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A three-state heat-denaturation of bovine α-lactalbumin

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The heat-denaturation of bovine α-lactalbumin (α-La) was studied using tryptophan and tyrosine fluorescence intensity measurements at 2–80°C. The solvent used was sodium cacodylate buffer (10 mM, pH 6·0) with 1 mM EDTA or 9 mM $CaCl_2$. The denaturation of apo-α-La conformed to a *two-state* reaction with a melting temperature (T_m) of 34·5 (± 0·7)°C. In contrast, the heat-unfolding of holo-α-La was consistent with a *three-state* reaction. The T_m for holo-α-La heat-unfolding was 47 (± 2·6)°C or 67·5 (± 1·3)°C as determined from tyrosine or tryptophan fluorescence changes, respectively. Such results suggest that Ca^{2+} binding preferentially stabilises one domain of α-La against heat-denaturation.

INTRODUCTION

The whey protein α-lactalbumin (α-La) is a control subunit for lactose synthetase and a calcium ion (Ca^{2+})-binding protein. Metal ion binding is essential for lactose synthesis *in vitro* and could be associated with the control of lactose synthesis *in vivo* (cf. Kronman, 1989; Mckenzie & White, 1991 for recent reviews). Therefore, the effect of calcium binding on the structure and stability of α-La is of continued interest.

Binding to Ca^{2+} ions stabilises the conformation of α-La (Hiroaka *et al.*, 1980) and reduces its hydrophobicity (Musci & Berline, 1985), electrophoretic mobility (Thompson *et al.*, 1988), lysozyme-like activity (White, 1992) and proteinase susceptibility (Schmidt & Poll, 1992). However, the different characteristics of apo- and holo-α-La seem to be due to the instability of the apo-form which is partially unfolded at room temperature. At low temperature, apo- and holo-α-La possess similar native conformations as monitored by ultraviolet circular dichroism (UV-CD). The stabilisation of α-La by Ca^{2+} appears to be largely entropic: Ca^{2+} binding increases the rigidity of α-La (Kuwajima *et al.*, 1986). At the same time, nuclear magnetic resonance and infrared spectra for the native conformations of apo- and holo-α-La are significantly different (Kuwajima *et al.*, 1986; Prestrelski *et al.*, 1991).

The denaturation of native (N) α-La by guanidine hydrochloride (GnHCl) is a three-state reaction: N ⇔ A ⇔ D, proceeding via intermediate (A) and denatured (D) states Kuwajima *et al.* (1976). The denaturation behaviour of α-La alters in the presence of excess calcium. In 9 mM Ca^{2+}, two GnHCl denaturation transitions (transitions I and II) were observed by ultraviolet difference or fluorescence spectrophotometry (Owusu, 1992*a,b*). Transition I was not recorded by UV-CD (Ikeguchi *et al.*, 1986). In view of such results the effect of Ca^{2+} ions on the heat denaturation of α-La, previously studied by UV-CD (Hiroaka *et al.*, 1980; Dolgikh *et al.*, 1981; Hiroaka & Sugai, 1984; Kuwajima *et al.*, 1986), was examined using very sensitive measurements of tryptophan (Trp) and tyrosine (Tyr) steady-state fluorescence intensity.

MATERIALS AND METHODS

Bovine α-La (Type III grade with less than 0·3 mole Ca^{2+}/mole of protein) was supplied by Sigma Ltd, (UK). AnalaR grade reagents were obtained from the British Drug Houses (BDH) Ltd (UK). Procedures were essentially as described previously (Owusu, 1992*a,b*). α-La (0·025 mg/ml; 1·8 μM) in sodium cacodylate buffer (10 mM, pH 6·0) was heated at 2–80°C. Fluorescence measurements were at an excitation wavelength of 280 nm and an emission wavelengths of 305 and 345 nm. The instrument used was a Perkin-Elmer 204 fluorescence spectrophotometer fitted with a heated cuvette block. All measurement were performed in triplicate.

RESULTS AND DISCUSSION

Figures 1 and 2 show graphs of Trp and Tyr fluorescence intensity (I305 and I345, respectively) plotted versus temperature for systems containing 1 mM ethylenediaminetetraacetic acid (EDTA) or 9 mM calcium chloride. The difference between I345 and I305 (i.e.

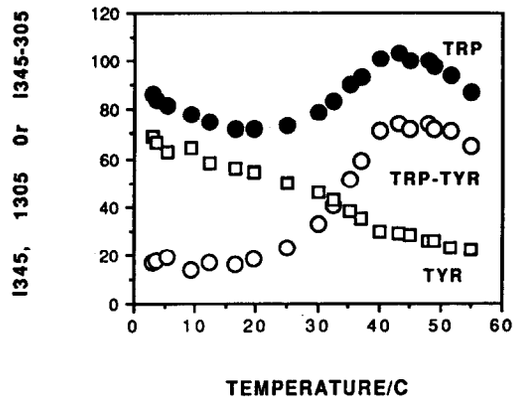

Fig. 1. Heat-denaturation of bovine α-lactalbumin in sodium cacodylate buffer (10 mM, pH 6·0) with 1 mM EDTA. Fluorescence excitation wavelength = 280 nm; tryptophan (Trp) or tyrosine (Tyr) residues were monitored at 345 nm (I345) and 305 nm (I305), respectively. I345–305 shows the difference between Trp and Tyr fluorescence.

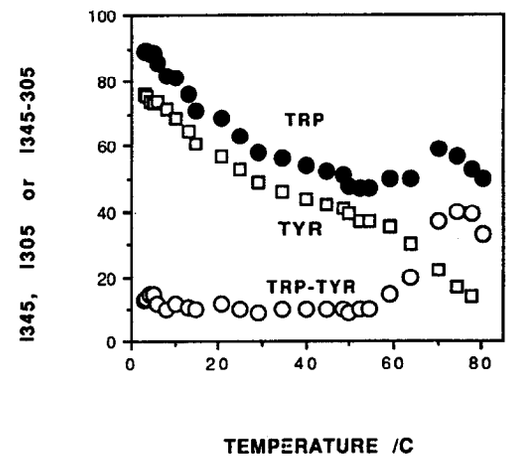

Fig. 2. Heat-denaturation of bovine α-lactalbumin in sodium cacodylate buffer (10 mM pH 6·0) with 9 mM $CaCl_2$. Fluorescence excitation wavelength = 280 nm; tryptophan (Trp) or tyrosine (Tr) residues were monitored at 345 nm (I345) and 305 nm (I305), respectively. I345–305 shows the difference between Trp and Tyr fluorescence.

I345–305) values is also shown. Figures 3 and 4 show the corresponding graphs for the percentage of protein molecules unfolded versus temperature. Pre- and post-denaturational thermal quenching of the N- and D-state, fluorescence intensity was corrected for after assuming a linear dependence of I345, I305 and I345–305 values on temperature (Permyakov *et al.*, 1984).

In a Ca^{2+}-free solvent, all indices of α-La heat-unfolding (I345, I305 and I345–305) were superimposable (Fig. 3). The temperature at which 50% of α-La molecules were unfolded (T_m) was 34·5 (± 0·7)°C in agreement with previous reports (Hiroaka & Sugai, 1984). The three overlapping thermal transitions in Fig. 3 suggest that the heat-unfolding of apo-α-La conforms to a *two-state* reaction. N ⇔ MG, where N is the native state and MG represents the molten globule state first described by Dolgikh *et al.* (1981).

In excess (9 mM) Ca^{2+}, heating α-La resulted in non-superimposable thermal transitions (Fig. 4). Such results

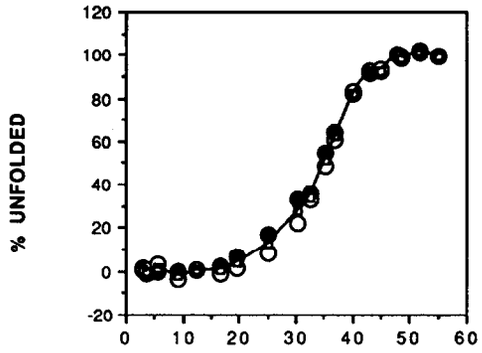

Fig. 3. Heat-denaturation profile for α-lactalbumin in sodium cacodylate buffer (10 mM pH 6·0, + 1 mM EDTA). Per cent of unfolded protein (% unfolded) plotted versus temperature (°C). Symbols are as in Fig. 1.

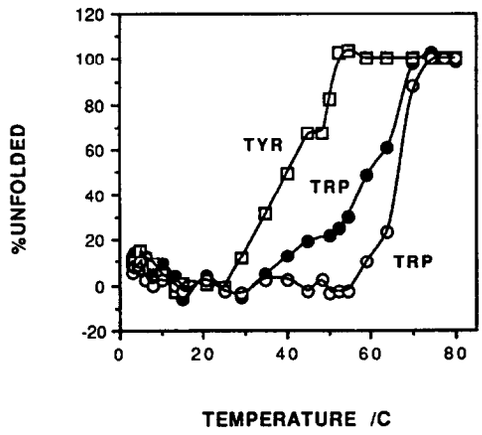

Fig 4. Heat-denaturation profile for α-lactalbumin in sodium cacodylate buffer (10 mM pH 6·0, + 9 mM). Per cent of unfolded protein (% unfolded) plotted versus temperature (°C). Symbols are as in Fig. 2.

are consistent with a *three-state* heat-unfolding reaction. Ca^{2+} binding increased the T_m for α-La from 34·5 (± 0·7)°C (Fig. 3) to 47 (± 2·6)°C (based on Tyr results) or 67·5 (± 1·3)°C (based on Trp results). The biphasic I345 (Trp) unfolding transition (Fig. 4) also points to two classes of Trp residues located in two parts of the holo-α-La molecule with different heat stability. Similar three-state heat-unfolding transitions were also observed for bovine or human α-La heat denatured in Tris–HCl buffer at pH 8·0. A full account of these results will be reported later (Owusu Apenten, 1995, in prep.).

Like hen egg white (HEW) lysozyme, α-La consists of two lobes or domains (Acharya *et al.*, 1991). Therefore, a two-state (all-or-none) heat-unfolding transition for bovine apo-α-La requires that the two halves of the protein have equal heat stability and/or form a strongly interacting co-operative unit (Kronman, 1989).

The three-state heat-unfolding of holo-α-La (Fig. 4) may be presented as the equation: N ⇔ SMG ⇔ MG. Here SMG is a stable intermediate, or 'semi-molten globule', state. It is thought that Ca^{2+} binds to one lobe of α-La (Stuart *et al.*, 1986; cf. Kronman, 1989, or Mckenzie & White, 1991, for extensive reviews) leading

to a localised 2°-structure change involving about 16% of residues in the protein (Prestreski *et al.*, 1991). It is possible that Ca^{2+} binding predominantly stabilises one region in the native conformation of α-La. Hence, reduction of the Cys 6–Cys 160 disulphide bond in Ca^{2+}-bound α-La generates 3 (S–S) holo-α-La with clearly differentiated high and low mobility regions within the native structure (Ewbank & Creighton, 1993). The SMG state of α-La may be a denaturation intermediate in which the Ca^{2+}-bound domain retains substantially more native-like structure or mobility than usually pertains to the MG state.

The three-state heat-unfolding reported above is most readily explained by supposing that Ca^{2+} binding predominantly stabilises one lobe of holo-α-La, resulting in two independently unfolding lobes with unequal heat stability. HEW lysoyme (like apo-α-La in this study; Fig. 3) shows two-state heat-denaturation (Mckenzie & White, 1991). Equine lysozyme, with Ca^{2+}-binding properties like α-La, however, shows a three-state thermal transition (Morozova *et al.*, 1991). Multi-phasic thermal transitions have also been observed for other calcium-binding proteins such as parvalbumin, troponin C and calmodulin (Permyakov *et al.*, 1985; Privalov & Talkova, 1985).

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