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Thermodynamic parameters of β-lactoglobulin and α-lactalbumin. A DSC study of denaturation by heating [1]

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Abstract

The thermodynamic parameters of β-lactoglobulin and α-lactalbumin heat denaturation, at acid and neutral pH respectively, were determined by differential scanning calorimetry using heating rates ranging from 2.5 to 15°C min^{-1}. At the high heating rates, the heat denaturation of bovine β-lactoglobulin (crystallized three times or obtained by microfiltration) at about 2–3 mM protein concentration, was found to be partly reversible. The corresponding temperatures and enthalpy changes are protein-concentration-dependent. α-Lactalbumin, in both free and bound Ca^{2+} forms, shows total reversibility. The effect of adding Ca^{2+} to the Ca^{2+}-free form was studied, and the thermodynamic parameters of the transition were determined and compared with published results obtained at lower protein concentrations.

INTRODUCTION

In the "native" state, β-lactoglobulin (β-lg) and α-lactalbumin (α-la) have globular conformations in aqueous solutions. Heat modifies this structure and has effects on the functional properties of these two major whey proteins which are used in food products. At concentrations higher than 5%, β-lg heat denaturation is irreversible because of the intermolecular bonds which give rise to the formation of a network [1,2]. Recently, we have studied the effect of protein concentration on the thermodynamic parameters of β-lg heat denaturation. At pH 3.2, we observed that this process is protein-concentration-dependent and that its half-life time, calculated according to the Borchard and Daniels method applied to

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calorimetric curves using a straight baseline, was not confirmed by an isothermal method for 1.9 mM protein concentration [1]. The half-life times were confirmed by using a sigmoidal baseline to the calorimetric curves obtained at scan rates of up to 7.5°C min^{-1}. This result may be due to the change of heat capacity following the denaturation which plays an important role in the case of relatively low protein concentrations: it deviates the baseline from the simple interpolation between the beginning and the end of the transition. However, the half-life times were not checked in the case of higher scan rates [3]. The hypothesis of partial denaturation was proposed to explain these experimental observations.

At acid pH, it is known that α-la has no tertiary structure [4]. At neutral pH, α-la may be in a Ca^{2+}-bound form (holo form) or in a Ca^{2+}-free form (apo form) [5]. The heat or chemical (guanidium hydrochloride) denaturation process has been extensively studied at low concentration (≈ 20 μM) by different methods including circular dichroism, nuclear magnetic resonance [5,6], spectrofluorometry [7] and, at about 1 mM concentration, by differential scanning calorimetry [4,8].

In this work, we investigate the reversibility of the β-lg heat denaturation and compare the corresponding thermodynamic parameters for β-lg and α-la at relatively high concentrations (2–3 mM) and at pH levels where they have a high thermal stability: acid pH (≤ 3.5) for β-lg and neutral pH (≈ 6.5) for α-la.

MATERIALS AND METHOD

The samples were dispersed in distilled water by dispersion of the proteins, without further purification. The pH (1.7 or 2.3) of the β-lg sample, S_1 (Sigma L0130, crystallized three times), was adjusted with HCl (0.1 M). The sample contained 1% salts and 95% proteins, of which 85% were β-lg. The β-lg sample, S_2, was fractionated and purified by the Laboratoire de Recherche de Technologie Laitière de l'INRA de Rennes [9]. The sample, obtained by ultra- and diafiltration, contained 95% protein of which 92% was β-lg and 3.5% α-la, with 3% salts, 0.5% fat and 1% lactose (dry matter, wt/wt); its pH was 3.5

α-La in the holo and apo forms, was purchased from Sigma (L5385 and L6010, respectively).

The protein concentrations were determined spectrophotometrically, using $A_1^{1\%}$ cm (278 nm) = 9.6 and $A_{1\,cm}^{1\%}$ (280 nm) = 20.1 for β-lg and α-la, respectively [4–8]. The monomer molecular weights were 18420 Da (β-lg) and 14020 Da (α-la).

A differential scanning calorimeter (Perkin–Elmer DSC7) was used in the scanning mode at various rates ranging from 2.5 to 15°C min^{-1}. The calibration (temperature and enthalpy change) and the thermal resistance R of the apparatus were deduced from the calorimetric curves of indium

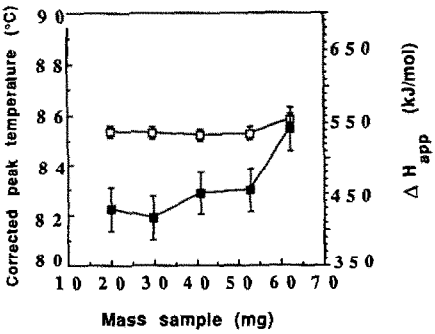

Fig. 1. Variation of peak temperatures (□) and enthalpy changes (■) as a function of sample mass (β-lactoglobulin, sample S_2, 10% wt/wt concentration, 10°C min^{-1}).

[10]. Stainless steel pans (75 μl volume and 0.158 J $°C^{-1}$ heat capacity) were used. The reference material was heat denatured β-lg (20% wt/wt).

The sample temperature T_S was assumed to be uniform in the pan, and was deduced from the programmed temperature T using the equation

$$T_S = T - \beta R m c_p \quad (1)$$

where β is the scan rate (°C s^{-1}), R the thermal resistance ($\approx$ 40°C W^{-1}) and mc_p the heat capacity of the sample before denaturation ($mc_p \approx 0.16$ J $°C^{-1}$). The corrected peak temperatures and enthalpy ΔH_{app} (Fig. 1) were constant within $\pm$0.25°C and $\pm$20 kJ mol^{-1}, respectively, when the sample mass varied in the range 20–55 mg. For an approx. 60 mg sample, the corrected temperatures and the enthalpy changes increased by about 0.6% and 20%, respectively (Fig. 1).

The thermal denaturation temperature T_D is determined by extrapolating the peak temperatures T_p to a 0°C min^{-1} rate. For a reversible two-state process, native protein ↔ denatured protein, the effective enthalpy change ΔH_{VH} was deduced from the Privalov approximation [11]

$$\Delta H_{VH} = 4RT_{max}^2 \, \Delta C_{max}/\Delta H_{app} \quad (2)$$

where R is the gas constant (8.31 J mol^{-1} K^{-1}) and ΔC_{max} is the maximum variation of molar specific heat capacity calculated from $(dH/dt)_{max}$, the maximum heat flux change determined from the baseline

$$(dH/dt)_{max} = m \, \Delta C_{max} \, dT/dt \quad (3)$$

In the case of reversibility, the cooperativity of the denaturation process is evaluated by the ratio $\Delta H_{app}/\Delta H_{VH}$.

RESULTS AND DISCUSSION

β-Lactoglobulin

After a first heating of a β-lg sample S_2 from 20 to 120°C at 2.5, 5, 10, 12.5 or 15°C min^{-1}, the sample was cooled to 20°C at 250°C min^{-1},

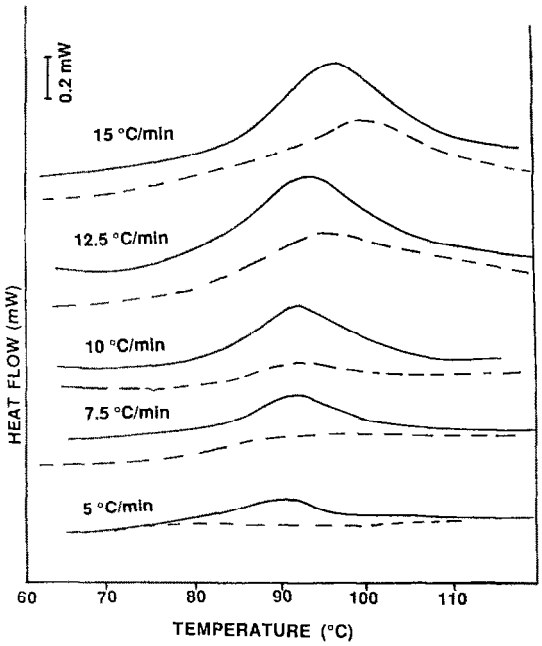

Fig. 2. Calorimetric curves corresponding to first and second heating of β-lactoglobulin at different scanning rates (sample S_1): ——, heating curves, — — —, cooling curves.

maintained 5 min at this temperature and reheated at the same scan rate. At scan rates of 7.5°C min^{-1} and lower, the second heating run does not show any endothermal peak. However, at higher scan rates, a symmetrical peak is observed (Fig. 2). Its area (straight baseline) is lower than that of the first heating thermogram and increases with scan rate (Table 1).

TABLE 1

Thermodynamic parameters of β-lactoglobulin heat denaturation as a function of scan rates (sample S_1, first run from 20 to 120°C)

dT/dt (°C min^{-1})	T_p (°C)	ΔH_{app} (kJ mol^{-1})	ΔH_{VH} (kJ mol^{-1})	$\Delta H_{app}/\Delta H_{VH}$	T_D (°C)	Reversibility [a]
0					87.6	
2.5	87.6	255	366	0.7		0
5	88.6	263	377	0.7		0
7.5	89.8	261	360	0.72		0
10	90.15	296	334	0.88		16
12.5	90.6	274	305	0.90		24
15	91.1	266	291	0.91		41

[a] The reversibility is calculated from the ratio of the second heating area peak to the first (cooling at 250°C min^{-1}).

Assuming that the thermal denaturation of β-lg is reversible at very low concentration [12], with 1.9 mM concentration, a partial reversibility was observed only for scan rates higher than 7.5°C min^{-1} and after rapid cooling. Recently, we have observed that, at higher concentrations ($\geqslant$ 8.8%), this protein undergoes an irreversible aggregation phenomenon if the heating time at a temperature higher than the denaturation temperature is sufficiently long [1,10]. β-Lg is composed of 162 amino acids, with two cysteins and one thiol group. During the heat treatment, if the heating time is sufficiently long, disulphure/sulphydryl interchanges give rise to the formation of intramolecular covalent bonds and then the denaturation process becomes irreversible [13]. Because the enthalpy changes during the second heating run did not depend on the scan rate, according to our experimental uncertainties (Table 1), it would seem that the partial reversibility observed at low heating times ($\leq$ 3 min) does not correspond to the denaturation of proteins which were not denatured during the first heating run. One of two hypotheses may be suggested: during the first heating run, the intramolecular S–H/S–S interchange reactions have not sufficient time to be completed and then the denaturation is partly reversible; soluble aggregates are formed and the peak observed during the second heating run corresponds to the dissociation of these aggregates.

Calorimetric curves obtained with S_1 samples at pH 1.7 and 2.3, with heating from 20 to 100°C, indicate more than 60% reversibility after cooling at rates higher than 2.5°C min^{-1}; an example is given in Fig. 3. The exothermal peak observed during cooling corresponds to the renaturation of the protein at acid pH. At pH 2.3, a partial reversibility (60%) is observed at 10°C min^{-1} and at pH 1.7, at 5°C min^{-1} (70%). This supports

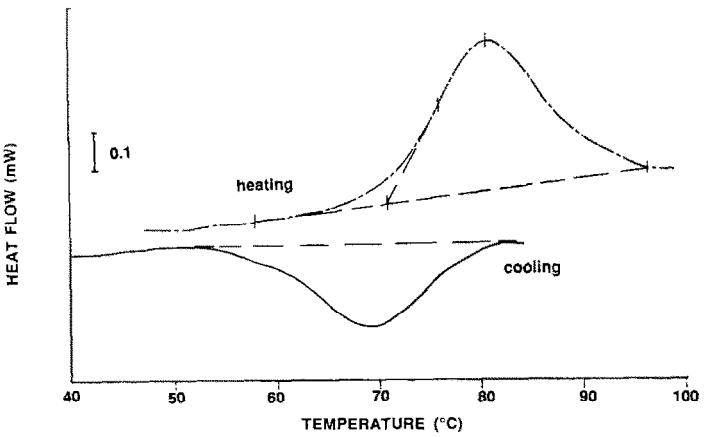

Fig. 3. Heating (10°C min^{-1}, from 20 to 100°C) and cooling (10°C min^{-1} from 100 to 10°C) calorimetric curves of β-lactoglobulin (sample S_2, pH 2.3).

TABLE 2

Temperature and enthalpy change of β-lg heat denaturation as a function of pH

Sample (β-lg)	Conc. (%)	pH	Scan rate (°C min^{-1})	T_S (°C)	ΔH_{app} (kJ mol^{-1})	Ref.
S_2, UF	3.5	3.5	Extr.	87.6±0.3	255±20	This work [a]
Sigma	1.1	3.5	1	88.55	214	Schwarz [15]
Salt-free	4.4	3.5	Extr.	84±0.5	–	Hegg [16] [b]
Sigma	1.1	2.3	1	81.3	205	Schwarz [15]
S_1, Sigma	3.5	2.3	Extr.	78.2±0.3	185±20	This work [a]
S_1, Sigma	3.5	1.7	Extr.	74.1±0.3	165±20	This work [a]

[a] The temperature transition is determined by extrapolation of the values obtained at different heating rates to 1°C min^{-1}.

[b] Salt-free β-lactoglobulin was prepared by precipitation with ammonium sulphate [16]. The denaturation temperature was determined as the intersection of the extrapolated low-temperature side of the calorimetric curve and the baseline (10°C min^{-1}).

an increase in thermal stability at pH 1.7. From the variation of ΔH_{app} versus the temperature of the onset of denaturation, the change in molar specific heat capacity upon denaturation is about 5 kJ mol^{-1} K^{-1}, as for major globular proteins [7,11,14]. This should be confirmed by further investigations. Using UV difference spectroscopy Kella and Kinsella [14] observed that the thermal stability of β-lg is enhanced at acid pH; however, in contrast to the present study, their temperature and enthalpy change values increased with decreasing pH up to pH 1.5. Nevertheless, more recently, Schwarz [15] used DSC to determine the temperature and enthalpy changes of β-lg heat denaturation at low concentrations (0.1%–1.16% and HCl–glycine buffer) and at pH values from 2.27 and 3.57. He considered the protein as a dimer and therefore its ΔH_{app} values were about twice our results at the same pH (88.5°C, 428 kJ mol^{-1} for pH 2.3 and 81.3°C, 410 kJ mol^{-1} for pH 3.5, respectively). For a β-lg sample S_2 at pH 3.5, our results seem to be somewhat higher than those of Schwarz (Table 2). This increase could be attributed to the presence of 3% salts in our solution. This discrepancy will be examined in future works. Hegg [16] has determined the denaturation temperature of β-lg (pH from 2 to 9) prepared from fresh raw milk by precipitation with ammonium sulphate. He determined the denaturation temperature by the intersection of the extrapolated low-temperature side of the calorimetric curve (10°C min^{-1}) and the baseline. Using the same extrapolation method, our result (80°C) is lower than that of Hegg (84°C).

α-Lactalbumin

Figure 4 shows examples of calorimetric curves obtained with α-la in the apo and holo forms. The samples were heated from 5 to 100°C at different

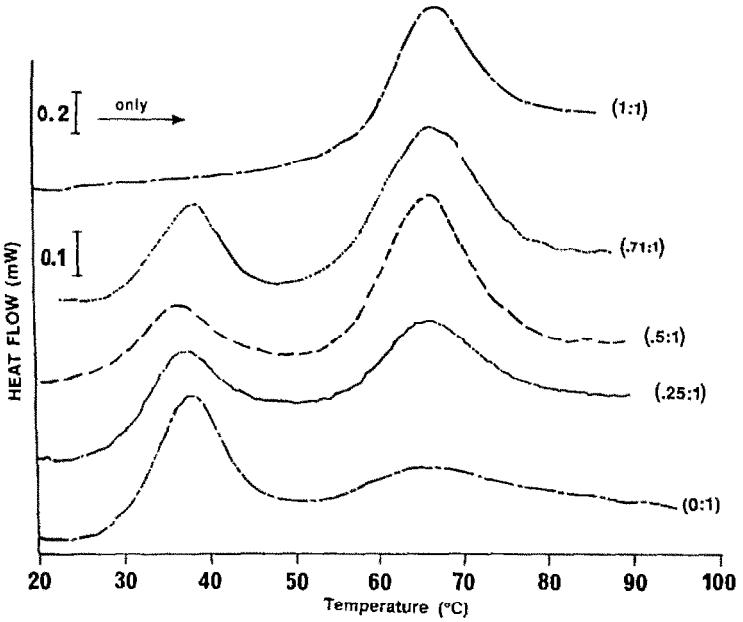

Fig. 4. Effects of added Ca^{2+} (mol Ca^{2+} per mol protein) on heat denaturation of α-lactalbumin in the apo form (10°C min^{-1}).

scan rates and then cooled down to 5°C at the same scan rates. The reversibility of the denaturation is controlled by a second heating run after 5 min at 5°C.

The thermogram obtained with the apo form shows two peaks at about 30 and 60°C. The first peak is attributed to the thermal denaturation of the protein in the apo form [4–8]. The second peak has a smaller surface than the first and could be due to the Ca^{2+}-bound form, because the commercial sample contains some traces of Ca^{2+} (less than 0.3 mol Ca^{2+} per mol protein). To verify this hypothesis, we added increasing Ca^{2+} concentrations to the commercial apo form sample (Fig. 5). The area of the first peak decreases and the area of the second increases linearly with added Ca^{2+} up to the molar ratio 1:1. This result agrees with circular dichroism results which indicate that one protein molecule binds one Ca^{2+} ion. An excess of Ca^{2+} did not significantly modify the denaturation parameters (Table 3).

For the holo form (Fig. 6), the enthalpy change (320 kJ mol^{-1}) and the variation of molar heat capacity (4.8 ± 1 kJ mol^{-1} K^{-1}) deduced from eqn. (3) agree with most published values [4–7].

The area of the second peak, once extrapolated to zero-added Ca^{2+} concentration (≈ 5 mJ) indicates that the commercial sample contains about 15% metalloproteins. The enthalpy change (201 ± 40 kJ mol^{-1}) of the apo form was deduced from the total peak areas observed with the

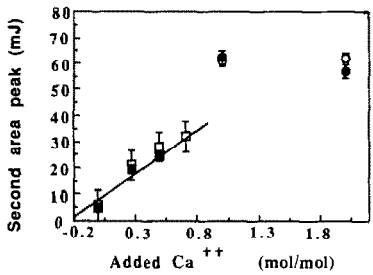

Fig. 5. Second peak area versus Ca^{2+} concentration (mol Ca^{2+} per mol protein). Open symbols, 10°C min^{-1}; solid symbols, 5°C min^{-1}.

TABLE 3

Thermodynamic parameters of α-lactalbumin heat denaturation (holo and apo forms, added Ca^{2+} in mol Ca^{2+} per mol protein)

Sample	T_D (°C)	ΔH_{app} (kJ mol^{-1})	ΔH_{VH} (kJ mol^{-1})	$\Delta H_{app}/\Delta H_{VH}$	ΔS (J mol^{-1} K^{-1})
Holo form (L5383)	60	303	308	0.98	909
Apo form (L6010)	30	201	–	–	663
Apo form (+Ca^{2+} 1:1)	58	320	330	0.97	968
Apo form (+Ca^{2+} 2:1)	60	295	301	1.02	974
Apo form (+Ca^{2+} 140:1)	61	310	304	0.98	928

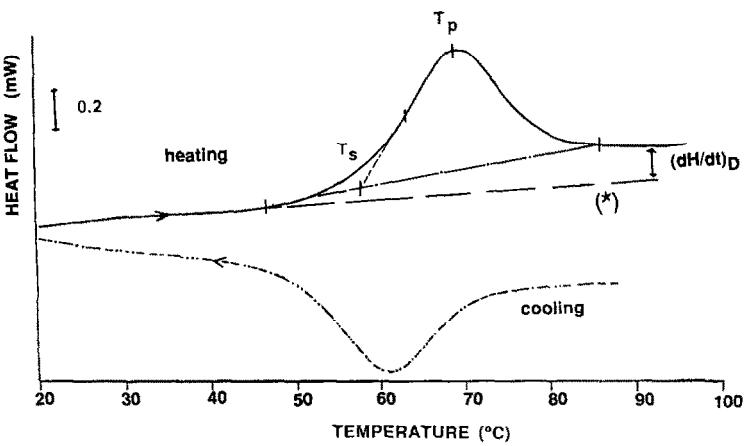

Fig. 6. Heating (10°C min^{-1}, from 5 to 100°C) and cooling (10°C min^{-1}, from 100 to 10°C) calorimetric curves of α-lactalbumin in the holoform. (*) is the baseline.

commercial apo form sample, using the hypothesis of enthalpy change additivity for both forms

$$\Delta H_T = 0.85\ \Delta H(\text{apo}) + 0.15\ \Delta H(\text{holo}) \quad (4)$$

The ratio values of enthalpy changes determined from the second and first heating indicate that the thermal denaturation of α-la in both apo and holo forms is more than 80% reversible whatever the heating and cooling rates. The cooperativity of the denaturation process evaluated from the ratio $\Delta H_{app}/\Delta H_{VH}$ (Table 3) is about 1. The values reported in Table 3 agree with published ones for α-la solutions at much lower concentrations [4–7]. They indicate that the thermal behaviour of α-la is the same whatever the concentration, ranging at least from 20 μM to 3 mM.

Recently [14], we have studied by DSC the effect of added Na^+ on apo α-la heat denaturation. We observed a linear increase in transition temperatures with $\ln(Na^+)$ and a linear relationship between enthalpy changes and transition temperatures, with a slope (4.6 kJ mol^{-1} K^{-1}) which corresponds to the variation of the molar specific heat capacity ΔC_p^d upon denaturation. This is about the same as the value determined for the holo form from eqn. (4).

From the dependence of the transition temperature on the enthalpy $\Delta H(T)$, entropy $\Delta S(T)$ and free energy $\Delta G(T)$ changes

$$\Delta H(T) = \Delta H(T_D) - 4.6(T_D - T) \quad (5)$$

$$\Delta S(T) = \Delta H(T_D)/T_D - \Delta C_p^d \ln(T_D/T) \quad (6)$$

$$\Delta G(T) = \Delta H(T) - T\,\Delta S(T) \quad (7)$$

the values of these thermodynamic functions were determined for the apo and holo forms at 25°C. The results, compared with those obtained for lysozyme [4,8], are reported in Table 4. Lysozyme and α-la have a primary structure composed of several identical amino-acid sequences, four disulphide bonds in the same position and no thiol groups [5]. The ΔH values calculated at 25°C are almost the same for both apo and holo forms. The ΔH value calculated at 110°C (40 J g^{-1}) is lower than for most globular proteins and for lysozyme (54 J g^{-1}) [11]. The free energy variation, ΔG

TABLE 4

Thermodynamic parameters (25°C) of α-lactalbumin heat denaturation (holo and apo forms): comparison with the corresponding values for lysozyme

Sample	ΔH_{app} (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG (kJ mol^{-1})	Ref.
α-La (Ca^{2+} 1:1)	168	485	35.5	This work
α-La (apo form)	178	586	3.2	This work
Lysozyme	239	589	60.7	11

(25°C) for the holo form is in the range of 30 ± 15 kJ mol^{-1}, as for most non-enzymatic globular proteins [11]. For the apo form, its value is slightly lower.

The thermodynamic parameters of heat denaturation of α-la in the apo and holo forms determined in this work agree with those obtained from other experimental methods [5–7] and with the DSC results of samples at much lower concentrations with Ca^{2+}-free protein [8].

CONCLUSIONS

At ph 6.5, the temperature and enthalpy change of apo α-la heat denaturation are about 30°C and 201 kJ mol^{-1}, respectively, and about 60°C and 320 kJ mol^{-1}, for the holo form. These thermodynamic parameters seem to be independent of protein concentration.

For β-lg purified by ultrafiltration (pH 3.5), after heating up to 120°C a partial reversibility is observed in a second heating after rapid cooling at 250°C min^{-1}. However, this reversibility is not observed when the scan rate of the first heating is lower than 10°C min^{-1}, and the degree of reversibility increases with increasing scan rate. At 15°C min^{-1}, it is about 40%. We have not observed the reversibility with cooling at low scan rate.

A crystallized β-lg sample (1.5–3 mM, pH 1.7 and 2.3) has a lower peak temperature and enthalpy change than a sample purified by ultrafiltration (73.6°C, 165 kJ mol^{-1} and 77.6°C, 185 kJ mol^{-1}, respectively). After a first heating from 20 to 100°C at 5°C min^{-1}, a partial reversibility was observed during cooling. No reversibility was observed after heating at 2.5°C min^{-1}, but at higher rates, it is about 70% and 60% for pH 1.7 and 2.3, respectively. With lower concentrations (1%) and scan rates (1°C min^{-1}), Schwarz recently observed the same type of results following heating up to 90°C [15].

β-Lg has a higher transition temperature than most globular proteins [11] and it is known that α-la does not undergo a cooperative transition [4] at acid pH, whereas at pH 6.5, the thermal transition is about 100% reversible. After heat denaturation, β-lg and α-la in both holo and apo forms [13] have about the same molar specific capacity, $\Delta C_p^d \approx 5$ kJ mol^{-1} K^{-1}. For α-la in both forms, the enthalpy, entropy and free energy changes calculated at 25°C (Table 4) indicate that α-la could be more flexible than the lysozyme. ΔH values, calculated at 110°C from eqns. (5)–(7), and using the transition temperatures and enthalpy changes given in Table 3 and considering that β-lg and α-la are both in monomeric form, are about 19 J g^{-1} for β-lg at the three pH values investigated and about 40 J g^{-1} for α-la. However, if β-lg at acid pH is a dimer [15], this characteristic parameter is almost the same for these two proteins.

The unfolding upon heating of the proteins alters their surface hydrophobicity, modifying their functional properties. For β-lg, interfacial

properties and protein association by hydrogen, electrostatic, van der Waals and disulphure bonds, enhanced by thermal unfolding, are well documented [2,17]. For α-la at 25°C, the small change in free energy following unfolding indicates that this protein is less structured or more flexible than lysozyme (Table 4). This globular protein is not thermally gelable at pH 6.5

For most globular proteins, it is accepted that the exposition of polar amino-acid residues into the aqueous phase and of the apolar ones to air or oil phase, generally decreases the interfacial pressure and thus stabilises foams or emulsions. It has been advocated that α-la may have some foaming capacity [13], but its role in emulsification stability is controversial. In addition to the compactness of the conformation, the kinetic rate of the rearrangement at the interface must be taken into account [18]. This parameter needs to be investigated in further studies.

REFERENCES

1 P. Relkin and B. Launay, Food Hydrocolloids, 4 (1990) 19.
2 D.M. Mulvihill and M. Donovan, Irish J. Dairy Res., 44 (1987) 145.
3 P. Relkin and B. Launay, Comm. to Symp. on Chem. Thermodynamics, Calorimetry and Thermal Analysis, 10–14 September 1989, Basel, Switzerland, to be published in Thermochim. Acta.
4 W. Pfeil, Biophys. Chem., 13 (1981) 181.
5 Y. Hiraoka, T. Segaya, K. Kuwajima, S. Sugai and N. Marai, Biophys. Res. Comm., 95 (1984) 1098.
6 Y. Hiraoka and S. Sugai, Int. J. Pept. Protein Res., 23 (1984) 535.
7 E.A. Permyakov, L.A. Morozova and E.A. Bursrein, Biophys. Chem., 21 (1985) 21.
8 W. Pfeil and S. Sugai, Studia Biophys., 100 (1985) 163.
9 J.L. Maubois, A. Pieer, J. Fauquant and M. Piot, Int. Dairy Fed., 212 (1987) 145.
10 S.C. Mraw, Rev. Sci. Instrum., 53 (1982) 2.
11 P.L. Privalov, Adv. Protein Chem., 33 (1979) 167.
12 P. Relkin, T. Liu and B. Launay, 7th Int. Conf. on Surface and Colloids Sci., July 7–13 1991, Compiègne, France.
13 P. Relkin, B. Launay and L. Eynard, J. Dairy Sci., in press.
14 N.K.D. Kella and J.E. Kinsella, Biochem. J., 255 (1988) 113.
15 F.P. Schwarz, Thermochim. Acta, 159 (1990) 305.
16 P.O. Hegg, Acta Agric. Scand., 30 (1980) 401.
17 A. Kiato, K. Komatsu, K. Fujimoto and K. Kobayashi, J. Agric. Food Chem., 33 (1985) 931.
18 J.E. Kinsella, D.M. Whitehead, J. Brady and N.A. Bringe, in P.F. Fox (Ed.), Developments in Dairy Chemistry—4, Functional Milk Proteins: Relationships of Structure and Function, Elsevier Applied Science, London, 1989.