1.5 mM DTAB. Protein concentration was 70 μM .

repeated twice, keeping the same sample solution in the cell. As can be seen from the superimposition of the DSC curves for a- α -LA, h- α -LA and m- α -LA forms, the unfolding transition were reversible for them (see Fig. 1, dashed curves).

Fig. 2(a) and (b) shows the temperature dependencies of partial molar heat capacities of the m- α -LA in 10 mM Tris at pH 8.0 with different relative concentrations of Cu^{2+} and DTAB. As the concentration of Cu^{2+} increases the major heat absorption peak increases in area giving rise to a second lower-temperature peak located at $T = 302.2$ K while the transition temperature of both peaks remained constant. This observation suggests that the influence of Cu^{2+} on the thermodynamic properties of α -LA depends on the concentration range of Cu^{2+} . At low concentrations of Cu^{2+} where the free Cu^{2+} in the solution is less than the protein concentrations and not all molecules of α -LA are bound to Cu^{2+} , two heat absorption peaks are clearly observed. At high concentrations of Cu^{2+} , we observe a large heat-absorption peak at high-temperature and a small peak at low-temperature. According to Fig. 2a, the addition of Cu^{2+} to the m- α -LA, decreases the partial heat capacity of small peak at low-temperature (a- α -LA) and increases the partial heat capacity of large peak at high temperature (h- α -LA).

Fig. 2b shows the effects of various concentrations of DTAB on the DSC profile of m- α -LA form. It is apparent that the addition of different concentrations of DTAB to the m- α -LA increases partial molar heat capacity of the peak at low-temperature (a- α -LA) and decreases partial molar heat capacity of the peak at high-temperature (h- α -LA). It is important to note that the molecular population of the a- α -LA has been increased in the presence of DTAB, whereas the molecular population of the h- α -LA decreased at the same

condition. Therefore, it can be clearly seen that the effect of DTAB on α -LA changes partial molar heat capacity. In addition, the enthalpy values change from the h- α -LA at high-temperature to the a- α -LA at low-temperature. The figures show that all the calorimetrically measured parameters of α -LA denaturation are in reasonable correspondence [28,36].

3.2. Fluorescence spectra

The intrinsic fluorescence emission spectra of bovine m- α -LA examined in this study were both dequenched and red shifted upon titration with DTAB. Fig. 3 shows the effect of DTAB on the fluorescence spectra of α -LA. According to Fig. 3, the addition of different concentrations of DTAB to α -LA causes an increase in the fluorescence intensity. Here, the interaction of α -LA with DTAB is consistent with the results obtained for a- α -LA and h- α -LA forms in the presence of several cations (e.g., Ca^{2+} and Mn^{2+}) as previously reported by Murakami et al. [31]. The addition of DTAB to the m- α -LA increased the peak area and peak maximum and correlated with an increase in DTAB concentration. On the other hand, emission spectra of interaction between α -LA and DTAB at pH 8.0 are shifted toward to red region and the relative intensity are increased markedly.

3.3. Intrinsic viscosity and Stokes radius

Table 1 shows intrinsic viscosity and Stokes radius (R_s) of the a- α -LA and h- α -LA forms, different structural states induced by various concentrations of DTAB and Cu^{2+} and the m- α -LA in 10 mM Tris at pH 8.0. The Stokes radii indicate different values for all states of α -LA and various structural

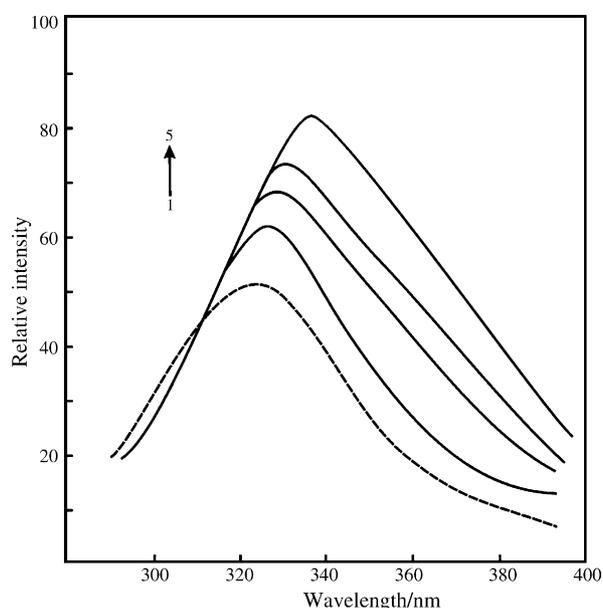
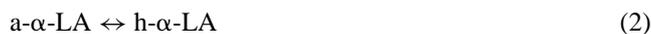


Fig. 3. Fluorescence spectra of α -LA with (relative intensity) plotted against wavelength at various concentrations of DTAB concentration at pH 8.0 ($\lambda_{\text{Excitation}} = 285 \text{ nm}$ and $\lambda_{\text{Emission}} = 325 \text{ nm}$). (1) dashed curve, 0 mM DTAB (m- α -LA); (2) 0.5 mM DTAB; (3) 1 mM DTAB; (4) 1.5 mM DTAB; (5) 2 mM DTAB. Protein concentration was $70 \mu\text{M}$.

forms induced by DTAB and Cu^{2+} . These results show the compaction of the different states of α -LA induced by Cu^{2+} relative to the native state for m- α -LA. On the other hand, the R_s values in Table 1 show the α -LA-DTAB complexes are expanded slightly compared with the native state of m- α -LA, and are much more compact than the a- α -LA form.

It is assumed from the above that the m- α -LA contains a mixture of the a- α -LA and h- α -LA forms at low and high temperatures, respectively. It should be mentioned that m- α -LA is not a new form of α -LA. We have used the term “m- α -LA” as protein solution with two different forms of α -LA (a- α -LA and h- α -LA) that are equilibrating with together

after dialyze experiment. If we take into account that these two forms differ drastically in stability and the denatured protein does not specially bind calcium, we can describe the observed process by the following scheme:



Such a process was analyzed in detail by Brandts and Lin [37] and Shrake and Ross [38]. Visiting tubing as the semipermeable membrane interchanges Ca^{2+} ions between the outside and inside of the dialyzed membrane. However, there are two forms of α -LA (a- α -LA and h- α -LA forms) in equilibrium in the protein solution. Thus, DSC profiles of m- α -LA and h- α -LA forms are different that show the presence of various molecular population in solution. It has been shown by Hiraoka and Sugai [6] that a- α -LA assumes a native like structure at low temperatures and an unfolded structure at $T = 298\text{--}302 \text{ K}$. Furthermore, it was observed that monovalent cations as Na^+ stabilize the apo-conformer in a similar way as the binding of one Ca^{2+} per α -LA [4,6]. The results conformed to the binding of one Na^+ per α -LA and the apparent binding energy was calculated. The effect of Zn^{2+} binding on the structure of α -LA was investigated. The α -LA binds Ca^{2+} and Zn^{2+} at different sites in a mutually non-exclusive manner. The structures of the metal-depleted form of α -LA (a- α -LA) and Ca^{2+} bound α -LA (h- α -LA) have been well characterized. The Zn^{2+} binding induces a local structural change on the h- α -LA, but it does not induce a large backbone conformational change [19,39]. The Cu^{2+} and DTAB play a similar role of Ca^{2+} and Na^+ in interaction with m- α -LA, respectively. The addition of Cu^{2+} to the m- α -LA, increases the partial heat capacity and enthalpy change of h-like- α -LA form at high temperature, meaning that Cu^{2+} can be bound at various sites of Ca^{2+} in the metal-depleted form of α -LA (a-like- α -LA) and equilibrium (a-like- α -LA \leftrightarrow h-like- α -LA) move to h-like- α -LA. Therefore, the molecular population of the h-like- α -LA increases in the presence of Cu^{2+} (see Fig. 2a). The addition of DTAB to the m- α -LA decreases partial molar heat capacity and enthalpy change values of h- α -LA form at high temperature. On the contrary, it increases these thermodynamics values for the a- α -LA at low temperature (see Fig. 2b). The DTAB is a cationic surfactant and its charge is similar to metal ions. The presence of DTAB causes the destabilization of protein and results in a decrease in the temperature of unfolding with an increase in the DTAB concentration [40,41]. The interaction between DTAB and the metal depleted form of α -LA (a- α -LA) causes the contiguous regions of the metal ions binding sites to be occupied by DTAB. Positive charges repulsion between DTAB and calcium metal ions is the most important factor for the non-binding calcium metal ions to the a- α -LA form. In addition to positive charges repulsion, hydrophobic tail of DTAB plays an important role to unfolding of h- α -LA, thus mixture of electrostatic and hydrophobic interactions can be caused in each binding site. Therefore, the equilibrium (a-like- α -LA \leftrightarrow h-like- α -LA) shifts to a- α -LA and enhances the molecular population of a- α -LA and stabilizes it.

Table 1

Intrinsic viscosity and Stokes radius of m- α -LA, a- α -LA and h- α -LA forms and various structural states of α -LA induced by different concentrations of Cu^{2+} and DTAB^a

Protein states	$[\eta]$ (M^{-1})	R_s^b (nm)	R_g^c (nm)
a- α -LA	30.9 ± 0.3	1.68 ± 0.02	1.72 ± 0.01
h- α -LA	23.1 ± 0.3	1.55 ± 0.02	1.57 ± 0.01
m- α -LA	28.3 ± 0.3	1.65 ± 0.02	NM
m- α -LA- Cu^{2+} (0.1 mM)	27.9 ± 0.3	1.63 ± 0.02	NM
m- α -LA- Cu^{2+} (1 mM)	27.6 ± 0.3	1.61 ± 0.02	NM
m- α -LA- Cu^{2+} (10 mM)	23.4 ± 0.3	1.57 ± 0.02	NM
m- α -LA-DTAB (0.5 mM)	28.5 ± 0.3	1.66 ± 0.02	NM
m- α -LA-DTAB (1.5 mM)	31.1 ± 0.3	1.69 ± 0.02	NM
m- α -LA-DTAB (2 mM)	31.6 ± 0.3	1.72 ± 0.02	NM

^a Cu^{2+} and DTAB were added to the m- α -LA.

^b The values were calculated from equation: $\frac{\eta_{sp}}{c} \cong [\eta] = \lim_{c \rightarrow 0} \left[\frac{\eta/\eta_0 - 1}{c} \right] = \frac{2.5N_A}{M} \left(\frac{4}{3}\pi R_s^3 \right)$.

^c The data are taken from Ref. [39]. NM, not measured.

The fluorescence of the aromatic amino acids in proteins offers a particular sensitive probe in detecting molecular conformational changes. Use of this technique, however, to define such change in terms of specific molecular processes has been comparatively limited due primarily to the fact that changes in the fluorescence characteristics of proteins potentially have such a wide variety of origins. This is particularly true of tryptophan fluorescence where solvent effects are known to have such a marked influence on the emission spectrum as compared with the absorption spectrum [42]. The fluorescence spectroscopic properties of the m- α -LA-DTAB complex strongly support the view that DTAB destabilizes the h- α -LA form. The fluorescence properties of the m- α -LA-DTAB complex closely resemble the metal depleted form of α -LA (a- α -LA), as reported by Kronman [42] and Murakami et al. [31], thus implying a close structural similarity. The addition of DTAB to the m- α -LA, an increase in the fluorescence is observed, and a small red shift occurs, indicating that DTAB starts to unfold the protein. That is to say DTAB increases the molecular population of a- α -LA and the equilibrium shifts to the metal-depleted form.

A key parameter for characterizing the h- α -LA form is compactness or globularity, but only limited data have been reported on the direct measurement of this quantity [43–46]. The intrinsic viscosity and Stokes radius, R_s , [47] are crucial measurements of the compactness of protein states. The R_s values in Table 1 show the most compact state is the h- α -LA form. The R_s value of the m- α -LA is less than the h- α -LA form. This means that the molecular population partition of h- α -LA form has been reduced by the dialyzing experiment (a part of h- α -LA changes to a- α -LA). Whereas, the m- α -LA consist mixture of a- α -LA and h- α -LA forms in solution, Stoke radii estimated are approximate of two equilibrium forms of the m- α -LA that are dependent to their molecular population partition. The addition of Cu^{2+} at various concentrations to the m- α -LA decreases the R_s values, it means that the equilibrium (a- α -LA \leftrightarrow h- α -LA) shifts to the h- α -LA and enhances the enthalpy change of calorimetric and molecular population of the h- α -LA (h- α -LA is more compact than a- α -LA). On the other hand, different concentrations of DTAB bound to protein increase R_s values, meaning to the equilibrium (a- α -LA \leftrightarrow h- α -LA) shifts to the a- α -LA and enhances the enthalpy change of calorimetric and molecular population of the a- α -LA (a- α -LA is less compact than h- α -LA) that is confirmed with fluorescence results (maximum wavelength shifts to red region). It is also noteworthy that the R_s values indicate the folding and unfolding of the m- α -LA during the addition of Cu^{2+} and DTAB at different concentration, respectively.

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