Cooperative thermal transitions of bovine and human apo-α-lactalbumins: evidence for a new intermediate state

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Abstract The thermal denaturation of bovine and human apo-α-lactalbumins at neutral pH has been studied by intrinsic protein fluorescence, circular dichroism (CD), and differential scanning microcalorimetry (DSC) methods. Apo-α-lactalbumin possesses a thermal transition with a midpoint about 25–30°C under these conditions (pH 8.1, 10 mM borate, 1 mM EGTA), which is reflected in changes in both fluorescence emission maximum and quantum yield. However, the CD showed a decrease in ellipticity at 270 nm with a midpoint at about 10–15°C, while DSC shows the transition within the region of 15–20°C. The non-coincidence of transition monitored by different methods suggests the existence of an intermediate state in the course of the thermal denaturation process. This intermediate state is not the classical molten globule state which occurs at higher temperature (i.e. denatured state at these conditions) [D.A. Dolgikh, R.I. Gilmanshin, E.V. Brazhnikov, V.E. Bychkova, G.V. Semisotnov, S.Y. Venyaminov and O.B. Ptitsyn, *FEBS Letters*, 136 (1981) 311–315] and has physical properties intermediate between the native and molten globule states.

Key words: α-Lactalbumin; Thermal denaturation; Tryptophan fluorescence; Scanning calorimetry; Circular dichroism

1. Introduction

α-Lactalbumin (α-LA) is a 14200 Da acidic milk protein which is the specifier component of lactose synthase in the lactating mammary gland [1]. A single, strong Ca^{2+}-binding site (which also binds Mg^{2+}, Na^{+}, K^{+}, and Mn^{2+}) stabilizes the native form of the protein [for review, see [2] and references therein], and has been identified in the 3-dimensional X-ray structure of the baboon [3] and human proteins [4]. Distinct Zn^{2+}-binding sites also exist which tend to destabilize the native calcium form [2]. One of the most interesting properties of α-LA is its transition to the molten globule state [see [5] and references therein]. At elevated temperatures above the thermal denaturation transition (ca. 50°C for apo-protein), at intermediate denaturant concentrations (3–4 M urea), or when Ca^{2+} dissociates from α-LA at acid 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 protein, but with fluctuating tertiary structure [for recent review, see [6]].

The apo-form of α-LA is significantly less stable then the Ca^{2+}-loaded protein, and is partially denatured in a variety of mild denaturing conditions (i.e. at low ionic strength and room temperatures), thus leading to the erroneous conclusion that apo-form is molten globule state [7]. The classical molten globule at acid pH bears a resemblance spectroscopically to apo-α-lactalbumin (apo-α-LA), but the two states differ strikingly in their thermal behavior [8,9]. While the acid-state possesses a virtually undetectable thermal transition (indicative of the absence of defined tertiary structure), the apo-form has consistently shown a rather well-defined cooperative thermal transition as studied by several methods [9–12]. The purpose of the current work was to compare and investigate the nature of the thermal unfolding phenomena for both bovine and human α-LA by several parallel physical/spectroscopic methods.

2. Materials and methods

Human α-LA was isolated and purified as described by Kaplanas and Antanavichius [13]. Bovine α-LA was from Sigma Chemical Co. (St. Louis, MO; lot 128F-8140). All other chemicals were reagent grade or better. Solutions were prepared from double-distilled demineralized water. Protein concentration was evaluated spectrophotometrically using extinction coefficients $E_{1\%,280nm} = 20.1$ for bovine [14] and 18.2 for human [15] α-LA. Apo-α-LA was prepared according to Blum et al. [16] in 10 mM borate buffer, 1 mM EGTA, pH 8.1, and used immediately for experiments. For Ca^{2+}-loaded α-LA, 10 mM borate buffer, 1 mM $CaCl_2$, pH 8.1, was used.

Heat sorption curves for bovine and human α-LAs were measured on a DASM-4M differential adiabatic scanning microcalorimeter (Institute for Biological Instrumentation, Russian Academy of Sciences, Russia). The heating rate was 1 K/min. Specific heat capacity of LA was calculated according to Privalov [17,18]. Partial specific volume of protein was calculated from the sequence of α-LA using the known partial volumes of amino acid residues [19]. Heat sorption curves were fitted using non-linear regression analysis [20] according to the simple two-state scheme based on van't-Hoff's equation and the assumption that the heat capacity difference between native and denatured protein states is temperature independent in the temperature interval studied [17].

Fluorescence spectra were measured both on a Perkin-Elmer LS-50B and a lab-made spectrofluorimeter described earlier [21], with an excitation wavelength of 280 nm. The fractional conversion from the native to the thermally denatured state was calculated from plots of the temperature dependence of emission intensity at a fixed wavelength as previously described [22].

CD spectra of α-LA in the peptide and aromatic regions were measured on a JASCO-500C spectropolarimeter at a 200 nm/min scan rate and 0.25 s time constant. Pathlength was 2 mm for peptide and 10 mm for aromatic regions. Each spectrum was an average of 16 scans.

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Temperature scans for both fluorescence and CD measurements were performed stepwise, allowing the sample to equilibrate at each temperature for at least 5 min. This is different from the calorimetry measurements, where heating at constant speed is a must. Heating at a constant speed may introduce artefacts due to kinetic of the unfolding reaction, but heating speeds of 1 K/min are considered slow enough to allow measurements at near-equilibrium conditions [18].

3. Results and discussion

3.1. Bovine apo-α-lactalbumin

Fig. 1 depicts combined results of the thermal denaturation of bovine apo-α-LA (pH 8.1, 10 mM borate, 1 mM EGTA) by differential scanning calorimetry (DSC), circular dichroism (CD), and intrinsic fluorescence, respectively. A distinct heat sorption peak maximum at about 22°C is evident in Fig. 1A. The transition was accompanied by pronounced changes in the environments of tryptophan residues (midpoint at ≈10–15°C) as reflected by the decrease in ellipticity at 270 nm (Fig. 1B) and changes in tryptophan emission fluorescence parameters (which shows up as a >10 nm red shift in Fig. 1C, ■), corresponding to an increased accessibility of tryptophan residues, i.e. a transfer of some tryptophan residues from the protein interior to the surface [21,23].

3.2. Human apo-α-lactalbumin

Fig. 2 depicts combined results of the thermal denaturation of human apo-α-LA by microcalorimetry, CA, and intrinsic fluorescence, respectively. Again here, a distinct heat sorption peak maximum at about 29°C was observed (Fig. 2A) which was also mirrored by changes in tryptophan emission intensity (Fig. 2C). The CD transition (Fig. 2B) also tended towards lower temperature. Ellipticities of Ca^{2+}-loaded α-LAs at low temperature on Fig. 1BFig. 2B are shown for reference. The temperature dependence of ellipticity of Ca^{2+}-loaded α-LA below the transition can be approximated as a horizontal line (data not shown).

Thermal denaturing experiments were done at different protein concentrations due to limitations of methods employed. No concentration dependence of transition position was observed by fluorescence (data not shown) in the range used in experiments (0.3–2.6 mg/ml), so we can compare transition curves obtained by different methods. Fig. 3 shows the fractional conversion from the native to thermally denatured state derived from the data of the different methods for bovine (A) and human (B) apo-α-LAs, respectively: CD (Fig. 3, ○,●), intrinsic fluorescence (Fig. 3, □), and calorimetry (Fig. 3, ■). The fractional conversion curve for intrinsic fluorescence was calculated based on the emission intensity data, since this parameter (but not the emission maximum parameter) is linearly related to the fractional conversion [22]. For CD data, the plateau region was taken for denatured state. There is a little ambiguity of what to take as ellipticity of the 'native state' under those conditions, thus we show transition curves calculated in the assumption that the first data point corresponds to the native apo-state (Fig. 3, ●), although it is clear that even at 4°C the transition is already in progress. Second approximation used was that ellipticity of 'native' apo-protein is that of the Ca^{2+}-loaded α-LA (Fig. 3, ○). Assuming the experimentally observed at low temperature ellipticity value instead of the 'true' value as an ellipticity of the native form shifts the calculated transition toward higher temperatures. Fitting of DSC data for bovine α-LA was based upon a

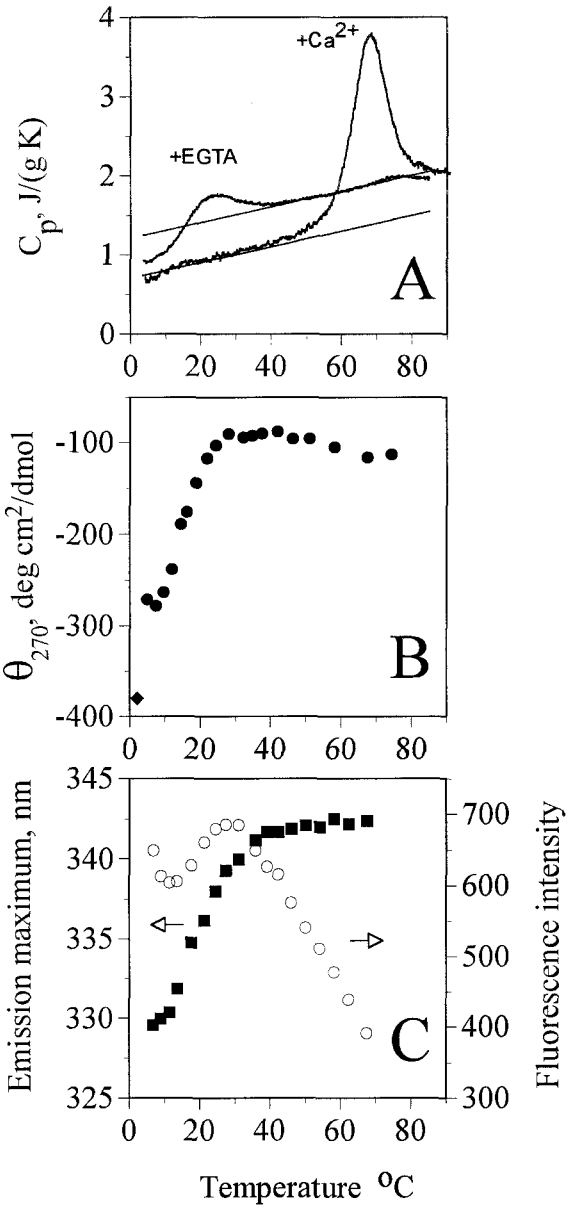

Fig. 1. Thermal denaturation of bovine apo-α-LA studied by (A) scanning calorimetry, [α-LA = 1.75] = 1.75 mg/ml (straight lines are heat capacities of the native and denatured states); (B) circular dichroism, 0.4 mg/ml, ellipticity at 270 nm (●, apo-α-LA, ♦, Ca^{2+}-loaded α-LA); and (C) intrinsic fluorescence, 2.6 mg/ml, spectrum position (■) and fluorescence intensity at 350 nm, arbitrary units (○).

heat capacity temperature dependence of denatured state, obtained by linear extrapolation of the part of heat sorption curve immediately after transition. In case of human α-LA DSC data such an approach seems to be incorrect due to supposed protein aggregation at elevated temperatures (the heat capacity dependence is curved downward there), and the heat capacity function of native Ca^{2+}-loaded human α-LA under analogous conditions was considered as the heat capacity of native apo-form. The following three parameters were varied for the fitting: the value of the heat capacity difference between the two protein states, half-transition temperature and transition enthalpy. The same results were achieved by the procedure described in [24].

The results obtained by intrinsic fluorescence, microcalorimetry, and CD do not coincide with each other, suggesting the existence of an *intermediate state* in the course of the thermal denaturation process [25]: different methods are sensitive to different protein states. This new state is not the classical MG state since this protein *intermediate* was partially denatured at best, as evidenced by the heat sorption/intrinsic fluorescence transition at higher temperature (ca. 20–30°C) compare to CD transition. Therefore the classical MG state exists somewhere above 50°C (see Fig. 3) as shown originally by Dolgikh et al. [26,27] who characterized this form by several methods. Another interesting feature of the heat sorption curve is a cold denaturation, which is more obvious for human α-LA. This observation is in line with the data of Xie et al. [28], although due to higher ionic strength (additional 0.2 M NaCl) they registered it only in the presence of guanidine hydrochloride.

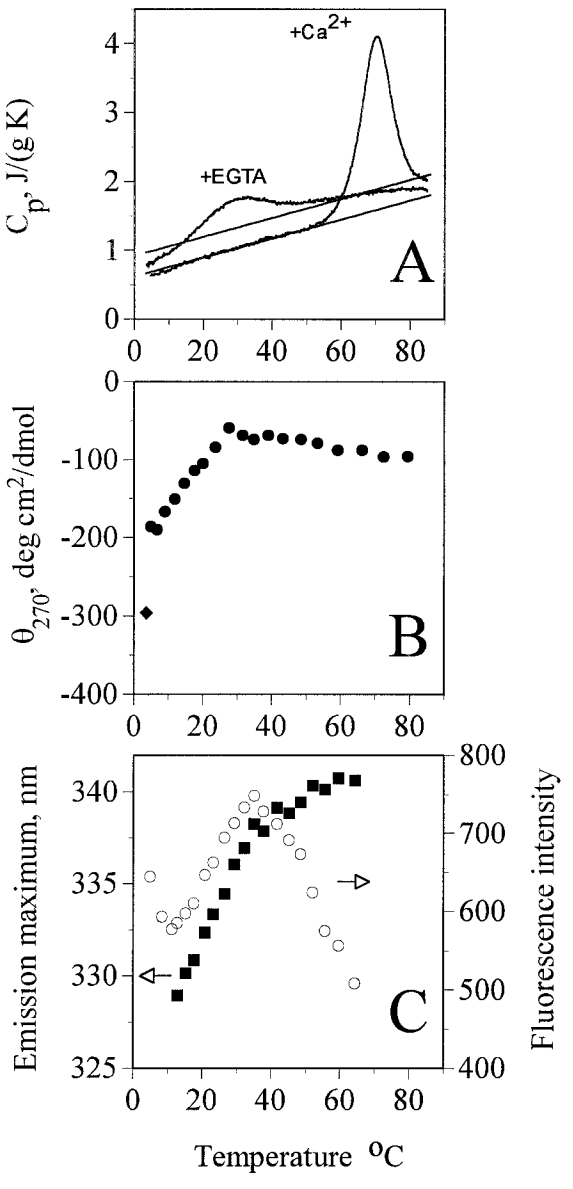

Fig. 2. Thermal denaturation of human apo-α-LA studied by (A) scanning calorimetry, [α-LA] = 1.37 mg/ml (straight lines are heat capacities of the native and denatured states); (B) circular dichroism, 0.4 mg/ml, ellipticity at 270 nm (●, apo-α-LA, ♦, native Ca^{2+}-loaded α-LA); and (C) intrinsic fluorescence, 0.3 mg/ml, spectrum position (■) and fluorescence intensity at a fixed wavelength, arbitrary units (○).

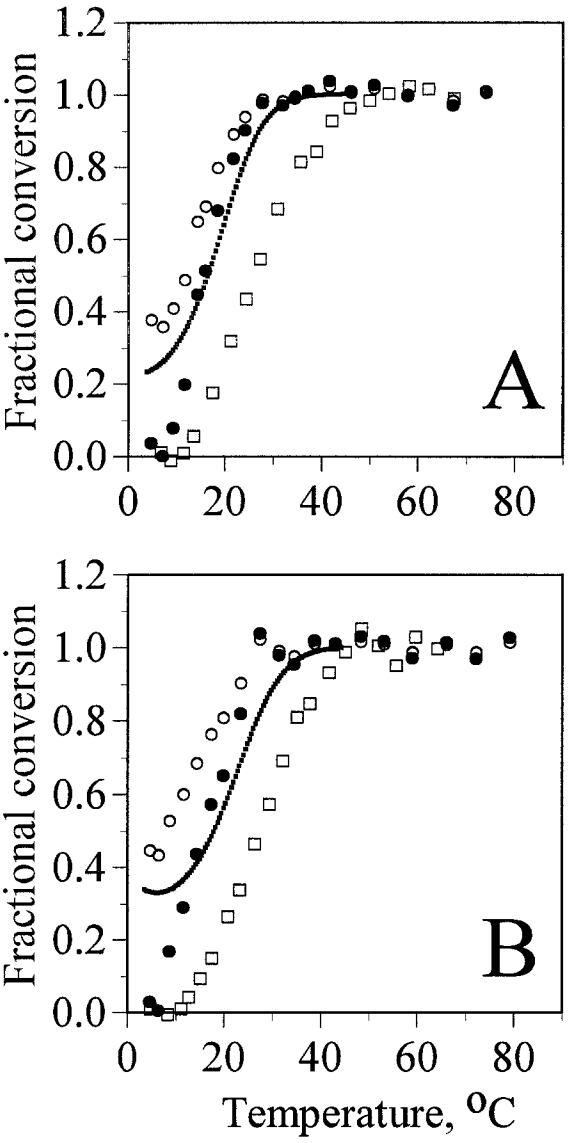

Fig. 3. Comparison of the temperature transition(s) of bovine (A) and human (B) apo-α-LA displayed as the fractional conversion from native to thermally denatured state by several methods: CD, (○, based on ellipticity of native Ca^{2+}-loaded α-LA for 'native' state; ●, based on first data point as ellipticity for 'native' state); intrinsic fluorescence emission, (□); and calorimetry, (■).

On the one hand, the intermediate state is characterized by increased rotational mobility of hiral residues (CD). On the other hand, accessibility of tryptophan residues to the molecules of the solution (water) in this state is close to that of the native state (fluorescence). Keeping in mind that the denatured state under these conditions is the molten globule, we can draw a scheme of the apo-α-LA denaturation at neutral pH and low ionic strength. First, the molecule swells giving a rotational freedom for such residues as tyrosines and tryptophans [29]. This process is accompanied by some hydration of hydrophobic residues (calorimetry), but tryptophan environment (Trp^2 and Trp^{104} of bovine α-LA belong to the hydrophobic core of molecule) still remains to be rigid. In the next

stage, the tryptophan-containing core becomes less rigid, exposing the aromatic residues to the solvent, although the hydration of hydrophobic residues is not so different from that of the molten globule state.

A distinct thermal transition for bovine apo-α-LA has been reported previously by other authors under conditions very similar to those used above [11,12] It is clear from these data that a *lower temperature* CD transition occurs; but the discrepancy between the aromatic ellipticity change and the scanning calorimetry results have been never reported to our knowledge. In addition, Hiraoka and Sugai [30] observed a distinct thermal transition by CD under conditions similar to those of Yutani et al. [7] who failed to detect this transition in borate buffer. This was perhaps due to the fact that their thermal measurements started at high temperatures (>20°C) where the thermal transition was substantially complete by CD (Fig. 1B), but only partially complete by calorimetry (Fig. 1A). The suggestion of intermediate states in α-LA is not unprecedented. Such a state was shown to exist in the native to acid transition using steady-state and circularly polarized fluorescence by Gussakovsky and Haas [31] and also from Raman spectroscopy by Wilson et al. [32]. Owusu Apenten [33] suggested a three-state thermal denaturation of both human and bovine Ca^{2+}-loaded α-LAs by intrinsic fluorescence measurements, but did not reveal intermediate states for the apo-forms. The work presented above emphasizes the importance of employing several physical and spectroscopic techniques to the analysis of multistate denaturation processes and has suggested a new intermediate state in the thermal unfolding of apo-α-LA.

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