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Thermochimica Acta 246 (1994) 371–386

thermochimica acta

Differential scanning calorimetry: a useful tool for studying protein denaturation

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Received 29 June 1993; accepted 20 April 1994

Abstract

Power-compensated differential scanning calorimetry is applied to the determination of the thermodynamic parameters of protein denaturation. The heating of relatively high mass samples ($\approx$40 mg) is programmed at relatively high scan rates (10°C min^{-1}). For these dynamic thermal measurements, attention is paid to the problems of sample thermal lag and to the construction of the baseline under the calorimetric curve. Simplified mathematical models are applied to the case of the denaturation transition of β-lactoglobulin and α-lactalbumin dispersed in distilled water at relatively high concentration ($\approx$5% w/w). The effects of Na^+ and Ca^{2+} addition on the conformational stability of the proteins upon thermal treatment are compared at pH 3.5 and 7, where these two major whey proteins display different thermal behaviours.

Keywords: Denaturation; DSC; pH; Protein; Thermodynamics

1. Introduction

Heat treatments are of great importance in the production, concentration and processing of food proteins. Changes in protein structures have desirable or undesirable effects on their functional properties as defined by Kinsella [1]. The thermal denaturation process may be reversible or irreversible and more or less cooperative as a function of the sequence of the amino-acid residues and of the chemical environment, such as pH, concentration, ionic strength, etc.

SSDI 0040-6031(94)01877-J

The polypeptide chain of β-lactoglobulin (β-lg) is composed of 162 residues (18 400 Da) and possesses two disulphide bridges and a free cystein group. It is in dimer form at pH 5–6 and dissociates into monomers at acid pH [2]. At basic pH, the –SH/–SS– interchanges play an important role in the formation of high molecular weight polymers during heat treatments [3]. X-ray crystallography shows that the core of the molecule consists of a β-barrel formed by eight-stranded anti-parallel β-sheets [4]. α-Lactalbumin (α-la), as lysozyme, has four disulphide bonds but no free thiol group. At neutral pH, the tertiary structure of its metallo- (holo-) form, composed of several regions [5] of regular secondary structure (four α-helices, five 3_{10}-helices and two β-structures), is stabilized by the binding of Ca^{2+} at a specific cation-binding site [6–9]. The location of the high-affinity binding site is known from the crystal structure but the nature of other specific binding sites for mono- or divalent cations remains controversial. This point is discussed in a review by Kronman [9]. Dissociation of the Ca^{2+} cation from this high-affinity site of the native form gives rise to conformation destabilization through the free metallo- (apo-) conformer. At acidic pH, α-la is known to be a "molten globule", without tertiary structure [10]. The thermal behaviour of these two globular whey proteins is known to be very different: at acid pH, β-lg has high conformational stability upon heat treatment [11–16]; at the low concentrations used for optical techniques, the heat denaturation is 100% reversible [11,12] but at the relatively high concentrations (≈ 3%–5%) needed for DSC techniques, its reversibility seems to be dependent on concentration, buffer and programmed scan rates [13–15]. At basic pH, the reversibility of the denaturation process has been observed by optical techniques [11,17,18] but no indication of reversibility was detected at the usual protein concentrations used for DSC [19]. In food applications, this protein has thermal gelation and surface properties [20–23] and α-la with its metal-binding porperties has a principally biological activity [9]. The thermodynamics of the binding properties of this latter protein to metal cations have been extensively studied by various tehniques including batch microcalorimetry [8] and DSC [15,19,24–26]. In food applications, α-la has poor surface properties and no thermal gelation properties at neutral pH [23].

The heating of globular proteins is accompanied by the destruction of some of the forces which stabilize the tertiary and/or secondary structures, e.g. hydrogen interactions between the polar groups and interactions of non-polar groups (hydrophobic interactions) through the surrounding water molecules which form cages around them. Electrostatic bonds and Van der Waals interactions [27] are also involved in this heat denaturation process, although to a lesser extent. Concerning β-lactoglobulin, the free sulphydryl group is known to become more reactive upon heating [28] and disulphide/sulphydryl exchange reactions, which are enhanced by heating, may also be involved in conformation change (see Liu et al., this issue) and in subsequent high molecular polymer formation [3].

Dynamic thermal measurements have also been extensively applied to the determination of the thermodynamic parameters of heat denaturation of globular proteins used in food systems for their gelation or surface properties. DSC, with its high sensitivity, is still the thermal analysis technique most often used, providing

qualitative and quantitative information to the thermodynamic properties of proteins [27,29,30]. As the protein sample must be diluted to avoid aggregation, the transition peak associated with the change in conformational state is small and the use of relatively high scan rates and high mass samples is necessary to minimize experimental uncertainties. Nevertheless, concerning power-compensated DSC, attention must be paid to the problem of sample thermal lag and the construction of the baseline.

In previous works, Mraw [31] published a mathematical model of the heat flow signal obtained from the melting of pure material, and Dumas [32] considered the case where the baseline deviates from the simple interpolation between the pre- and post-denaturation temperatures because the specific heat capacity of the pure sample before the transition is different from that observed after the transition ($C_D > C_N$). Heuvel and Lind [33] published a model of the baseline curve which is also relevant in the case of rapid transition where an appreciable difference in heating rate between the sample and the reference is observed. More recently, Hemminger and Sarge [34] have shown that the enthalpy change of ice melting, calculated from the area under the transition peak and the various forms of the baseline, is not dependent on the baseline equation, within experimental uncertainties. Schwarz [16] has also considered the effect of a sigmodial baseline on the thermodynamic parameters of the heat denaturation of β-lg at acid pH and, independently, Relkin and Launay [13,14] observed that the heat capacity change between the native and denatured state of β-lg (pH 3.2, 3.5%) could require the use of a sigmoidal baseline for the calculation of the kinetic parameters of heat denaturation.

The purpose of this work is to apply simplified models for the correction of sample thermal lag and of the sigmoidal baseline equation, to the determination of the thermodynamic parameters of β-lg and α-la heat denaturation in some physicochemical conditions.

2. Material and methods

2.1. Sample temperature lag

In DSC systems, the heat flow to the sample or to the reference is

$$W_{s,r} = (T_{s,r} - T)/R \tag{1}$$

where $T_{s,r}$ is the temperature of the sample (reference), T the programmed temperature at constant heating rate β (°C s^{-1}) and R the thermal resistance (°C W^{-1}) between the sample and the heater. If T_0 is the starting temperature, the programmed temperature at time t, is

$$T = T_0 + \beta t \tag{2}$$

Considering a sample with heat capacity equal to mC_s, the heat flow between sample holder and sample pan is

$$W_s = mC_s(dT/dt)_r \tag{3}$$

Using Eqs. (1) and (3), the solution of the differential equation

$$mC_s\, dT_s/dt = (T_s - T_0 - \beta t)/R$$

is

$$T_s = T_0 + \beta t - \beta R m C_s (1 - e^{-t/mC_sR}) \quad (4)$$

where the exponential part is negligible for $t \gg mC_sR$ and, therefore, the sample temperature is then given by the approximation

$$T_s = T - \beta m C_s R \quad (5)$$

The sample thermal lag or the difference between the sample temperature and the programmed temperature is not negligible if any of the following three factors is large: heating rate, thermal resistance, heat capacity of sample + pan.

In this work we used a differential scanning calorimeter (Perkin-Elmer DSC7) with stainless steel pans (75 μl volume and 0.158 J $°C^{-1}$ heat capacity).

The calibration (onset temperature and enthalpy change) and the thermal resistance R of the apparatus were deduced from the melting curves of pure indium as indicated in Fig. 1. The thermal resistance deduced from the slope ($1/R$) of the linear increase of the heat flow (Fig. 1) is $\approx 40°C\ W^{-1}$, whatever the scan rate between 1 and 15°C min^{-1}.

The calculation of the heat capacity, mC_s, of the protein solution ($m = 41$ mg, β-lg 3.5%) before the thermal transition ($T = 60°C$) is made relative to the heat capacity of bidistilled water $m'C'$ from the equation

$$mC_s = m'C'(W_s - W_{ep})/(W' - W_{ep}) \quad (6)$$

where W_s, W_{ep} and W' are the heat flow signal measured at 60°C for protein solution, empty pan and pure water, respectively, see Fig. 2. The corresponding value of heat capacity of the protein solution is 0.17 J $°C^{-1}$, which is about that of a water sample.

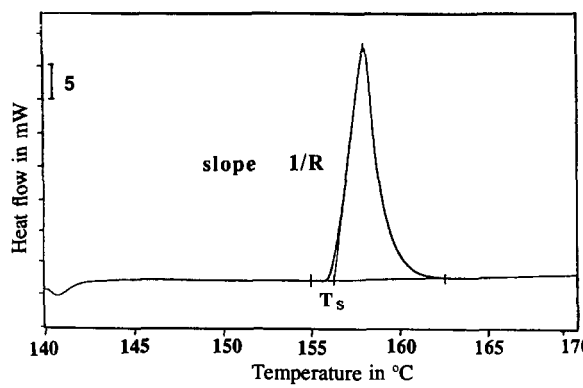

Fig. 1. Example of melting curve of pure indium 15.16 mg in a stainless steel pan, empty pan as reference; 10°C min^{-1} from 140 to 170°C.

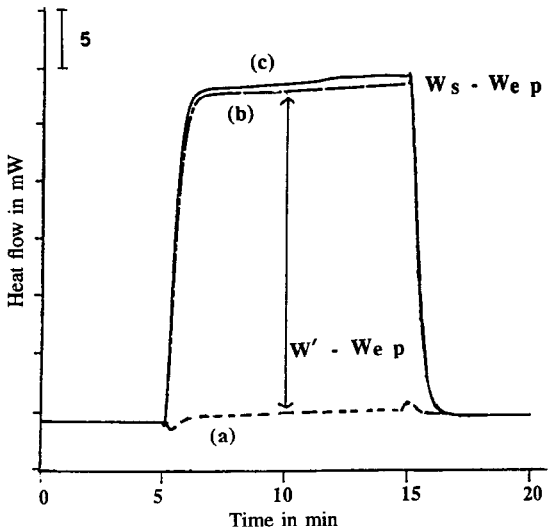

Fig. 2. Principle of heat capacity measurement: empty pan as reference, 5 min at 20°C, heating at 10°C min^{-1} from 20 to 120°C and, 5 min at 120°C. Curve a, empty pan; curve b, pure water (40 mg); curve c, protein solution (10% w/w, 41 mg).

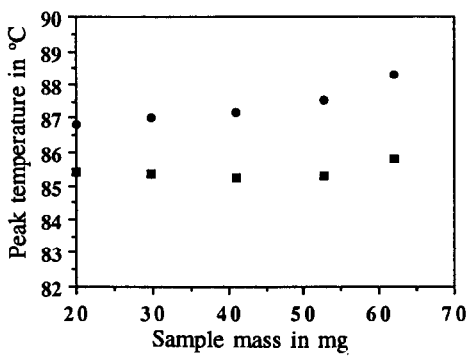

Fig. 3. Variation of peak temperature as a function of sample mass: ●, peak temperature without sample thermal lag correction; ■, peak temperature corrected with Eq. (5), see text.

Curve (●) in Fig. 3 shows the effect of sample mass on peak temperature obtained by heating β-lg dispersed in distilled water (10% w/w, pH 6.6, 10°C min^{-1}) from 20 to 110°C. Curve (■) is obtained using the sample thermal lag correction (Eq. (5)). For protein solutions with sample masses between 20 and 55 mg, the corrected peak temperature and the enthalpy change seem to be independent of sample mass within experimental uncertainties, i.e. 0.25°C and 20 kJ mol^{-1}, respectively, defined from the reproducibility of three different thermograms.

2.2. Sigmoidal baseline

The enthalpy change ΔH_{app} is calculated from the area under the peak using a straight baseline between the beginning and the end of the transition and on the basis of a monomeric unit-molecular-weight of the protein.

The difference between powers delivered by the heaters to both the sample and the reference [35] is

$$W = W_s - W_r = (T_r - T_s)/R \tag{7}$$

or

$$W = C_s \, dT_s/dt - C_r \, dT_r/dt \tag{8}$$

then

$$dW/dt = (dW/dT_r)(dT_r/dt)$$
$$= (dT_r/dt - dT_s/dt)/R \tag{9}$$

Using Eqs. (7) and (9) the relation between the heating rates of the sample and of the reference is

$$dT_s/dt = dT_r/dt[1 - R \, dW/dT_r] \tag{10}$$

For $T_1 < T < T_2$, the heat flow between the sample and the reference is

$$W = dT_r/dt[(\alpha(\Delta C_N^D) + C_N)(1 - R \, dW/dT_r) - C_D] \tag{11}$$

where α is the percentage of denatured protein at temperature T_s, and the heat capacity of the sample is [36]

$$C_s = \alpha C_D + (1-\alpha)C_N = \alpha(\Delta C_N^D) + C_N \tag{12}$$

where C_D and C_N are the heat capacity of denatured and native protein, respectively, and ΔC_N^D is the heat capacity change between the post- and pre-denaturation process. In a first approximation, α the percentage of reactive products is deduced from the ratio $[A(T_s)]/A$ where A is the total area under the transition peak and $[A(T_s)]$ is the partial area up to the temperature T_s. Because we used denatured protein as a reference, $C_r = C_D$ and then, the equation of the sigmoidal baseline in the temperature range $T_1 < T_s < T_2$ is

$$W(T_s) = \{[A(T_s)/A]\Delta W_{T1}^{T2}(1 - R \, dW/dT_r)\} - [RW_{T1} \, dW/dT_r] \tag{13}$$

where

$$\Delta W_{T1}^{T2} = (dT_r/dt)\Delta C_N^D$$

2.3. Thermodynamic parameters

The denaturation process is defined as a transition during which the spatial distribution of the polypeptide backbone changes toward a more disordered one but without hydrolysis of the primary covalent bonds [37,38]. The thermodynamic

characterization of the perturbation in the conformation of "native" state through the change of only one intensive thermodynamic parameters, the temperature, has been established by Brandts [29] and Privalov and Khechinashivali [27] and DSC was confirmed as a powerful technique in this application. For most globular proteins, the "reversible two-state" model from native (N) to denatured (D) conformation is relevant [27] and its criterion is defined by the equality of the calorimetric apparent enthalpy change, ΔH_{app}, and the effective enthalpy change ΔH_{VH}, determined from the van't Hoff equation

$$\Delta H_{VH} = 4RT^2_{max} W_{max}/(A\ dT/dt) \tag{14}$$

where R is the gas constant (8.31 J mol^{-1} K^{-1}) and W_{max} is the maximum deviation of heat flow over the baseline. In the case of proteins with a conformation stabilized by specific interactions as covalent disulphide bonds that join various domains, $\Delta H_{app}/\Delta H_{VH}$ is higher than unity.

The value of the width at half-height peak, $\Delta T_{1/2}$, is indicative of the degree of cooperativity of the denaturation process: the smaller this value, the higher the cooperativity.

The equilibrium constant, $K(T)$, between the fractions of protein in the N and D states is determined from the fractional area α of the transition curve up to the temperature T, over the total area above the baseline

$$K(T) = [D(T)]/[N(T)] = \alpha/1-\alpha \tag{15}$$

The dependence of K on temperature is obtained from the van't Hoff equation

$$d\ln K/d(1/T) = -\Delta H/R \quad \text{or} \quad \Delta H = -R[d(\ln K/d(1/T)] \tag{16}$$

The effective enthalpy change in heat-induced denaturation is deduced from the linear variation of $\ln K = f(1/T)$.

In the case of small globular proteins, the heat capacity change between the native and denatured state, ΔC^D_N is independent of temperature [27] and thus the enthalpy, entropy and free energy variations as a function of temperature are deduced from the equations

$$\Delta H(T) = \Delta H(T_D) - \Delta C^D_N (T - T_D) \tag{17}$$

$$\Delta S(T) = \Delta H(T_D)/T_D - \Delta C^D_N \ln(T_D/T) \tag{18}$$

$$\Delta G(T) = (1 - T_D)\Delta H(T_D) - \Delta C^D_N[(T_D - T) + T \ln(T/T_D)] \tag{19}$$

T_D, the transition temperature, $\Delta H(T_D)$, the enthalpy change and ΔC^D_N, the heat capacity change upon heat transition, are determined from the heating thermogram.

The thermodynamic parameters of the transition are mainly dependent on the concentration, on the scanning rate and on the heating temperature range. The dependence on concentration is due to intermolecular interactions and also, to some degree, on the impurity of the protein solutions; the dependence on scan rate is an indicator of a kinetically controlled or an irreversible process [39].

The use of DSC for the determination of the thermodynamic parameters of a heat-induced transition assumes that a reaction equilibrium exists. The reversibility

of the denaturation process is generally checked by a second heating of the sample solution which should show a similar peak transition to that observed in the first heating curve. If this second heating curve does not show peak, the thermal reaction could follow the model

$$N \underset{k'}{\overset{k_1}{\rightleftharpoons}} D \xrightarrow{k} A$$

If $k \ll k_1$, the irreversible step of aggregation giving rise to species A is slow compared to the rate of the denaturation transition $N \rightarrow D$, and the thermal behaviour of the solution (an overall irreversible process) is similar to that of a reversible process [40]. If $k \gg k'$, most of the denatured proteins D are converted into series A and, if the rate of denaturation is comparable to the programming scan rate, the temperature of maximum deflection increases with increasing scan rates [14].

The denaturation temperature T_D is generally determined by extrapolation to a very low scan rate of the peak temperature obtained at various scan rates with a given protein concentration [42]. The temperature of denaturation is also determined by the intersection with the baseline of the extrapolated leading edge of the heating curve [41]. This temperature is called the onset temperature T_s.

In this study, β-lg in powder form was kindly given by Le Laboratoire de Recherches de Technologie Laitière, INRA-RENNES, France. It was prepared by ultrafiltration and diafiltration from acid whey protein concentrates, following industrial-scale purification [43]. The isolate contained 95% dry matter of which 86% is β-lg and less than 4% α-la, 3% salt, 0.5% fat, and 1% lactose. The protein concentration was determined by the Folin–Lowry method with BSA as a standard.

In the study of the effects of Na^+ and Ca^{2+} addition on the thermodynamic parameters, the protein sample was dialysed (24 h) against NaCl and $CaCl_2$ (Prolabo-France) solutions at various ionic strengths. The pH was adjusted with HCl or NaOH (6 N) and the solutions were kept at 4°C, 24 h before use.

α-Lactalbumin in the apo- (Ca^{2+} free) form was purchased from Sigma Chemical Co., St Louis, MO, L6010, and dispersed, without further purification, in distilled water ($\approx$5% to 8% w/w, pH $\approx$6.5) at various concentrations of NaCl or $CaCl_2$. The protein concentration was determined spectrophotometrically, using $E^{1\%}_{1cm} = 20.1$ and molecular weight 14 000 Da.

3. Results and discussion

3.1. β-*Lactoglobulin*

Examples of heating curves (20–120°C, 10°C min^{-1}) obtained with β-lg samples (4.95% concentration, mass sample $\approx$40 mg, pH 3.5 and 7) are shown in Figs. 4a and 4b. The correction from the sample temperature lag (Eq. (5)) is about 1.15°C and the sigmoidal baselines are drawn using Eq. (13), where we have considered $dT_r/dt = dT/dt = \beta$.

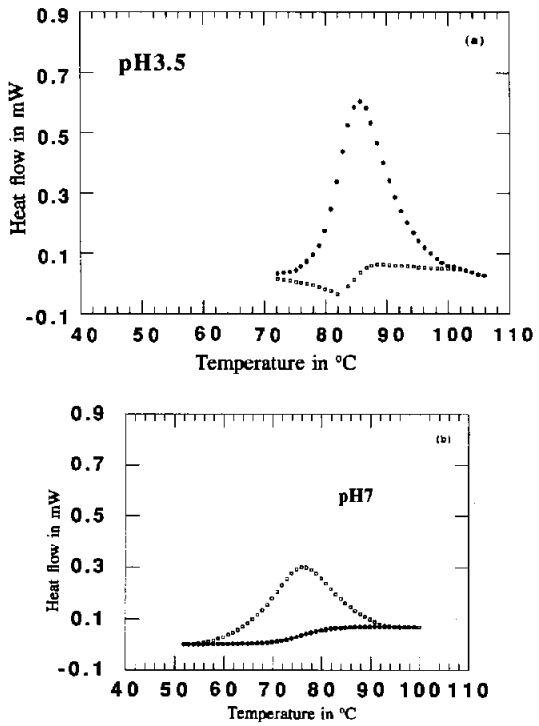

Fig. 4. Examples of β-lactoglobulin heating curves (upward curves) (4.95% concentration, $\approx$40 mg, 10°C min^{-1} from 20 to 120°C) and sigmoidal baseline (downward curves), drawn following Eq. (13) (see text): a, pH 3.5; b, pH 7.

Table 1
Examples of thermodynamic parameters of heat denaturation of β-lactoglobulin: at pH 3.5 (4.95% concentration in 0.05 NaCl) and pH 7 (4.9% concentration in 0.01 NaCl)

pH	Parameters	
3.5		
	$T_s = 78$°C	$\Delta T_{1/2} = 8.75$°C
	$\Delta H_{linear} = 316$ kJ mol^{-1}	$\Delta H_{sigmoidal} = 380$ kJ mol^{-1} (Eq. (13))
	$\Delta H = 345$ kJ mol^{-1} (Eq. (16))	$\Delta H_{VH} = 436$ kJ mol^{-1} (Eq. (14))
7		
	$T_s = 62.5$°C	$\Delta T_{1/2} = 14.5$°C
	$\Delta H_{linear} = 258$ kJ mol^{-1}	$\Delta H_{sigmoidal} = 240$ kJ mol^{-1} (Eq. (13))
	$\Delta H = 274$ kJ mol^{-1} (Eq. (16))	$\Delta H_{VH} = 225$ kJ mol^{-1} (Eq. (14))

Examples of apparent enthalpy change values, obtained from the area under the calorimetric peak and the straight, or sigmoidal baseline (Eq. (13)) and those (effective enthalpy changes) calculated from Eqs. (14) and (16) are summarized in Table 1. These transitions were observed with solutions at pH 3.5 (0.05 M NaCl) and pH 7 (0.01 M NaCl). We obtained a higher value of ΔH_{app} when we applied

a sigmoidal baseline to the transition peak corresponding to a more cooperative process, i.e. with smaller value of $\Delta T_{1/2}$, and for which we also observed a more pronounced curvature in the sigmoidal baseline (Fig. 4a). This phenomenon, observed for solutions at pH 3.5, is indicative of the relatively high difference between sample and reference heating rates and also of a higher cooperativity degree of the denaturation process. We observed the same trend in $\Delta T_{1/2}$ and in curvature of sigmodial baseline with increased ionic strengths (results not shown).

For dialysed solutions at both pH 3.5 and 7, the ratio $\Delta H_{app}/\Delta H_{VH}$ was about unity, but the other thermodynamic parameters of the heat-induced transition were different: temperature, enthalpy change and cooperativity degree are higher at pH 3.5, where we also observed some degree of reversibility (small endothermic peak in the second heating curve). The highest thermostability of β-lg conformation at acid pH is in agreement with the results of Mills [44] determined by tryptophan spectrophotometric measurements, and with those of Kella and Kinsella [11] using a UV-difference spectrophometric technique. The lower thermodynamic parameters observed at pH 7 and the absence of any peak in checking some partial reversibility by a second heating run [15,26] probably correlate with the slight decrease in the sulphydryl group concentration that we observed after heat treatment (Liu et al. in this volume). The –SH groups may be oxidized into disulphide bridges or participate in sulphydryl–disulphide exchange reactions, implying steric impossibility for renaturation.

The variations of the onset temperature, T_s, and of the width at half-peak height, $\Delta T_{1/2}$, as a function of added NaCl or $CaCl_2$ are shown in Figs. 5 and 6. The onset temperature is determined by the intersection with the linear straight baseline (Fig. 1).

At pH 7, the addition of Na^+ or Ca^{2+} causes a sudden increase in T_s at an equivalent ionic strength of about 0.03. At higher equivalent ionic strengths, the increase is linear ($\Delta T_s \approx 10°C$ with $\Delta I \approx 0.6$). We observe a slight decrease in enthalpy change (≈ 13 kJ mol^{-1}) with addition of Na^+ and a more important effect (56 kJ mol^{-1}) with Ca^{2+} addition. These observations are probably due to the

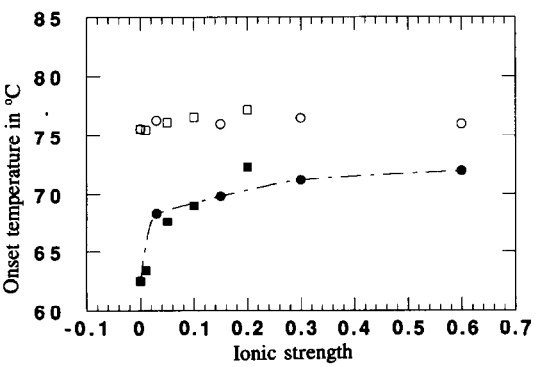

Fig. 5. Variation of onset temperature as a function of equivalent ionic strength (open symbols, pH 3.5; full symbols, pH 7; ●,○, Ca^{2+}, ■,□, Na^+).

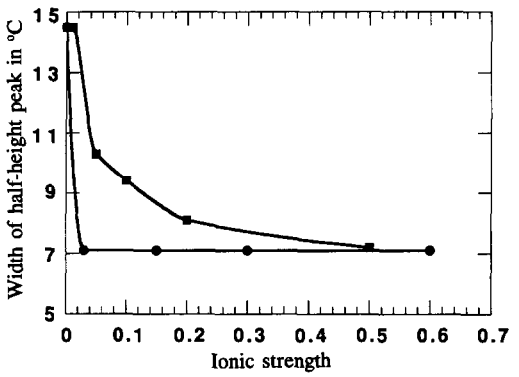

Fig. 6. Variation of width of half-height peak as a function of equivalent ionic strength at pH 7: ●, Ca^{2+}; ■, Na^{+}.

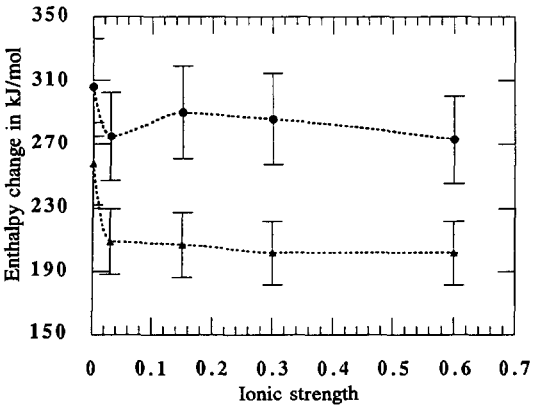

Fig. 7. Evolution of apparent enthalpy change as a function of equivalent ionic strenght at pH 7: ▲, Ca^{2+}; ●, Na^{+}.

screening effects of salts on the electrostatic forces of the protein (more shielding of negatively charged amino groups) and more strengthening of hydrophobic interactions. This more pronounced effect with Ca^{2+} than with Na^{+} suggests an improvement of intermolecular interactions in the presence of Ca^{2+} counterion as predicted by the lyotropic effect on the stability of macromolecules [45].

At pH 3.5 and with equivalent ionic strengths up to 0.3, the cation-dependent variation of T_s and ΔH_{app} was not observed, within experimental uncertainties. This result means that there is less screening effect of salts at low concentration and also, that there is no binding of cation at acid pH, according to Baumy and Brule [46] who observed that β-lg binds 3.5 mol Ca^{2+} cations per mol protein, at pH 6.6 and low ionic strength (<0.01), and that binding affinity decreases with decreasing pH or increasing ionic strength.

The addition of Ca^{2+} at higher concentration (>0.1 M) gives rise to a decrease (≈ 30 kJ mol^{-1}) in ΔH_{app} but does not change the onset temperature (Fig. 7). This

is probably due to some collapse effect of those hydrophobic interfaces more exposed to the solvent at high ionic strength.

3.2. α-Lactalbumin

As expected from the published results [10,24] we have not observed a transition peak with α-la solution at acid pH.

Fig. 8 shows an example of the heating curves of apo-α-la with 1 mol Ca^{2+} per mol protein (pH 6.5). The protein samples were heated from 5 to 100°C. By cooling to 5°C at 10°C min^{-1}, we observed an exothermic peak due to the renaturation process [15,26]. The degree of reversibility of the denaturation, evaluated from the ratio of the area of the second peak (following 5 min at 5°C) to the area of the first one, was about 100%. The thermodynamic parameters of the conformational change are summarized in Table 2. The values of heat denaturation enthalpy change as evaluated from a linear baseline (ΔH_{linear}) or a sigmoidal baseline ($\Delta H_{sigmoidal}$), or from the van't Hoff enthalpy (Eqs. (13), (14) and (16)) are in fair agreement within experimental errors. Those observations from protein solutions at lower concentration indicate, as hypothesized by Pfeil [24] and Pfeil and Sadowski

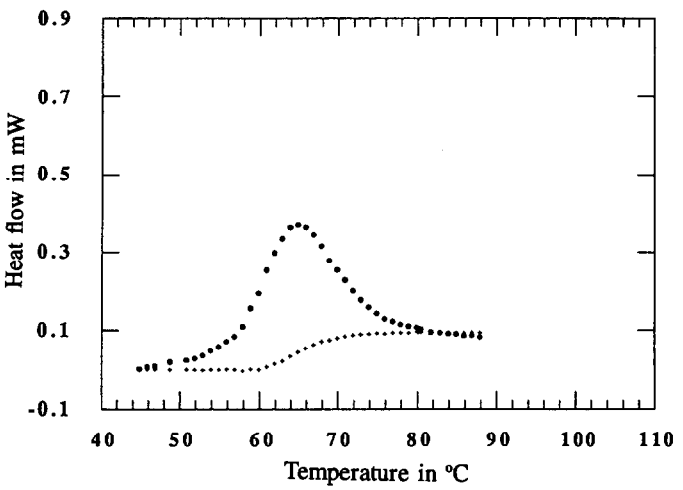

Fig. 8. Example of heating curve of apo-α-lactalbumin with 1 mol Ca^{2+} per mol protein pH 6.5; 4.45% concentration; 10°C min^{-1} from 5 to 100°C (upward curve) and sigmoidal baseline following Eq. (13) (downward curve).

Table 2
Thermodynamic parameters of heat denaturation of apo-α-lactalbumin with 1 mol Ca^{2+}/mol protein (4.5% concentration, pH 6.5)

$T_s = 58$°C	$\Delta T_{1/2} = 12.15$°C	$\Delta C_N^D \approx 5$ kJ mol^{-1}
$\Delta H_{liniear} = 320$ kJ mol^{-1}		$\Delta H_{sigmoidal} = 302$ kJ mol^{-1} (Eq. (13))
$\Delta H = 332$ kJ mol^{-1} (Eq. (14))		$\Delta H_{VH} = 330$ kJ mol^{-1} (Eq. (16))

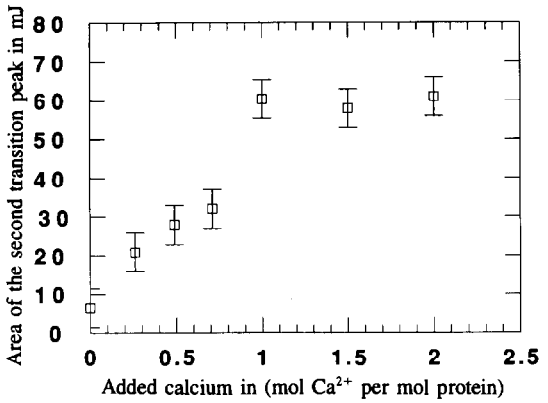

Fig. 9. Evolution of the area under the second peak transition as a function of added Ca^{2+}: ≈40 mg of apo-α-lactalbumin, 4.5% concentration.

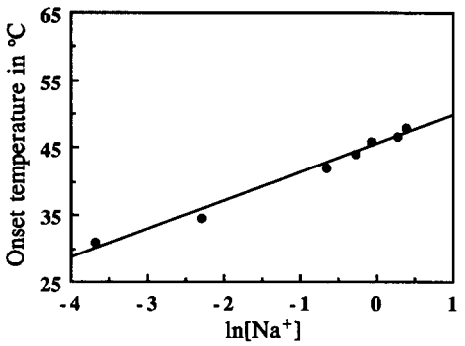

Fig. 10. Evolution of onset temperature as a function of $\ln[Na^+]$: apo-α-lactalbumin, 4.5%, 10°C min^{-1}.

[25], that the "two-state model" between native and denatured forms is relevant ($\Delta H_{app}/\Delta H \approx 1$) for the holo-form. The shift in the baseline between the pre-denaturation and the post-denaturation temperature range, corresponding to an increase in heat capacity ΔC_N^D after thermal denaturation, is about 5 kJ mol^{-1} K^{-1}. This shift is due to an increased exposure to the aqueous phase of polar amino-acid residues initially buried in the interior of the native protein.

The heating of commercial α-la in the apo-form (Ca^{2+}-free) gives rise to a transition curve with a first main peak ($T_s = 30°C$) and a second peak, the former decreasing and the latter increasing in area with added Ca^{2+} from 0:1 to 10:1 molar ratio of Ca^{2+} to apo-α-la. By adding Ca^{2+} up to 1:1 molar ratio, ΔH_{app} increased to a plateau value (Fig. 9) following saturation of the specific binding site. With addition of Na^+ from 0.01 to 1 ionic strength, T_s increased linearly with $\ln[Na^+]$ (Fig. 10) and ΔH_{app} also increased linearly with T_s, with a slope of about 4.6 J K^{-1} mol^{-1}. This value agrees with ΔC_N^D determined from the shift of the

baseline after thermal denaturation of holo-α-la (≈ 5 kJ K^{-1}) and with the published results [24,25]. The presence of two peaks in the heating curve of commerical apo-α-la dispersed in water, indicate that Ca^{2+} cations still present in the sample (less than 0.2 mol/mol protein) affect the total conformational change. Assuming additivity of the enthalpy change of the apo- and holo-forms, we deduced the enthalpy change of the apo-form ($\Delta H_{app}(30°C) = 201$ kJ mol^{-1}). The thermodynamic functions calculated at 25°C using Eqs. (17)–(19) and $\Delta C_N^D \approx 4.6$ kJ mol^{-1} K^{-1} are

Apo-form $\Delta H = 178$ kJ mol^{-1} $\Delta S = 586$ J mol^{-1} K^{-1} $\Delta G = 3.2$ kJ mol^{-1}

Holo-form $\Delta H = 168$ kJ mol^{-1} $\Delta S = 485$ J mol^{-1} K^{-1} $\Delta G = 23.5$ kJ mol^{-1}

They are in fair agreement with the results of Sadowski and Pfeil [25] ($\Delta H_{app} = 175$ kJ mol^{-1}) obtained by DSC, but somewhat higher than the results ($\Delta H_{app} = 142$ kJ mol^{-1}) of Desmet et al. [8], who used batch microcalorimetry and assumed competition of binding of Ca^{2+} and Na^+ to the same site. These authors did not observe a significant difference in ΔH conformational change in binding Ca^{2+} or Na^+ cations by apo-α-la (pH 8) prepared according to the procedure of Hiraoka and Sugai [47] and initially containing lower Ca^{2+} and Na^+ levels (0.03 mol Ca^{2+} and 0.07 mol Na^+/mol protein) than our commercial sample. Their total enthalpy change ($\Delta H = 145$ kJ mol^{-1}), considered as a result of conformational change (endothermic) and metal binding (exothermic) determined at 25°C with added Ca^{2+} (1 mol Ca^{2+} per mol protein), was somewhat lower than our calculated value (168 kJ mol^{-1}).

The affinity constants of binding monovalent and divalent cations, competitively or not, at specific binding sites are discussed in the review by Kronman [9]. Using our experimental values of the thermodynamic parameters, we calculated the binding constant, $K(Ca^{2+}) \approx 4 \times 10^6$ M^{-1} and $K(Na^+) \approx 10^3$ M^{-1} which are smaller than those obtained by Permyakov et al. [48], Murakami et al. [49], and Kuwajima et al. [50], but which agree with the results of Kronman [9] and Hiraoka and Sugai [47].

4. Conclusions

In the first part of this study, we applied a simple mathematical model for the determination of sample thermal lag and for the equation of a sigmoidal baseline under the transition peak of protein denaturation in solution. The former parameter is about 1.15°C for a 10°C min^{-1} scan rate, mass sample ≈ 40 mg, and thermal resistance ≈ 40°C W^{-1}. In the case of a highly cooperative process, i.e. when the heating rates of the protein sample and the reference are different, the cooperativity degree of the reaction is distorted by more curvature in the sigmoidal baseline and the choice of baseline equation has an effect on the value of the enthalpy change.

In the second part of this study, the usefulness of DSC for assessing the thermodynamic stability of protein conformation is illustrated using the example of

two globular proteins which have different behaviours on heating at acidic or basic pH and which bind, specifically or not, metal cations. The specific binding of Na^+ and Ca^{2+} to apo-α-la results in an enhancement of conformational stability through the apo-form → holo-form transition, as shown in previous studies [6–9,24–26]. The effects of these cations on the conformational stability of β-lg solutions are pH- and equivalent ionic strength-dependent: at pH 7, we observe a decrease in ΔH_{app} and $\Delta T_{1/2}$ and a sudden increase in T_s, in comparison with dialysed solution (Figs. 5–7); and, at pH 3.5, where β-lg is known to have maximum heat stability, the effects of both salts on T_s are negligible, and Ca^{2+} addition implies a decrease is ΔH_{app} and $\Delta T_{1/2}$. This thermal behaviour distorts specific conformational changes of β-lg in the presence of this cation.

Acknowledgment

The author thanks Professor B. Launay who stimulated this study and provided helpful discussion.

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