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Revised equilibrium thermodynamic parameters for thermal denaturation of β-lactoglobulin at pH 2.6

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Abstract

Thermodynamic parameters for thermal denaturation of β-lactoglobulin (β-lg) should account for the dissociation coupled unfolding (DCU) transitions, Dimer⇌Monomer⇌Unfolded state. Purified β-lg (0.4–4 mg ml^{-1} in 50 mM glycine–glycine–HCl buffer, pH 2.6) was heated and monitored by UV-difference spectrophotometry. The Monomer⇌Unfolded state transition occurred at 65–95°C with T_m equal to 82°C and a Gibbs free energy change (ΔG^0_U) of 51 kJ mol^{-1}. Such results were combined with parameters for β-lg dissociation leading to the Gibbs free energy change for DCU (ΔG^0_{DCU}) of 128 (±8.3) kJ mol^{-1}. The enthalpy and entropy change for DCU was (ΔH^0_{DCU}) equal to 373 kJ mol^{-1} and (ΔH^0_{DCU}) 824 J mol^{-1} K^{-1}. Thus, the room temperature stability of β-lg is 76 kJ mol^{-1} higher than reported previously. The possible significance of such results for protein stability function relations (PSFR) is discussed.

Keywords: Dissociation coupled unfolding transitions; β-Lactoglobulin; Protein stability function relations

1. Introduction

Protein thermodynamic stability can be considered without concern for the detailed pathway(s) of denaturation. In contrast, equilibrium measurements require a careful consideration of initial and final reference states. The stability of proteins with multiple subunits is partly determined by interactions between the units. Native β-lg is a dimer having two identical subunits [5]. The effect of monomer–monomer interactions on the thermal stability of this protein has not received much attention [1–4]. For that reason, the thermodynamic stability of β-lg may have been underestimated. Dissociation destabilizes β-lg and activates the SH-group in this protein [6]. Apenten and Galani reported thermodynamic parameters for β-lg dissociation over a broad temperature range and at pH 2.6 and 7 [7].

In this paper, we report the thermal stability of native β-lg. Purified β-lg (0.4–4 mg ml^{-1} in glycine–glycine–HCl buffer (50 mM, pH 2.6) was heated at temperatures from 7 and 90°C. Structural changes were monitored by UV-difference spectrophotometry [5]. β-Lactoglobulin unfolded at 65–95°C with a Gibbs free energy change ($\Delta G^0_{N,U}$) of 51.6 kJ mol^{-1}. By comparison, the Gibbs free energy change (ΔG^0_{DCU}) for the Dimer⇌Monomer⇌Unfolded state transition was 128 kJ mol^{-1}. The stability of β-lg is thus ∼76 kJ mol^{-1} greater than expected when β-lg is treated as having one subunit. Standard enthalpy and

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entropy changes for DCU are also reported. The possible significance of the new thermodynamic parameters on protein stability function relations (PSFR) are discussed.

2. Materials and methods

β-Lactoglobulin was prepared by ammonium sulfate precipitation from milk and purified by preparative filtration as described previously [5]. Protein was dissolved in 0.05 M glycine–glycine–HCl buffer (pH 2.6 at 25°C) containing 0.02% NaN_3 as preservative.

For thermal denaturation studies, exactly 0.2 ml of protein solution (6.4 or 64 mg ml^{-1}) was added to a cuvette containing 3 ml of buffer or 8 M urea. The final protein concentrations was 0.4 or 4 mg ml^{-1}. Protein denaturation was monitored from UV-difference absorbance measurements at 293 nm using a double beam Pye Unicam (Model SP-8000) UV-Vis spectrophotometer. Sample cuvettes containing protein were heated via the thermostated cuvette holder coupled to a Grant water bath. The reference cuvette contained protein dissolved in 8 M urea. A third cuvette was fitted with a thermocouple in order to monitor sample temperatures. In a typical study, samples were heated at selected temperatures for 10 min and UV-difference measurements were recorded. All studies were repeated at least 3–4 times. UV-difference absorbance readings were transformed to the molar quantities using the dimer molecular weight for β-lg (36,800).

3. Results and discussion

UV-difference absorbance changes at 293 nm (ΔA_{293}) for β-lg samples incubated at 7–90°C are shown in Fig. 1. Protein samples were equilibrated at each temperature for 10 min. The ΔA_{293} readings were recorded for samples before and after heating. In this way, it was established that denaturation changes were >90% reversible except at temperatures above 95°C. The maximum ΔA_{293} value was $-2290\ M^{-1}\ cm^{-1}$ when samples were heated to 95°C. Prolonged heating at such temperatures led to protein aggregation.

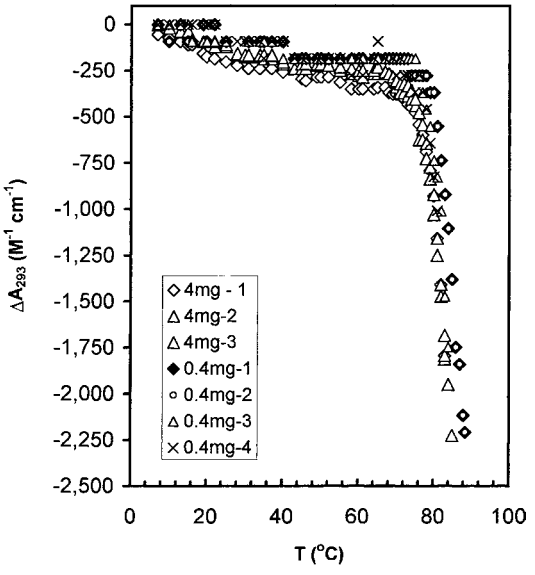

Fig. 1. Effect of temperature on UV-difference absorbance change for β-lg. 0.4 or 4 mg ml^{-1} Protein was dissolved in glycine–glycine–HCl buffer (0.05 M, pH 2.6). Boxed legend shows protein concentrations and number of replicates.

3.1. Determination of thermal denaturation profiles for β-lactoglobulin

Fig. 2 shows the fraction of β-lg denatured at different temperatures. The effect of increasing temperatures on the dissociation of β-lg dimer is shown as the dotted line. The thermal-dissociation profiles were determined as described in a previous study [7]. Clearly, β-lg dimer dissociates at 20–60°C (Fig. 2). By contrast, the monomer undergoes a structural transition at temperatures above 65°C. The denaturation process for β-lg can be summarized by a two-stage process involving the dimer (D_2), monomer (N) and partially unfolded monomer (U),

$$D_2 \rightleftharpoons 2N \rightleftharpoons 2U \quad (1)$$

As the $D_2 \rightleftharpoons 2N$ transition complete by 60°C (Fig. 2) then the experimental ΔA_{293} changes recorded at 70–90°C in this study (Fig. 1) are due to the β-lg unfolding transition,

$$N \rightleftharpoons U \quad (2)$$

These results are similar to those observed for urea denaturation studies. β-lg showed two equilibrium denaturation transitions with increasing urea concen-

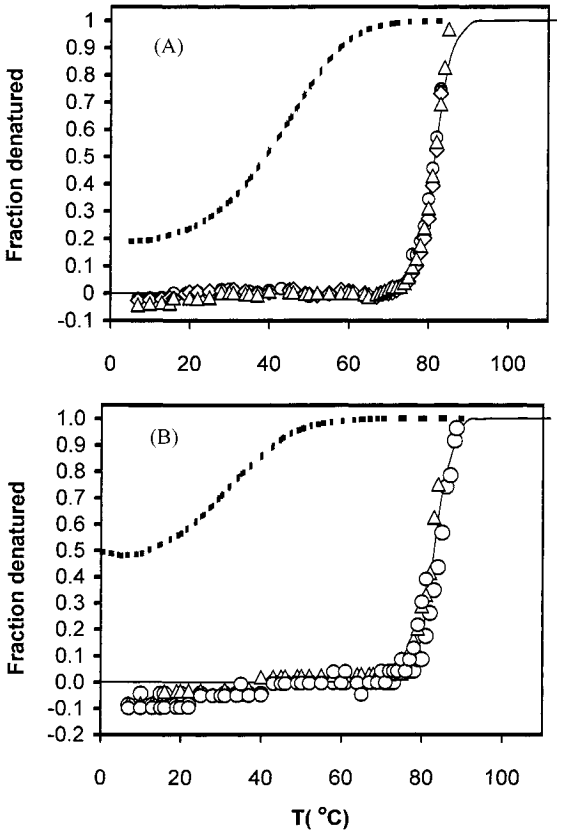

Fig. 2. The effect of temperature on the fraction of β-lg denatured. The protein concentration is (A) 4 mg ml^{-1} or (B) 0.4 mg ml^{-1}. Experimental points are shown. The continuous line is a calculated profile based on data in Fig. 3 using Eq. (8). See text for details. Dashed line shows the fraction of β-lg dimer dissociated at each temperature (Data is from [7]).

trations. First, the dimer dissociates producing two folded subunits at 0–4 M urea. Next, each subunit unfolds with a loss of tertiary (3°) structure in 4–8 M urea [5].

The fraction of protein denatured (F_{den}) and the equilibrium constant for denaturation

(K) was calculated from:

$$F_{den} = \frac{Y_{OB} - Y_N}{Y_N - Y_D} \quad (3)$$

$$K = \frac{Y_N - Y_{ob}}{Y_{ob} - Y_D} \quad (4)$$

where Y_{OB} is the observed ΔA_{293} value at any temperature, Y_N and Y_D are the ΔA_{293} value for the native and denatured protein, respectively. Values for Y_N for "native" β-lg monomer at 50–60°C were fitted to a linear equation; $Y_N = mx + C$. Thereafter, values for Y_N at 60–90°C were found by extrapolation. A fixed value for Y_D of –2290 M^{-1} cm^{-1} was used in calculations involving Eqs. (3) and (4). These procedures have been described before [8].

3.2. Thermodynamic parameters: the thermal denaturation of β-lactoglobulin monomer

The dissociation and unfolding transitions for β-lg are clearly separated and can be analyzed separately. To find ΔG for the process described by Eq. (2), ln K was plotted versus $1/T$ using only results for $F_{den} = 0.05-0.9$. Outside of this range the ratio of N:U is too large or too small to allow an accurate analysis of results. From the slope and intercept of the Van't Hoff graphs (results not shown) were determined enthalpy (ΔH_m), entropy (ΔS_m) at the temperature for 50% denaturation (T_m) [7,8]. As summarized in Table 1, the precision of results obtained at 4 mg ml^{-1} protein was higher (±5%) than that obtained at 0.4 mg ml^{-1} (±9–10%).

The values for T_m, ΔH_m and $\Delta S_m$for β-lg presented in Table 1 are in broad agreement with results from differential scanning calorimetry (DSC). For 0.8–3 mg ml^{-1} β-lg in 0.1 M KCl–HCl buffer (pH 2), DSC results showed $T_m = 78$°C, $\Delta H_m = 313$ and $\Delta S_m = 889$ J mol^{-1} K^{-1}. With 0.1 M sodium phosphate buffer (pH 2.0) as solvent then $T_m = 85$°C, $\Delta H_m = 340$ kJ mol^{-1} (±4.5%) and $\Delta S_m = 947$ J mol^{-1} K^{-1}. Finally, using 0.2 M sodium phosphate buffer (pH 2.0) as solvent gave $T_m = 91$°C, $\Delta H_m = 371$ and $\Delta S_m = 1019.2$ J mol^{-1} K^{-1}. From DSC studies, the calorimetric and Van't Hoff enthalpy ratio was 1.03. Thus, β-lg monomer unfolds via a two-

Table 1
Thermodynamic parameters for the thermal unfolding of β-lg monomer

Parameter	Protein concentration	
	4 mg ml^{-1}	0.4 mg ml^{-1}
T_m (°C)	81.3 (±0.21)	83.1 (±1.1)
ΔH_m (kJ mol^{-1})	475.5 (±24)	528 (±54)
ΔS_m (J mol^{-1} K^{-1})	1342 (±68)	1483 (±147)
ΔC_p (J mol^{-1} K^{-1})[a]	6000	

[a] Value from the literature [1,9].

stage process at temperatures near the T_m [9]. *However, this study is concerned with evaluating standard thermodynamic parameters of β-lg at room temperature where this protein exists as a dimer* [7].

The Gibbs free energy change for denaturing β-lg monomer according to Eq. (2), i.e. ΔG_U was estimated from the following relations:

$$\Delta H_U = \Delta H_m - \Delta C_P (T - T_m) \quad (5)$$

$$\Delta S_U = \Delta S_m + \Delta C_P \ln\left(\frac{T}{T_m}\right) \quad (6)$$

$$\Delta G_U = \Delta H_U - T\,\Delta S_U \quad (7)$$

The subscripts indicate that we are dealing with the N/U transition (Eq. (2)). The thermal capacity change (ΔC_p) value of 6000 J mol^{-1} K^{-1} applies for the following solvent conditions; pH 2.0–6.5 and ionic strength of 3.2 mM–0.2 M [2,3,9].

Fig. 3 shows temperature–ΔG_U profiles for β-lg monomer. The continuous line in the middle of the graph is an average of seven replicate experiments. Data collected at a β-lg concentration of 4.0 mg ml^{-1} (dashed lines) are clustered around the average curve. Experiments using 0.4 mg ml^{-1} (continuous (thin) line) yield a greater number of ''outliers''. To check

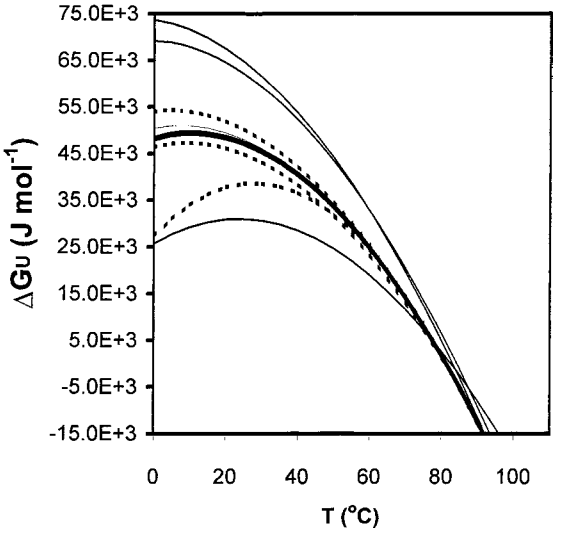

Fig. 3. The effect of temperature on the average Gibbs free energy for unfolding β-lg monomer (bold line). Dashed lines — results using 4.0 mg ml^{-1} protein, Continuous (thin) line — results using 0.4 mg ml^{-1}. Continuous (bold) line — show average values for all data.

the reliability of ΔG_U estimates unfolding profiles were calculated using:

$$F_{den} = \frac{\exp(-\Delta G_U/RT)}{1 + \exp(-\Delta G_U/RT)} \quad (8)$$

These thermal denaturation profiles, displayed as continuous lines in Fig. 2, agree closely with experimental results.

Using previous ΔH_m, ΔS_m, T_m and ΔC_p values from DSC measurements with KCl–HCl buffer (0.1 M pH 2.0), sodium phosphate buffer (0.1 M, pH 2.0) or sodium phosphate buffer (0.2 M, pH 2.0) solvent, Eqs. (5)–(7) lead to ΔG_U^0 estimates of 23.6, 25 and 32 kJ mol^{-1}, respectively [9]. Kella and Kinsella [1] reported ΔG_U^0 between 16 kJ mol^{-1} and 22 kJ mol^{-1} for β-lg heated in 3.2–20 mM HCl solvent (pH 2.0–2.6). The current ΔG_U^0 estimate of 51.6 (±5.1) kJ mol^{-1} suggests that glycine–glycine buffer stabilizes β-lg monomer.

DSC measurements of ΔC_p were obtained with a precision of ±12.5% [2,3,9]. Thus, it is important to evaluate the extent to which errors in ΔC_p affect present results. It was found that ΔG_U^0 changes linearly with the assumed value for ΔC_p according the empirical relation:

$$\Delta G^0(\text{J mol}^{-1}) = 79.1 \times 10^3 - 4.78\,\Delta C_p \quad (9)$$

Thereafter, using ΔC_p (±2 S.D.) within the range 4500–7500 J mol^{-1} K^{-1} did not alter the present results significantly. Assuming $\Delta C_p \geq 8000$ J mol^{-1} K^{-1} resulted in β-lg monomer low-temperature unfolding transition (Fig. 4). Thus, cold-denaturation can be expected if two conditions are met for β-lg (a) $\Delta C_p \geq 8000$ J mol^{-1} K^{-1} and (b) the protein does not associate to form a dimer. Pace and Tanford [10] and also Griko and Privalov [9] observed cold-denaturation transitions for β-lg in the presence of 2–4 M urea. Urea (a) dissociate β-lg dimer and (b) increases ΔC_p to a value very close to 8000 J mol^{-1} K^{-1}.

3.3. Thermodynamic parameters: the thermal denaturation of β-lactoglobulin dimer

At 25°C β-lg exists as the dimer. Using a low pH solvent in conjunction with a low protein concentrations will not justify treating this protein as a monomer. At pH 2–8, significant levels of dimer formation occur at β-lg concentrations ≥1 mg ml^{-1} [5,7]. The

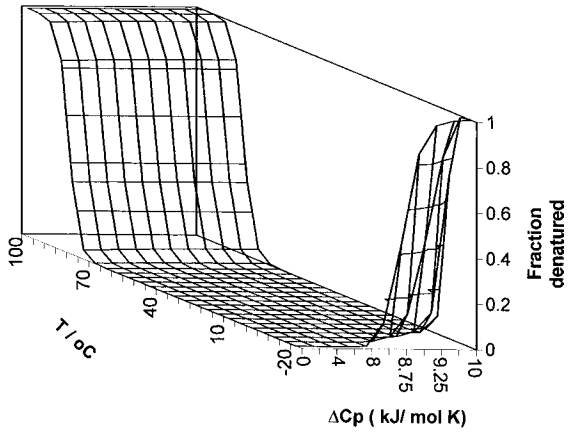

Fig. 4. Simulated results showing the effect ΔC_p on the thermal unfolding profile for β-lg monomer. The ΔC_p scale is expanded between 8 and 10 kJ mol^{-1} K^{-1}.

apparent T_m for β-lg dissociation increases with protein concentration. Thus the relative positions of dissociation and unfolding transitions in Fig. 2 change with increasing β-lg concentrations. To obtain standard thermodynamic parameters for β-lg dimer we combined thermodynamic parameters of the N⇌U transition (this study) and corresponding parameters for dissociation [7]. From the stoicheometry of Eq. (1):

$$\Delta G_{DCU} = \Delta G + 2\,\Delta G_U \quad (10)$$

where ΔG_{DCU} is the free energy change for dissociation-coupled unfolding and ΔG is the Gibbs free energy change β-lg dissociation [5,7]. Standard enthalpy and entropy values for DCU were determined using the same additive principle (Table 2). From Eq. (10) and values for ΔG and ΔG_U we find that ΔG^0_{DCU} is 128 kJ mol^{-1} (±6.4%). The final results (Table 2) are based on seven replicate experiments involving 4 and 0.4 mg ml^{-1} protein. β-lg Dimer is 76 kJ mol^{-1} more stable compared to the monomer. As further support, urea denaturation studies showed that ΔG^0_U was 36 (±1.9) kJ mol^{-1} and ΔG^0_{DCU} was 97 kJ mol^{-1} (±5%) [1]. Higher ΔG^0_{DCU} values are obtained using a non-linear extrapolation method as described in [5].

Table 2

Parameter	Dissociation[a] (D_2⇌N)	Unfolding[b] (N⇌U)	DCU[c] (D_2⇌2U)
ΔH^0 (kJ mol^{-1})	57 (±13)	158 (±11)	373
ΔS^0 (J mol^{-1} K^{-1})	108 (±6.3)	358.0 (±27)	824
ΔG^0 (kJ mol^{-1})	24.8 (±0.35)	51.6 (±2.6)	128 (±8.3)

[a] Data from Apenten and Galani [7].
[b] This work, average for all data (calculated from Table 1).
[c] Calculated from Eq. (10).

3.4. Significance of present results to protein stability and function relations

There is growing interest in protein stability function relations (PSFR). For example, protein stability and digestibility are negatively correlated. Sites for proteolysis are usually hidden by the intact tertiary (3°) and quaternary (4°) structure. Native proteins with higher values for ΔG_U or ΔG_{DCU} are less digestible in vitro.

Generally, stability–digestibility correlations are quite refined. Thermal unfolding of lysozyme can be measured from the rate of digestion by pepsin [11] or pronase [12]. Lysozyme, β-lg and casein were studied using immobilized pronase [13]. Structural changes in myosin at 5–40°C were determined using papain, chymotrypsin or trypsin digestion [14]. Unstable and highly flexible regions in thermolysin were revealed as sites for auto-digestion [15]. Stability–proteolysis correlations have been demonstrated for whole protein extracts from bacteria [16]. Proteolysis may be used as a probe for unstable regions in a protein molecule [17].

In foods, protein ingredients may be selected on the basis of their stability. Kato et al. [18,19] demonstrated a correlation between proteinase susceptibility, ΔG_U and protein function as foaming and emulsifying ingredients. In vitro digestibility and the allergenic potential for genetically modified proteins may be related [20]. Accurate measurements of protein stability are necessary to help to establish PSFR.

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