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A combined scanning dilatometric and differential scanning calorimetric study of the thermal unfolding of bovine serum albumin

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Abstract

Here we report the first results of combined scanning calorimetric and dilatometric investigations carried out on bovine serum albumin in aqueous solution at 30–97°C. Comparison of experimental data obtained by the two techniques suggests a "steps" model for the thermal denaturation of the protein.

Moreover, the results in the high-temperature region, after aggregation of the unfolded chain, show a subsequent, previously unreported, spatial rearrangement of the polypeptidic chain network, during which the order of the system increases with an increase in temperature.

The results indicate that scanning dilatometry is a very useful method of detecting phenomena which are not seen by calorimetry. The results also demonstrate the determinant role of water in the unfolding process.

INTRODUCTION

Understanding the conformational stability of proteins in solution is fundamental to understanding their biological activity because proteins are active only in their native state in water [1]. The water of hydration plays an important role in the functioning of biological macromolecules. Changes in hydration play a critical role in the process of protein folding and assembly, in which solvent-mediated solute–solute interactions (such as hydrophobic interactions) make a major contribution to the total driving force [2]. Measuring the stability of a protein consists of studying its destruction [3, 4], or unfolding, a process in which the spatial disposition of the polypeptide chain passes from a folded state, bound by intramolecular interactions, to a random-coil state. The enthalpic effects of unfolding can be detected by a differential scanning calorimeter (DSC) [4], while the

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volumetric effects can be seen by scanning dilatometric measurements (SD) [5].

The apparent specific volume of a protein is a parameter that has been used to elucidate several processes depending on protein conformation or during which protein conformation changes [6, 7], but these experimental data are available only for 25°C [8]. The lack of data on the volumetric behaviour of small compounds in solution as a function of temperature prevents the formulation of a model for the volumetric effects associated with the temperature-induced denaturation of proteins.

The purpose of this work is to determine the possibility of studying the thermal denaturation of proteins by means of a technique generally used for other purposes [5]. Our investigation, carried out from 30 to 97°C for both techniques (DSC and SD), shows new phenomena in the range 80–97°C in the thermal denaturation of bovine serum albumin. These new experimental data allowed us to advance some hypotheses on the thermal unfolding process of this protein. The experimental model of the entire process is in agreement with the predictions of other studies [3, 4, 6, 9–12].

MATERIALS AND METHODS

Materials

Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, MO, USA); potassium phosphate analytical grade was obtained from Fluka Chemie AG (Buchs, Switzerland).

Scanning dilatometric measurements

A Mettler TC10A thermomechanical analyser, previously calibrated for temperature and length, was used to obtain measurements of length as a function of temperature at constant pressure. This commercially available instrument was modified with a quartz cylinder with a tight, but freely movable piston as the sample holder. The movement of the piston was measured as a change in length of the sample. The thermomechanical analyser was interfaced with an IBM-XT computer with a sampling rate of 0.329 points per second. Further details are reported in refs. 13 and 14. The scanning dilatometer, in the configuration described has a detection limit and reproducibility of about 3.2×10^{-7} ml and 0.2%, respectively. The temperature accuracy and stability were ± 0.2 and ± 0.01°C, respectively. Scanning was from 30 to 97°C at 0.5°C min^{-1}. About 20 mg of protein per ml were dissolved in 100 mM phosphate buffer at pH 7.03. The ionic strength was adjusted to 0.1 M by sodium chloride. The volume of the sample was about 0.600 ml each for the volumetric and calorimetric experiments. The volumetric measurements were corrected for the thermal expansion of pure electrolyte solution using the equation reported in ref. 15.

The volumetric curve has no physical significance above 97°C because of the formation of micro-bubbles in the solution.

Differential scanning calorimetric measurements

Calorimetric measurements were carried out with a differential scanning calorimeter (microDSC, Setaram, France), with stainless steel sample cells, interfaced with a BULL 200 Micral computer. The data sampling rate was 1 point per second. The solutions were prepared in the same way as for the dilatometric experiments. The same solution without protein (about 0.600 ml) was used in the reference cell. Both the sample and reference solutions were scanned from 30 to 97°C, with temperature accuracy and stability of ± 0.08°C and ± 0.01°C respectively, at a rate of 0.5°C min^{-1}. The average noise was about $\pm 0.4\ \mu W$ and the reproducibility at refilling was about 0.1 mJ K^{-1} ml^{-1}. Electrical calibration was achieved with a Setaram EJ2 Joule calibrator.

RESULTS AND DISCUSSION

The apparent partial molar volume ΔV_s of albumin relative to that at 25°C is plotted against temperature in Fig. 1(a). The apparent partial molar heat capacity C_p of albumin is shown against temperature in Fig. 1(b). To simplify the discussion, the volumetric effects of the hydrophilic residues will be neglected. This assumption is reasonable because we are dealing with changes in the molar volume from the molar volume at 25°C, and the hydrophilic surface area of the protein exposed to water does not change during unfolding. Thus, hydrophilic groups are not expected to cause appreciable changes in volume as a consequence of unfolding.

The volumetric changes in a protein in aqueous solution during heating can be ascribed to two positive and three negative contributions. The positive volumetric variations with increasing temperature can be ascribed to: (a) intrinsic expansion of the protein backbone as a consequence of the increase in conformational degrees of freedom; and (b) increase in the hydration cospheres as a consequence of exposure of apolar groups to the solvent [6, 7].

The "clathrate-like" or "ice-like" water is in a lower energy state (with stronger interactions between water molecules) and a lower entropy state than for bulk water [2]. This water in the hydrophobic hydration cosphere also has a higher molar volume [6]. When the temperature is raised, the more ordered, low-entropy water of hydrophobic hydration converts into less structured bulk water.

The negative volumetric variations with increasing temperature are: (a) progressive destruction of the hydration cosphere; (b) irreversible intermolecular aggregation and the release of water from the hydration cosphere

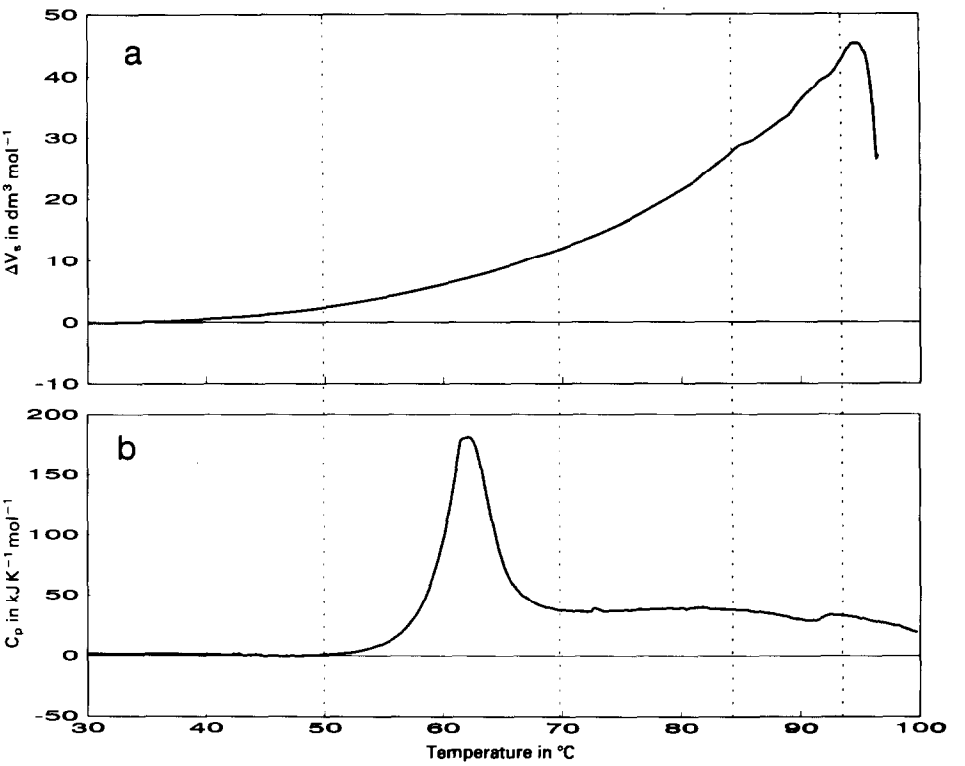

Fig. 1. (a) Change in apparent partial molar volume ΔV_s/(in dm^3 per mol of protein of bovine serum albumin) versus temperature (in °C) at constant pressure in phosphate buffer at pH 7.03. (b) Temperature dependence of the change in apparent partial molar heat capacity of albumin in phosphate buffer at pH 7.03.

to the bulk; and (c) rearrangement of aggragates into more ordered structures. This last phenomenon is hypothesised by us in the light of the measurements reported in this work. All these effects (positive and negative) contribute to the total variation of the volume with temperature.

The volumetric and calorimetric curves (Fig. 1(a) and (b)) show the following. In the range 30–50°C, C_p does not vary appreciably while the volume progressively increases. This slight increase in volume is probably purely a result of intrinsic expansion of the protein caused by the increase in internal motions of the polypeptide chain. In the range 50–70°C, the breakdown of the intramolecular protein bonds occurs, as shown by the endothermic peak in the calorimetric curve for which ΔH is 874.5 kJ mol^{-1}. The unfolding of the protein gives rise to a marked increase in the heat capacity, $\Delta C_p = 37.17$ kJ K^{-1} mol^{-1} [3]. In the same temperature range, the dilatometric curve shows a progressive increase in the molar volume with an ever increasing slope. In this range the volumetric increase should be due to the sum of the positive contributions (a) and (b). In the range 70–80°C, C_p

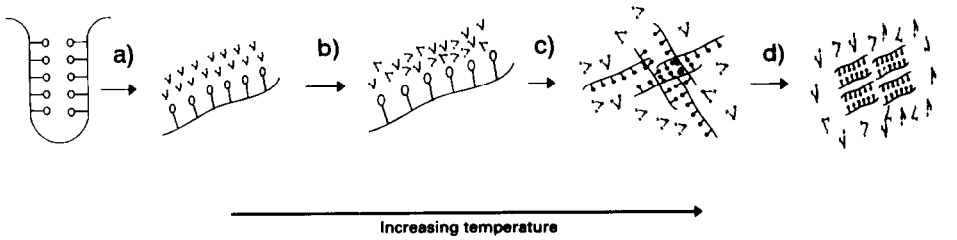

Fig. 2. Schematic diagram showing the thermal transitions in bovine serum alumbin. (a) Breakdown of intermolecular interactions in the folded structure and exposure of apolar groups to the solvent, with formation of hydration cospheres. (b) Partial destruction of the hydration cospheres. (c) Aggregation of the polypeptide chains. (d) Ordering of the aggregated polypeptide chains.

initially increases linearly and then commences to decrease. This trend is well interpreted by Privalov's model [3] which predicts, after unfolding, a progressive fusion of "ice-like" water previously coordinated by hydrophobic groups. A comparison of calorimetric and dilatometric profiles in this range seems to indicate that even if the energetic aspects end at 70°C, the process of unfolding probably continues to temperatures a few degrees higher. The major contribution to the increase in the volume is still the positive contributions (a) and (b). In the range 85–93°C, aggregation occurs [16–18], characterised by a broad exothermic peak in the C_p profile with a minimum at 91°C and $\Delta H = -20\ \mathrm{kJ\ mol^{-1}}$. The formation of intermolecular cross-links, with release of part of the water of the hydration cosphere, produces a decrease in volume which contrasts with the progressive increase in volume caused by internal motions of the polypeptide chain that are not yet blocked by the intermolecular cross-links. The result is a decrease in the slope of the dilatometric curve.

Finally, at temperatures higher than 93°C, the system undergoes a considerable spatial rearrangement with an abrupt reduction in specific volume. This rearrangement, which decreases the entropy in the polypeptide chain, is partially compensated by the increase in the entropy of the water released to the bulk.

A recent, detailed calorimetric study of the thermal denaturation of human and bovine albumins [19] is in good agreement with this study. In particular, Barone et al. [19] hypothesised that in concentrated solutions (about 2.2×10^{-4} M), disordered aggregates are formed. Our dilatometric analysis, carried out in very similar conditions, confirms this. We also hypothesise a subsequent ordering of these incoherent structures at higher temperatures. This latter phenomenon has previously been observed for some polypentapeptides [2] which undergo an inverse temperature transition in solution when the temperature is raised, i.e. the intra- and intermolecular orders increase with increasing temperature. The hypothesised denaturation "steps" are shown schematically in Fig. 2.

TABLE 1

Columns two to four report the coefficients of the polynomial $V(T) = a + bT + cT^2$ for each range of the dilatometric curve; the fifth column gives the temperature derivative of the cubic expansion coefficient calculated from the fitted curves; the last column gives the standard deviation of the difference between the experimental and fitted curves

Range/°C	a	b	c	$\frac{\partial\alpha/\partial T}{K^{-2}}$	S.D.
30–50	0.02916	0.00284	0.00616	24.2×10^{-5}	3.4%
50–70	1.33534	−0.14914	0.01071	43.1×10^{-5}	0.4%
70–84	44.5376	−2.22090	0.03557	14.3×10^{-5}	0.4%
84–93.2	200.615	−7.40690	0.07802	31.4×10^{-4}	0.9%

In order to characterise better the different volumetric effects occurring in the various temperature ranges defined above, the dilatometric curve was fitted to a second-order polynomial. The results which confirm the occurrence of different steps in the thermal denaturation are reported in Table 1.

The combined use of calorimetric and dilatometric techniques confirms the unfolding behaviour of bovine serum albumin up to 70°C, as reported previously by others. Our measurements up to a temperature of 97°C show a series of phenomena not previously reported. Dilatometric investigations should be applied more frequently to the study of proteins in a solution. We know of no other reports of scanning dilatometry being applied to proteins in solution. The technique is conceptually simple and provides information that other measuring techniques do not give, for example the "quasi-thermal" transitions accompanied only by entropy changes.

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