Thermochimica Acta, 199 (1992) 197–205
Elsevier Science Publishers B.V., Amsterdam

DSC studies on the denaturation and aggregation of serum albumins [1]

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(Received in final form 14 September 1991)

Abstract

Denaturation temperatures and enthalpies of bovine serum albumin (BSA) and human serum albumin (HSA) measured by DSC at different pH values and protein concentrations are compared with the literature data on BSA, or SH-blocked BSA. Both parameters display a trend in the region of pH 6.0–8.5 with a maximum at pH 7.1–7.2, in agreement with the literature data. An exothermic peak related to protein aggregation and precipitation was found at higher temperatures. For BSA, at lower pH values, this peak moves towards lower temperatures and overlaps the denaturation endothermic peak at pH < 5.5. The maximum temperature of aggregation, however, depends markedly on the protein concentration; therefore a separation of the two peaks was attempted. At pH 4.0, a shoulder appears at a temperature lower than that of denaturation. A gelification phenomenon also occurs and partially interferes with the other thermal processes.

INTRODUCTION

As part of a program on the thermal stability of homologous proteins from different sources, a preliminary DSC study is presented on the thermal denaturation of Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA), a pair of well known proteins with 80% of homology, displaying, in the same temperature range, aggregation phenomena superimposed on the thermal denaturation process.

BSA and HSA are characterized by overall oblate shape, with three domains, each stabilized by an internal network of disulphide bonds, and each bearing a high number of ionizable groups of opposite sign. They are versatile carrier proteins, active against a wide set of substances with widely differing properties (hydrophobic or hydrophilic, anionic or cationic, etc.).

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[1] Presented at the 12th National Conference on Calorimetry and Thermal Analysis, Bari, Italy, 11–13 December 1990.

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The DSC method provides direct values of denaturation enthalpy ($\Delta_d H$), the temperature of the mid-point of the denaturation corresponding to the maximum of the heat emission plot (T_d), and the change of the molar specific heat ($\Delta_d C_p$) concerning the transition from the native to the unfolded state. Some DSC investigations have been carried out on BSA in the past, especially in the neutral pH range [1–8]. Its thermal denaturation has also been studied by Raman spectroscopy [9]. This and other kinds of conformational changes have been reviewed by Foster [10] and more recently by Peters [11]. Depending on different conditions of pH, ionic strength and temperature, various kinds of transformation have been described. BSA bears 101 carboxylic groups (on 582 residues) and HSA 99 (on 585 residues), 40 of them becoming titrable at pH 4.0–4.5. The highly charged form **F** (that moves "fast" upon gel electrophoresis at pH 3.0–4.0 [10]) is converted reversibly into the expanded (**E**) forms at lower pH (< 2.7) and into the "normal" (**N**) form at pH 4.3 [10–12]. At higher pH (8–9), the **N** form converts in a reversible manner into the highly protonated "basic' (**B**) form. The lysine ε-amino groups (59 residues) all become protonated in a relatively small pH range. The cooperativity of this transition, however, is poor with respect to that at pH 4.3. An "aged" form (**A**) has also been described at higher pH values, after a few days [13]. Owing to the very high number of glutamic and aspartic acid residues on the one hand, and of lysine and asparagine (23 and 24 for BSA and HSA respectively) on the other, many changes in properties have been attributed to rupture of the intramolecular ionic pairs with changing pH [8].

Current interpretations of the processes involved in the thermal denaturation, however, are somewhat inadequate. For this reason, we have undertaken a systematic study of serum albumins from different sources to characterize all the thermal phenomena encountered and to determine the mechanisms (reversible or irreversible) of denaturation, aggregation, etc., concerning this family of proteins.

Here we report a preliminary series of measurements for BSA and HSA at pH 6.0–8.5 and at constant ionic strength and protein concentration. A detailed study at pH 5.0 with changing protein concentration is also reported for BSA to deconvolute the aggregation and denaturation thermal phenomena. Finally, preliminary measurements at pH 4.0 are reported. The data are discussed in the light of the literature values.

EXPERIMENTAL

Materials

The BSA used was a Sigma product, sample A 7511 from fraction V (prepared according to Cohn et al. [14]), defatted to less than 0.005% [15] and finally crystallized and lyophilized [16]: mol. weight, 66300 g mol^{-1};

$\varepsilon_{279} = 44\,200$ l cm^{-1} mol^{-1} [11]. HSA was also a Sigma product, sample A 1887 from fraction V, treated following the same procedure: mol. weight, 66 500 g mol^{-1}, $\varepsilon_{279} = 35\,300$ l cm^{-1} mol^{-1} [11]. The purity of the samples was first tested by UV measurements ($\varepsilon_{279}/\varepsilon_{255} \gg 2$) [11] and then by HPLC, in ammonium acetate 0.1 M, at pH 5.0 using a Protein-pak 125 column from Waters. The chromatograms for both proteins show a pair of peaks at a molecular weight about twice those of serum albumins, independent of protein concentration. This was attributed to the presence of some percent of globins. However no further purifications were attempted.

The BSA and HSA solutions were prepared by weight in 0.2 M tris–HCl buffer or 0.1 M acetate buffer according to the pH range chosen, using double distilled and degassed water. For each measurement, the pH was controlled and adjusted, if necessary, with acetic acid or HCl. The protein concentrations were finally monitored spectrophotometrically.

Instrumentation

The microcalorimeter used was a second-generation Seteram Micro-DSC apparatus, expressly designed for studies on dilute aqueous solutions of biological macromolecules. Its temperature program covers the range 298–398 K and the scan rate can be changed to suit widely differing conditions. After some trial experiments, a scan rate of 0.5 K min^{-1} was chosen for the present study.

RESULTS AND DISCUSSION

The direct DSC signal, normalized according to the electrical calibration, gives the values of several important parameters: the initial (T_i) and final (T_f) temperatures of the denaturation process, the temperature of the maximum heat emission (T_d) (assumed to correspond to the mid-point denaturation) and the change in denaturation enthalpy ($\Delta_d H$) and apparent molar specific heat (C_p). The denaturation enthalpy is obtained directly by integrating the area under the denaturation peak

$$\Delta_d H = \int_{T_f}^{T_f} C_p \, dT - \int_{T_i}^{T_d} C_p^N \, dT - \int_{T_d}^{T_f} C_p^D \, dT \qquad (1)$$

where C_p^N and C_p^D are the apparent molar specific heats below and above the denaturation range and are extrapolated into this range up to T_d. Their difference gives the important parameter $\Delta_d C_p$.

The first set of DSC measurements, concerning the thermal denaturation process in the pH range 6.0–8.5, is reported in Figs. 1–3 and Tables 1 and 2, for both BSA and HSA. The T_d and $\Delta_d H$ curves show maxima at pH 7.2 and 7.1 for BSA and HSA respectively (Fig. 1). These results agree

TABLE 1

Thermodynamic characteristics of the thermal denaturation of bovine serum albumin (BSA) in 0.2 M tris-HCl buffer

pH	$\Delta_d H$ (kJ mol^{-1})	$\Delta_d C_p$ (kJ mol^{-1} K^{-1})	T_d (K)
6.3	683 (16)	38.1 (1.8)	336
6.7	690 (17)	36.3 (0.1)	337
7.0	720 (3)	32.4 (2.2)	337.5
7.3	811 (15)	27.8 (0.8)	338
7.5	742 (6)	26.1 (1.7)	336
8.0	623 (20)	22.3 (0.3)	334
8.5	505 (6)	18.0 (0.9)	330

BSA concentration 1×10^{-4} M; uncertainty limits in parentheses.

TABLE 2

Thermodynamic characteristics of the thermal denaturation of human serum albumin (HSA) in 0.2 M tris-HCl buffer

pH	$\Delta_d H$ (kJ mol^{-1})	$\Delta_d C_p$ (kJ mol^{-1} K^{-1})	T_d (K)
6.5	562 (5)	60.6 (2)	333
7.0	688 (4)	52.5 (1)	338
7.3	595 (3)	46.3 (4)	337
7.7	571 (10)	37.5 (1)	335.5
8.0	480 (4)	34.5 (2)	334
8.5	446 (5)	26.9 (3)	333

HSA concentration 1×10^{-4} M; uncertainty limits in parentheses.

with those found in earlier works [6,8]. No substantial differences are observed in the T_d values with respect to the BSA bearing the sulphydryl group in position 34 protected with iodoacetamide at comparable ionic strength [8]. However our results give $\Delta_d H$ values lower by about 30–100 kJ mol^{-1}. A marked dependence on the ionic strength (at lowest values)

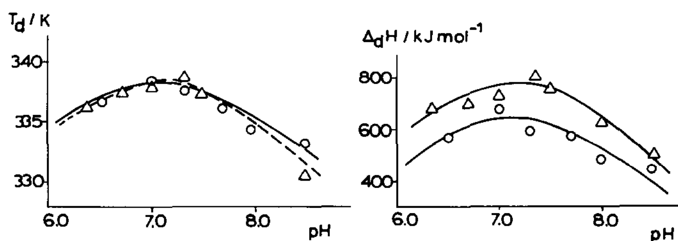

Fig. 1. Trends of T_d and $\Delta_d H$ as functions of pH: △, BSA; ○, HSA. Tris-HCl buffer 0.2 M. Protein concentration 1×10^{-4} M.

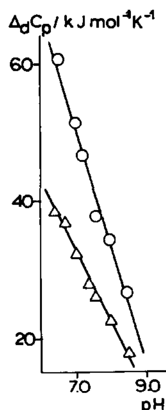

Fig. 2. Trends of $\Delta_d C_p$ for BSA and HSA as functions of pH: same experimental conditions as in Fig. 1.

was observed in the absence of buffers [8]. Changes in the $\Delta_d H$ values and a maximum at pH 5.0 were observed for BSA in the presence of other milk proteins [6].

For HSA, the T_d trend is strictly similar to that of BSA, but with $\Delta_d H$ values 50–100 kJ mol^{-1} lower. There is a marked dependence of $\Delta_d C_p$ on pH, approximately linear in this range (Fig. 2). An increase in protein deprotonation on increasing the pH, promotes a more extensive solvation and a little swelling of the native BSA. As a consequence, the $\Delta_d C_p$ decrease could be attributed to an increase in C_p^N. Despite these wide changes, the dependences of $\Delta_d H$ and T_d on the pH largely compensate each other, giving an approximately linear increase of $\Delta_d H$ with T_d.

For both BSA and HSA, heating at temperatures higher than T_f generates the irreversibility of the denaturation process. Despite this, no practical dependence of the parameters on scan rate has been revealed. Application of the van't Hoff analysis of incremental areas [17], moreover, gives $\Delta_d H$ values in good agreement with the calorimetric determinations, as though a two-state equilibrium was operative in this pH range. If the scanning range is extended to $\approx 100°C$, an exothermic peak is always

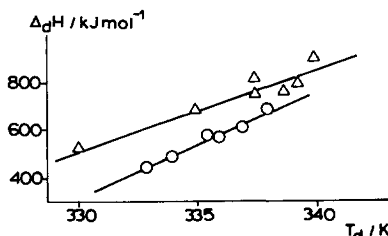

Fig. 3. Correlation of $\Delta_d H$ and T_d. Symbols and experimental conditions as in Figs. 1 and 2.

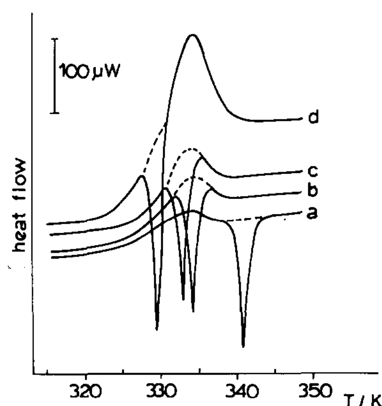

Fig. 4. DSC plots for BSA in acetate buffer, 0.1 M at pH 5.0 for different protein concentrations: a, 2.9×10^{-5} M; curve b, 7.5×10^{-5} M; curve c, 1.0×10^{-4} M; curve d, 2.2×10^{-4} M.

revealed and the precipitation of protein occurs. This precipitate cannot be resolubilized. Thermal denaturation of SH-blocked BSA has been described as reversible [8]. In our case, it may be assumed that the free –SH in position 34 exchanges with some of the numerous disulphide bridges, giving rise to "scrambled" structures that cannot be reconverted into normal ones by recooling the solution. This can also result in a quasi-athermal formation of irreversible dimers or trimers, etc., promoting the aggregation and precipitation of these proteins. In higher alkaline ranges, rupture and resettlement of the disulphide bridges are catalysed by OH^- ions and favoured by high temperatures [18,19].

At pH < 5.5 denaturation overlaps with an aggregation process (at higher pH, this occurs at temperatures well above those of the denaturation range, as discussed before), so that the thermodynamic properties cannot be easily determined. The maximum aggregation temperature, however, depends on protein concentration. In Fig. 4, for instance, the corrected DSC signals are reported as a function of the temperature inside the cell at different concentrations of BSA and at constant pH 5.0. The results are summarized in Fig. 5 and Table 3. Constant values for the denaturation parameters are obtained by extrapolating the denaturation trend through the aggregation temperature range. In some simple cases (Fig. 4, curves a and d), the normalized data were fitted with a polynomial from T_i to T_d, or from T_f to T_d, respectively, excluding those in the overlap region. In other cases (Fig. 4, curves b and c), the fit was carried out by means of pairs or triplets of gaussians, each used only in a limited temperature range. The arbitrariness of this choice of ranges and parameters proved to be not significant. The $\Delta_{ag}H$ (obtained by the deconvolution of the area of the exothermic peak from that of the denaturation endothermic peak) and the temperature of the maximum of the exothermic peak

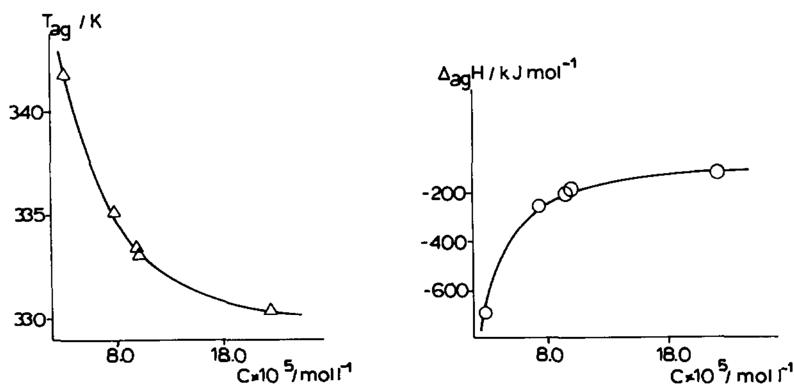

Fig. 5. Behaviour of T_{ag} and $\Delta_{ag}H$ as functions of BSA concentration. Acetate buffer 0.1 M at pH 5.0.

show a marked dependence on the BSA concentration. Then, by changing the protein concentration, it is possible in principle, at least partially, to distinguish the two phenomena. (The homogeneity of the samples and their purity degree, however, are very important for any reproducible comparison.) The temperature of the maximum of the peak diminishes rapidly on decreasing the protein concentration. The aggregation enthalpy per mole also diminishes. This can only be explained by assuming that in very dilute solutions the efficiency of the intermolecular contact is higher, whereas in less diluted solutions incoherent formation of less intensive contacts promotes precipitation at lower temperatures. This is in agreement with the phenomena observed at lower pH.

Fig. 6 shows a typical DSC-profile output at pH 4.0. At this pH a gelification process occurs, depending in turn on the protein concentration. Gelification is observed even in very dilute solution, but its thermal effects are limited and not very reproducible. Another endothermic effect is encountered very close to room temperature. The DSC profile is repro-

TABLE 3

Thermodynamic characteristics of the thermal denaturation of Bovine Serum Albumin (BSA) in 0.1 M acetate buffer

C (mol l^{-1})	$\Delta_d H$ (kJ mol^{-1})	$\Delta_{ag} H$ (kJ mol^{-1})	$\Delta_d C_p$ (kJ mol^{-1}K^{-1})	T_d (K)	T_{ag} (K)
2.9×10^{-5}	524	−685	49.1	335	341.5
7.5×10^{-5}	528	−257	51.5	335	335
9.7×10^{-5}	518	−198	49.8	335	333.5
1.0×10^{-4}	551	−190	49.2	335	333
2.2×10^{-4}	540	−114	49.2	335	330.5

pH 5.0.

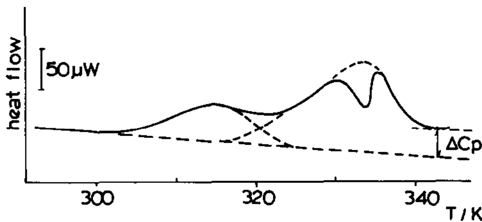

Fig. 6. Deconvolution of the DSC plot for BSA at pH 4.0 (acetate buffer 0.1 M).

ducible showing, at this pH, a smoothed endothermic shoulder that can be deconvoluted, giving a maximum at about 314 K. Gelification (revealed by the exothermic peak superimposed on the denaturation peak) cannot be easily analysed. This peak is much less sharp than the aggregation peak. Its shape seems to depend on protein concentration and kinetic factors. We suspect that in many cases the use of less sensitive instruments or of high scanning rates has led some authors to ascribe their results to two distinct endothermic peaks, instead of to the overlap of an exothermic and an endothermic peak. The exothermic effect represents only about 8% of the sum of the two endothermic effects, whereas the lower temperature shoulder gives about 30% of the total endothermic effect. Its nature remains to be clarified, even though predenaturation is the most obvious suggestion [8]. For this effect we found no reversibility in the scanning, unless the temperature program was stopped at 315–320 K. Moreover, in some cases the shoulder disappears some days after the preparation of the solution. Work is in progress to analyse more quantitatively the thermal behaviour of the serum albumins across the pH ranges in which overlapping takes place. In our opinion, however, this preliminary work is the first systematic attempt to separate aggregation and denaturation processes in different ranges of pH and temperature.

ACKNOWLEDGEMENTS

This work was financially supported by the Italian Consiglio Nazionale delle Ricerche (C.N.R.), Rome, and Ministero dell'Università e della Ricerca Scientifica e Tecnologica (M.U.R.S.T.).

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