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Thermochimica Acta 262 (1995) 1–12

thermochimica
acta

Thermodynamic parameters for 3-state thermal denaturation of human and bovine α-lactalbumin

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Received 28 July 1994; accepted 3 February 1995

Abstract

Thermodynamic parameters were determined for the thermal denaturation of Ca^{2+}-bound (holo) and Ca^{2+}-free (apo) α-lactalbumin from human and bovine milk. Thermal denaturation profiles were determined from changes in the intrinsic fluorescence emission intensity (FI) as a function of temperature (T). Human apo α-lactalbumin was heat-denatured in a 2-state process with $T_m = 25°C$, $\Delta H = 167$ kJ mol^{-1}, $\Delta S = 7700$ J mol^{-1} K^{-1} and $\Delta C_p = 15400$ J mol^{-1} K^{-1}. The corresponding values for bovine apo α-lactalbumin were: $T_m = 20°C$, $\Delta H = 180$ kJ mol^{-1}, $\Delta S = 9000$ J mol^{-1} K^{-1} and $\Delta C_p = 5100$ J mol^{-1} K^{-1}.

Derivative plots of d(FI)/d(T) versus T revealed that both human and bovine holo α-lactalbumin were heat-denatured via a 3-state process. Thermal denaturation transitions were associated with a T_m value of 67°C or 42°C, based on changes in tryptophan or tyrosine FI results, respectively. Apparently Ca^{2+}-bound α-lactalbumin possesses two regions (domains) with significantly different conformational stability. Based on tryptophan fluorescence measurements, $\Delta H = 330$ kJ mol^{-1}, $\Delta S = 4600$ J mol^{-1} K^{-1} and $\Delta C_p = 8200$ J mol^{-1} K^{-1} for human or bovine holo α-lactalbumin. From tyrosine fluorescence emission changes, $\Delta H = 54$–103 kJ mol^{-1}, $\Delta S = 300$–2000 J mol^{-1} K^{-1} and $\Delta C_p = 3000$–4000 J mol^{-1} K^{-1}.

Keywords: Heat unfolding; α-Lactalbumin; Milk protein; Protein stability

1. Introduction

α-Lactalbumin is the predominant globular protein in human milk. In bovine milk, it is the second most abundant globular protein after β-lactoglobulin. α-Lactalbumin is a metallo-protein with a strong affinity for calcium (Ca^{2+}) ions ($K_d = 10^{-6}$–10^{-9} M); other metal ions are also bound but with a reduced affinity. The binding of Ca^{2+} ions

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SSDI 0040-6031(95)02292-9

may affect the role of α-lactalbumin as the control subunit or specifier protein for lactose synthetase (see Refs. [1] and [2] for recent reviews). The Ca^{2+}-free (apo) and Ca^{2+}-bound (holo) proteins exhibit differences in their thermal stability, protease susceptibility, electrophoretic mobility and surface hydrophobicity [3–6]. However, it is not yet certain that Ca^{2+} binding leads to a pronounced change in the structure of α-lactalbumin. Apo α-lactalbumin denatures near room temperature and the holo protein appears to be susceptible to cold denaturation (see below). There is a limited temperature range over which native forms of the apo and holo proteins are stable and may be compared. Circular dichroism (CD), nuclear magnetic resonance (NMR) and time-resolved fluorescence polarisation measurements indicate that a subtle conformational change commences on Ca^{2+} binding to α-lactalbumin [5, 7–9].

The denaturation of α-lactalbumin was one of the first recognised cases of protein denaturation involving a stable "molten globule" intermediate [10]. Another subtle conformational change can be seen as a Ca^{2+}-induced change in the guanidine hydrochloride denaturation profile for α-lactalbumin [11, 12]. To further elucidate the effect of Ca^{2+} ions on α-lactalbumin structure, the thermal denaturation of apo and holo α-lactalbumins was investigated using very sensitive measurements of protein intrinsic fluorescence.

Several workers have examined the thermostability of α-lactalbumin using ultraviolet (UV) difference and fluorescence spectrophotometry [13–16] as well as CD [17] and differential scanning calorimetry [18]. However, the level of Ca^{2+} employed in these studies may have been insufficient to saturate all binding sites [2]. A comparative study of the effect of Ca^{2+} ions on the thermodynamics of human and bovine α-lactalbumin thermal denaturation has yet to be reported. Another unique feature of the present work is that thermal denaturation profiles derived from changes in the environment of tyrosine (Tyr) and tryptophan (Trp) residues are compared. Evidence is presented to suggest that the thermal denaturation of human and bovine holo α-lactalbumin is a 3-state reaction. By contrast the Ca^{2+}-free protein undergoes a 2-state thermal denaturation. The results are discussed in terms of a possible effect of Ca^{2+} binding on α-lactalbumin domain stability.

2. Materials and methods

Human and bovine α-lactalbumin samples and tris (hydroxymethylamino)methane (THAM) were from Sigma Chemical Co. Ltd., Poole, UK. Calcium chloride, disodium ethylene diaminetetraacetic acid (EDTA) and all other chemicals were AnalaR grade from BDH Ltd. (UK). Distilled-deionized water was used throughout.

Fluorescence measurements were performed on a Perkin-Elmer 204 fluorescence spectrophotometer fitted with a digital output and a thermostated twin-cuvette holder, essentially as described before [11, 12, 19]. Protein samples (0.025–0.05 mg ml^{-1} or 1.76–3.5 μM) were dissolved in THAM–HCl (10 mM, pH 8.0 containing 9 mM $CaCl_2$; ionic strength = 0.037 M) or THAM–HCl (10 mM, pH 8.0 with 1 mM EDTA; ionic strength = 0.012 M) buffer. Solutions of α-lactalbumin were stored at 5°C overnight before use.

To determine thermal denaturation profiles, protein samples (2.5 ml) were placed in a quartz cuvette bearing a Teflon stopper, and heated at about 2–85°C. The sample was allowed to equilibrate at each temperature for 10 min before fluorescence readings were taken. The fluorescence excitation wavelength was 280 nm and the emission wavelength was 305 nm (FI305: tyrosine fluorescence) or 345 nm (FI345: tryptophan fluorescence). After each experiment the heated protein sample was cooled to 25°C in order to establish that thermal denaturation was reversible. After a single use, the quartz cuvettes were exhaustively washed with deionized water, soaked in 0.1 M HCl overnight, and rinsed successively with water and THAM–HCl buffer (10 mM, pH 8.0) before reuse. All thermal denaturation measurements were repeated at least twice.

3. Results and discussion

The temperature dependence of FI305 and FI345 values for calcium-free human (apo) α-lactalbumin can be seen in Fig. 1. Corresponding results for the holo protein are shown in Fig. 2. The difference between FI345 and FI305 values, i.e. FI(345 – 305), are also shown. There are background decreases in FI arising from thermal quenching. FI changes due to protein denaturation are superimposed on this background FI decrease. Thermal quenching effects are usually a linear function of temperature [14]. The

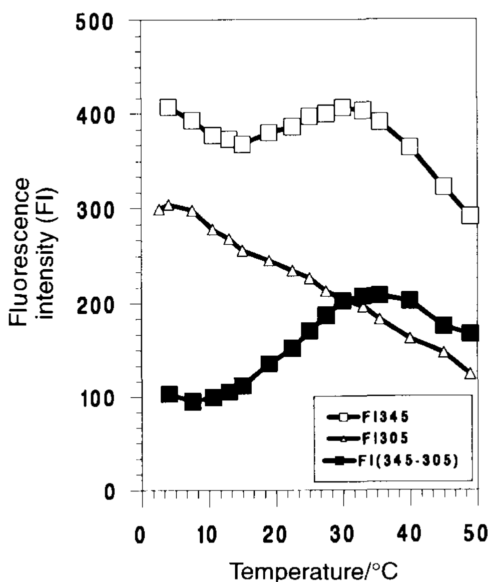

Fig. 1. The effect of temperature on human α-lactalbumin intrinsic fluorescence emission intensity (FI). Fluorescence excitation wavelength = 280 nm. Fluorescence emission wavelength = 305 nm (FI305) or 345 nm (FI345). The difference between FI345 and FI305 results is shown as FI(345 – 305). Conditions: calcium-free (apo) α-lactalbumin in THAM–HCl buffer (0.01 M, pH 8.0 with 1 mM EDTA).

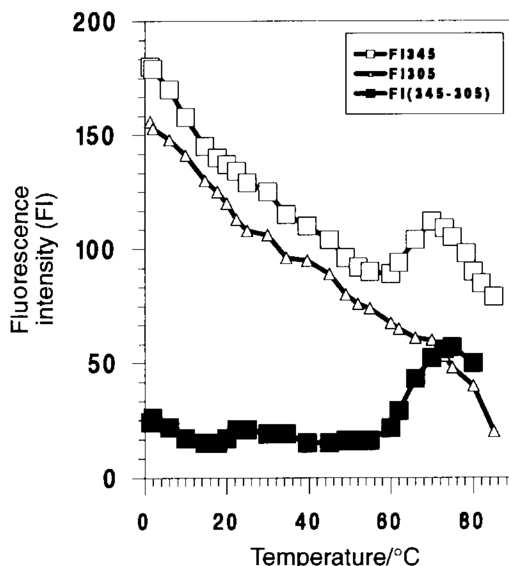

Fig. 2. The effect of temperature on human α-lactalbumin intrinsic fluorescence emission intensity (FI). Symbols as in Fig. 1. Conditions: calcium-bound α-lactalbumin in THAM–HCl buffer (0.01 M, with 9 mM $CaCl_2$).

two contributions to the observed FI can be resolved using a first derivative plot of d (FI345)/dT versus T(Figs. 3 and 4).

For apo α-lactalbumin, the FI345 derivative plot (Fig. 3) shows a single peak. Single peaks were also obtained for derivative plots based on FI305 and FI(345 – 305) readings (data not shown). This means that the thermal denaturation of Ca^{2+}-free α-lactalbumin involved a single transition between native (N-) and denatured (D-) protein conformations. The thermal denaturation reaction can be presented as a 2-state process with no stable intermediate

$$N \leftrightarrow D \quad (1)$$

The D-state for heat denatured α-lactalbumin is described as a "molten-globule" state. This is a compact globule-like structure with an N-like secondary structure and a disrupted tertiary structure [10].

The FI345 derivative plot, in the case of Ca^{2+}-bound α-lactalbumin shows two peaks (Fig. 4). Therefore, human or bovine α-lactalbumin appears to undergo thermal unfolding via a 3-state reaction where X is a stable intermediate (see below)

$$N \leftrightarrow X \leftrightarrow D \quad (2)$$

3.1. Thermal denaturation profiles for α-lactalbumin

The percentage of denatured portein is given by the relation

$$100(FI_T - FI_N)/(FI_D - FI_N) \quad (3)$$

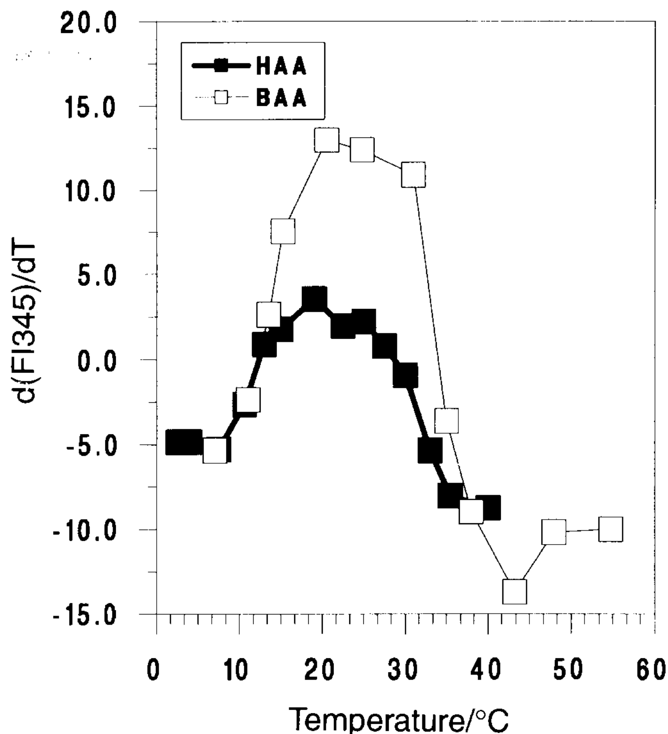

Fig. 3. A derivative fluorescence graph for human apo α-lactalbumin (HAA) and bovine apo α-lactalbumin (BAA). Conditions as in Fig. 1.

where FI_T, FI_N and FI_D are the FI values at any temperature T, FI for the native (N) protein and FI for the denatured protein, e.g. $FI_D = FI$ at $T > 40°C$, see Fig. 1. Due to the effects of thermal quenching, FI_N and FI_D will both be linear functions of temperature [14]

$$FI_N = m_N T + C_N \tag{4}$$

$$FI_D = m_D T + C_D \tag{5}$$

where m is the slope and C the intercept values of FI. Using Eqs. (4)–(5), one may extrapolate values for FI_N and FI_D over the whole temperature range of interest. The above methods were further refined to allow for the minority status of N- or D-state at high or low temperature, respectively. Above the melting temperature (T_m), defined as the temperature necessary to produce a thermal unfolding of 50% of all native protein molecules, the concentration of N-state declines. Eq. (4) is then not an accurate description of FI_N. The same consideration applies to Eq. (5) when $T > T_m$. It can be shown that where $T > T_m$ or $T < T_m$, then Eqs. (4) and (5) can be replaced by Eqs. (6)

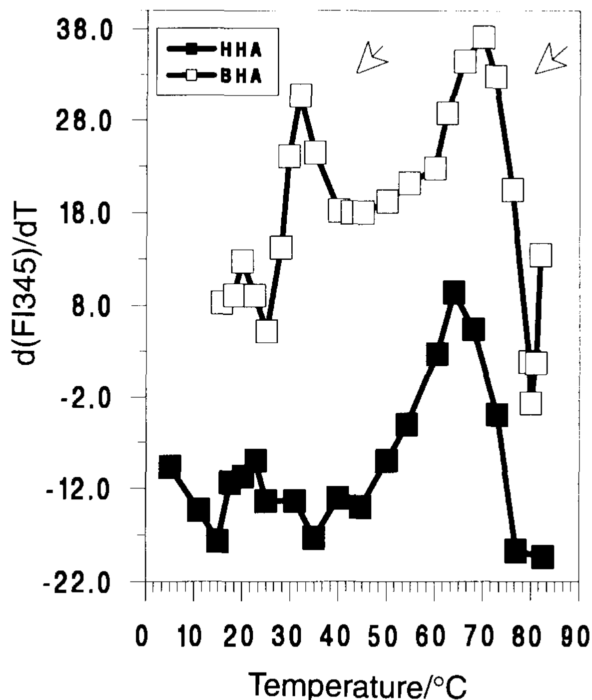

Fig. 4. A derivative fluorescence graph for calcium-bound (holo) α-lactalbumin (HHA) and bovine holo α-lactalbumin (BHA). Arrows show two thermal denaturation transitions occur between 15 and 80°C. Conditions as in Fig. 2.

and (7) respectively

$$FI_N = m_D T + T_m(m_N - m_D) + C_N \tag{6}$$

$$FI_D = m_N T + T_m(m_D - m_N) + C_D \tag{7}$$

In practice it was first necessary to use Eqs. (4)–(5) to obtain initial estimates of T_m before applying the refinements described by Eqs. (6) and (7). The thermal denaturation profiles for apo and holo α-lactalbumins are shown in Figs. 5–7.

The thermal denaturation profile for Ca^{2+}-free α-lactalbumin (Fig. 5) conforms to a 2-state transition. Denaturation transitions for each apo-protein were superimposable when either FI345, FI305 or FI(345–305) changes were monitored. Changes in the environment of Tyr (using FI305 as index) or Trp (using FI345 and FI345 – 305 as indices) residues occurred at the same temperature. For Ca^{2+}-bound α-lactalbumin the thermal denaturation profile based on Tyr-FI data preceded Trp-FI changes. Furthermore, there seemed to be two classes of Trp residue because FI345 and FI345 – 305 transitions were non-superimposable (Figs. 6 and 7). Such results are in accordance with a 3-state thermal denaturation process as described by Eq. (2).

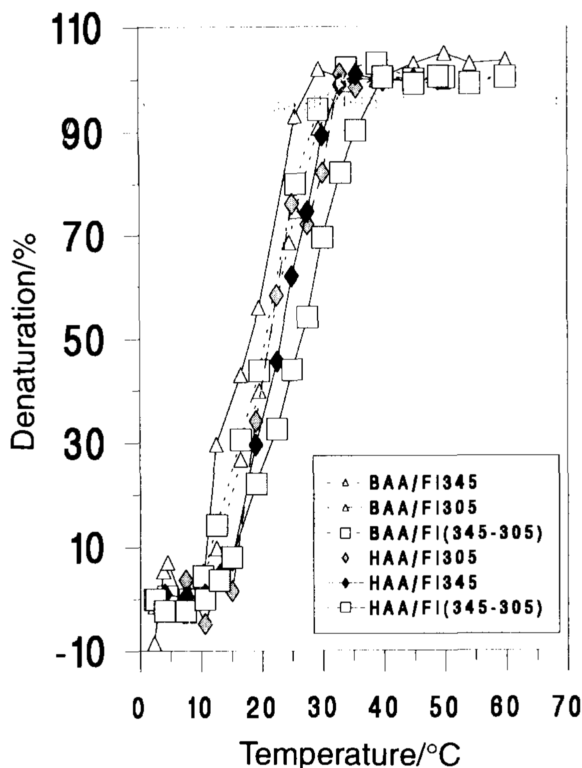

Fig. 5. Thermal denaturation profiles for human apo α-lactalbumin (HAA) and bovine apo α-lactalbumin (BAA). Symbols as defined in the text.

3.2. Thermodynamic parameters for α-lactalbumin thermal denaturation

Changes in the Gibbs free energy ΔG, enthalpy ΔH, entropy ΔS and heat capacity ΔC_p for α-lactalbumin thermal denaturation were determined from the temperature dependence of the equilibrium constant for denaturation K_{eq} ($K_{eq} = [D]/[N]$). A second-order Van't Hoff equation (Eq. (8)) was employed

$$\ln K_{eq} = A + BT^{-1} + CT^{-2} \tag{8}$$

The parameters A, B and C were determined by non-linear regression. In all cases the regression coefficient (r) was ≥ 0.98. The constants A, B and C are related to thermodynamic parameters, e.g. $d(\ln K_{eq})/d(1/T) = \Delta H/R$ and $d(\Delta H)/dT = \Delta C_p$. Consequently, $\Delta H/R = 2CT^{-1} + B$ and $\Delta C_p = 2CT^{-2}$. ΔG and ΔS were obtained from $\Delta G = RT \ln K_{eq}$ and $\Delta S = (\Delta G - \Delta H)/T$. Finally, at T_m, $\ln K_{eq} = 0$, therefore T_m is one of the solutions to the quadratic function (Eq. (8)); $T_m = 2C/(B + \sqrt{(B^2 - 4AC)})$. Results from the preceding analysis are given in Tables 1 and 2.

Thermodynamic parameters for human α-lactalbumin thermal denaturation do not appear to have been reported previously. For the thermal denaturation of bovine apo

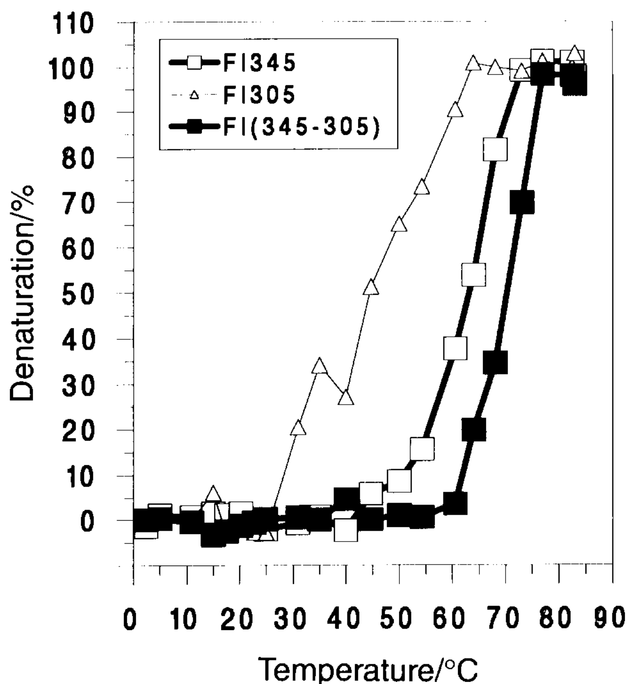

Fig. 6. Thermal denaturation profiles for calcium-bound human α-lactalbumin. FI305 = tyrosine fluorescence. FI345 and FI(345 – 305) = tryptophan fluorescence. Data from Fig. 2.

α-lactalbumin, reported values for T_m, ΔH and ΔC_p are respectively, 25°C, 130–180 kJ mol^{-1} and 4–5 kJ mol^{-1} K^{-1} [17]. For Ca^{2+}-bound bovine α-lactalbumin, the reported thermodynamic parameters for thermal denaturation are $T_m = 57$–64°C, $\Delta H = 222$–276 kJ mol^{-1} and $\Delta C_p = 4$–6.5 kJ mol^{-1} K^{-1}, [15, 16, 18]. Such results are in good agreement with those reported in Tables 1 and 2.

It may be concluded from the present results that bovine and human holo α-lactalbumin have nearly equal thermostabilities (Table 2). However, the human apo protein had a slightly (5°C) greater T_m value compared to bovine apo α-lactalbumin. There was also a significantly greater ΔC_p value for human apo α-lactalbumin. Apparently the greatest changes in the solvent accessibility of non-polar amino acid residues, during thermal denaturation, occurred for human apo α-lactalbumin compared to the other three systems examined, i.e. bovine holo or apo α-lactalbumin and human holo α-lactalbumin.

3.3. *Three-state thermal denaturation of α-lactalbumin*

When exposed to a high concentration of guanidine hydrochloride (GnHCl), α-lactalbumin undergoes 3-state unfolding involving a stable intermediate D (Eq. (9)). The D-state can also be generated at extremes of pH and by thermal denaturation

$$N \leftrightarrow D \leftrightarrow U \quad (9)$$

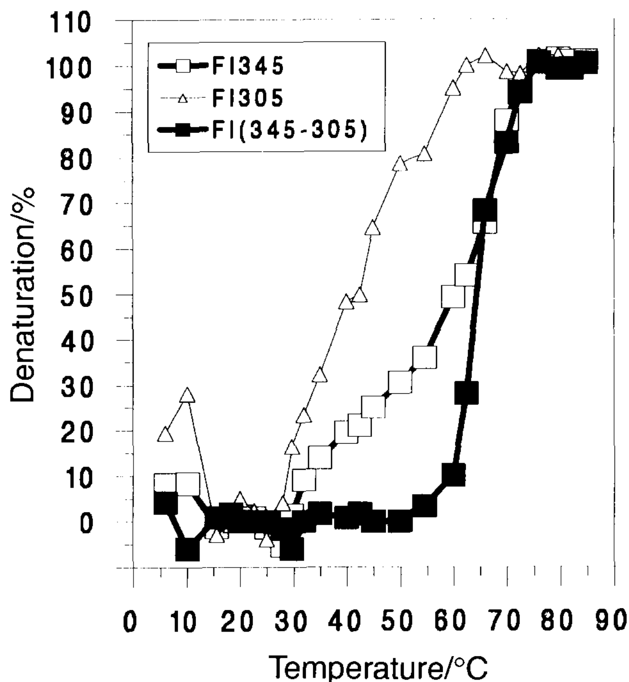

Fig. 7. Thermal denaturation profiles for calcium-bound bovine α-lactalbumin. FI305 = tyrosine fluorescence. FI345 and FI(345 − 305) = tryptophan fluorescence.

Table 1
Thermodynamic parameters for the thermal denaturation of calcium-free (apo) α-lactalbumin [a]

Protein	ΔH/(kJ mol^{-1})	ΔS/(kJ mol^{-1} K^{-1})	ΔC_p/(kJ mol^{-1} K^{-1})	T_m/(°C)	ΔG [b]/(kJ mol^{-1})
Human	167(±25)	7.7(±1)	15.4(±0.6)	25(±0.9)	4.0(±0.2)
Bovine	180(±20)	9.0(±0.3)	5.1(±3)	20(±1.5)	5.5(±3)
	166(±31) [c]	5.4(±0.7) [c]	3.6(±1.7) [c]	31(±2.7) [c]	9.1(±2.3) [c]

[a] Mean values at the T_m, $n = 6$ data points (SD in parentheses). Solvent, THAM–HCl buffer (10 mM, pH 8.0 with 1 mM EDTA). Tyrosine (Tyr) and tryptophan (Trp) thermal denaturation transitions are assumed to be 2-state. [b] ΔG value at 10°C. [c] Solvent, sodium cacodylate buffer (10 mM, pH 6.0 with 1 mM EDTA).

In Eq. (9), U is a disulphide-bond-constrained random polymer [20, 21]. In the presence of Ca^{2+} ions, there was a 4-fold increase in the value of ΔG for the N → U transition which increases from 11 (±0.5) kJ mol^{-1} to 41(±4) kJ mol^{-1} [12]. Comparing the latter value with the ΔG values reported in Table 2 shows that thermal denaturation ($\Delta G = 18$ kJ mol^{-1}) of holo α-lactalbumin involves a less extensive

Table 2
Thermodynamic parameters for the thermal denaturation of calcium-bound (holo) α-lactalbumin [a]

Protein	ΔH/(kJ mol^{-1})	ΔS/(kJ mol^{-1} K^{-1})	ΔC_p/(kJ mol^{-1} K^{-1})	T_m/(°C)	ΔG^{b}/(kJ mol^{-1})
Human					
TRP [c]	320(±4.5)	4.9(+0.1)	8.0(±3)	66.0	18(±3)
TYR	88	2	3.7	43	3.6
Bovine					
TRP [c]	340(±10)	4.5(+1.0)	6.0	67(±2)	16
	311 [d]	4.5 [d]	10.5 [d]	68(±1) [d]	–
TYR	103	0.3	4.0	41	4.0
	54 [d]	2.0 [d]	3.0 [d]	47 [d]	–

[a] See footnotes to Table 1. Solvent, THAM–HCl buffer (10 mM, pH 8.0 with 9 mM calcium chloride. [b] Maximum ΔG values at 19°C (Tyr results) or 40°C (Trp results). [c] Data from FI (345 – 305) measurements only, F345 shows a biphasic transition. [d] Sodium cacodylate buffer (10 mm, pH 6.0 with 9 mM calcium chloride).

disruption of protein structure compared to GnHCl denaturation. The 3-state thermal denaturation process alluded to previously (Eq. (2)) is not to be confused with the 3-state denaturation process in GnHCl (Eq. (9)). The stable intermediate (X) was not detected by UV difference spectrophotometry or CD studies of α-lactalbumin heat-unfolding [16, 17]. Arguably, these techniques are less sensitive than the method used here, judging by the 10–100-fold lower amount of protein required for the current study.

From Figs. 6 and 7, it appears that some Tyr residues are located in a relatively thermolabile region of α-lactalbumin with an apparent T_m value of 41–43°C (Table 2). By contrast two classes of Trp residue were evident with T_m values of 41–43°C and 66–67°C. Non-superimposable Tyr and Trp transitions have also been reported for bovine α-lactalbumin heat-denatured in sodium cacodylate buffer at pH 6.0 [19]. From Fig. 7, it can be seen that bovine holo α-lactalbumin was susceptible to (cold) denaturation at $T < 10$°C.

The above results will now be discussed in relation to the structure of α-lactalbumin. In common with lysozyme, with which there is a substantial structural homology, α-lactalbumin possess 2 lobes or domains separated by a deep cleft. The larger of the two lobes (Lobe 1) consists of amino acid residues 1–39 and 85–129 in the α-lactalbumin polypeptide chain. Lobe 1 also accommodates 6 of the 8 intrinsic fluorescence chromophores (Tyr-18, Tyr-36 and Tyr-103, as well as Trp-26, Trp-104 and Trp-118) monitored in this study. Lobe 2 consists of amino acid residues 40–80; this lobe contains two aromatic chromophores (Trp 60 and Tyr 50) and a single binding site for a Ca^{2+} ion. In the human α-lactalbumin, Trp 26 is replaced by a leucine residue [1, 2].

The origins of FI changes during thermal denaturation of α-lactalbumin are also known with some certainty [14]. In the N-state, there is an intermolecular energy transfer from Trp 26 and Trp 104 to Trp 60 which is quenched by the proximity of two disulphide groups. Increasing the distance between Trp 60 and the two cystine groups

produces the characteristic FI345 increases associated with α-lactalbumin denaturation. The environment of Trp-118 does not change upon thermal denaturation; the residue is fully accessible to solvent in the native protein [13]. It is generally accepted that Trp 60 is the major contributor to FI345 changes observed during α-lactalbumin denaturation [14].

The 3-state thermal denaturation of α-lactalbumin might result from a difference in the thermal stability of the two domains found within the calcium-bound protein. X-ray crystallography results (*B*-value plots) show that amino acid side-chains in Lobe 1 have a higher average intrinsic mobility than those in (Ca^{2+} binding) Lobe 2. The chemical reactivities of the three Trp residues in Lobe 1 are also significantly higher than the reactivity of Trp 60 located in Lobe 2 [22, 23]. Ca^{2+} binding to Lobe 2 might preferentially stabilise this region of the protein. In contrast, a 2-state thermal denaturation suggests that the two halves of the apo α-lactalbumin molecule possess equal thermostability. Equine and pigeon lysozymes, with strong affinity for Ca^{2+} ions, are more α-lactalbumin-like than other lysozymes. Both equine and pigeon lysozymes undergo a three-state thermal denaturation in the presence of an excess of calcium ions [24, 25].

Acknowledgement

The author is grateful to the AFRC for financial support.

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