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Thermochimica Acta 266 (1995) 365–372

thermochimica acta

Calorimetric study of the thermal unfolding of Kunitz-type soybean trypsin inhibitor at pH 7.0 ☆

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Abstract

Excess heat capacity changes during the thermal unfolding of Kunitz-type soybean trypsin inhibitor were measured in an adiabatic differential heat capacity calorimeter at pH 7.0 at various heating rates. It was found that, unlike most globular proteins, the thermal unfolding was kinetically limited even at the heating rate of 0.5 K min^{-1}. The apparent enthalpy change of unfolding observed with various buffers was almost independent of the buffer ionization heat of each buffer component. The number of protons released from the protein during unfolding was 0.18 ± 0.21.

The van't Hoff enthalpy change was estimated on the basis of an ideal two-state transition model from the DSC tracings which were observed at a heating rate slower than 0.25 K min^{-1} and was found to agree with the calorimetric enthalpy within a deviation of 10%. A curve fitting analysis showed that the DSC signals fitted well to a two-state unfolding model. The net enthalpy change of unfolding was determined to be $\Delta H_0 = 429 \pm 6$ kJ mol^{-1} or $200 + 0.3$ J g^{-1} at 59°C. The corresponding heat capacity change was $\Delta C_p^d = 11.0 \pm 0.5$ kJ K^{-1} mol^{-1} or 0.50 ± 0.04 J K^{-1} g^{-1}.

Keywords: Denaturation; DSC; Thermal unfolding; Trypsin inhibitor

Abbreviations

STI, Kunitz-type soybean trypsin inhibitor; DSC, differential scanning calorimetry; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; MOPS, 3-(*N*-morpholino)

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☆ Dedicated to Hiroshi Suga on the Occasion of his 65th Birthday.

SSDI 0040-6031(95)02451-4

propanesulfonate; ACES, *N*-(2-acetamido)-2-aminoethanesulfonate; Tris, tris(hydroxymethyl)aminomethane.

1. Introduction

Kunitz-type soybean trypsin inhibitor (STI) is a well-known proteinaceous protease inhibitor which specifically binds to trypsin to inhibit its catalytic activity [1]. The amino acid sequence of STI [2] and the three-dimensional structure of the STI–trypsin complex[3] are known. The thermodynamics of the specific binding of the inhibitor to trypsin has been studied by both isothermal [4, 5] and scanning [6] calorimetries. However, it seems that the thermal unfolding of STI at neutral pH has not yet been quantitatively studied.

The highly sensitive measurement of the changes in heat capacity of dilute aqueous solutions of biopolymers has provided a quantitative description of protein denaturation, and the thermodynamic stability of various proteins in solution has been examined using this technique. According to the results obtained by Privalov and his co-workers [7, 8], many compact globular proteins undergo thermal transition via a simple two-state process. This conclusion was drawn based on the result showing the equality of the van't Hoff and the calorimetric enthaplies which are both obtained from the analysis of excess heat capacity curves during the transition observed by DSC measurement. However, there are some proteins, such as papain [9], hexokinase [10], chymotrypsin [11], Taka-amylase A [12], pepsinogen [13], glucoamylase [14], and phaseolamine [15] which have a van't Hoff enthalpy smaller than the calorimetric enthalpy. The unfolding of these proteins is considered to involve one or more additional intermediate states during the transition; the process is thereby characterized by a multi-state transition model. Thus it is a general view that a very precise measurement of the excess heat capacity values with a good signal-to-noise ratio provides not only the values of thermodynamic quantities for protein denaturation but may also give information on the mechanistic process of the transition in view of equilibrium thermodynamics, if appropriate analysis can be made.

This paper reports the thermodynamic properties of the thermal unfolding of STI in aqueous solution at pH 7.0 measured by differential scanning calorimetry (DSC).

2. Material and methods

2.1. Materials

Lyophilized trypsin inhibitor from soybeans (STI) prepared chromatographically was purchased from Sigma Chemical Co., and used without further purification. The sample solution used for the study of the buffer effect was prepared by dissolving the lyophilized STI and dialyzing against doubly deionized water at 4 °C for 12 h followed by filtration through a Millipore filter (HAW PO2500, 0.45 μm; Millipore Co., Bedford, MA). The filtrate stored as a stock solution usually had a protein concentration of

about 12 mg ml^{-1} and was used for DSC measurements after appropriate dilution with buffer solutions of pH 7.0. The buffers employed were 17 mM HEPES, 17 mM MOPS, 17 mM ACES, 10 mM Tris, 13 mM triethanolamine, and 10 mM phosphate. No salt was added to adjust the ionic strength. The DSC studies except for the buffer effect were conducted in phosphate buffer; the solution was prepared by dialyzing exhaustively against 20 mM phosphate buffer at pH 7.0 and 4 °C. The pH of each buffer was adjusted to 7.0 at 60 °C, which is very close to the midpoint temperature of unfolding.

The protein concentrations were measured spectrophotometrically on the stock solution using a value of specific absorption of 0.994 cm^2 mg^{-1} [16].

2.2. Differential scanning calorimetry

The DASM-1M and DASM-4 adiabatic differential heat capacity calorimeters developed by Privalov et al. [17–19] were used for the DSC measurement after some modification of the electronic circuit for feedback control (DASM-1M) and for scan rate control (DASM-4). The volumes of the sample cell were 1.008 cm^3 (DASM-1M) and 0.450 cm^3 (DASM-4). The scan rates employed were 1.0, 0.5, 0.25, 0.125, and 0.05 K min^{-1}. In all the measurements, the buffers or the dialyzates were used as the reference solution. The calorimetric enthalpy of unfolding was estimated from each DSC tracing after establishment of appropriate baselines according to the method described previously [20–22]. The STI concentration used for the DSC measurement ranged from 2 to 2.5 mg ml^{-1}.

3. Results and discussion

In Fig. 1, typical recordings of the excess heat capacity during unfolding of STI in 20 mM phosphate buffer of pH 7.0 at various heating rates ranging from 0.05 to 1.0 K min^{-1} are shown. All the curves exhibit a typical endothermic feature with a single peak which is characteristic of the unfolding of many compact globular proteins [7, 18]. A rescan of the heated sample in phosphate buffer after cooling revealed that the unfolding was quantitatively reversible. The same behavior was also observed for the DSC tracings with different buffer components given in the experimental section.

3.1. The effect of heating rate

From Fig. 1 it can be seen that the peak temperature shifts slightly toward a lower temperature range as the heating rate is lowered. The same observation was reported by Varfolomeeva et al. [6]. This behavior becomes more evident when the apparent peak temperature is plotted as a function of the heating rate (inset of Fig. 1). Because the instrumental time constant was very small compared to that of the protein unfolding, no correction for this was made on the observed DSC signals. The result shown in Fig. 1 obviously indicates that the apparent excess heat capacity curve is kinetically limited at a heating rate higher than 0.5 K min^{-1} and that the unfolding process is thermodynamically in an equilibrium state when the STI solution is heated at a heating

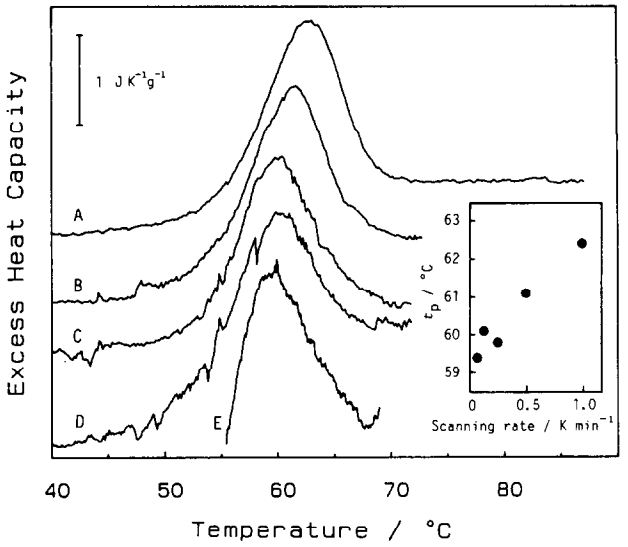

Fig. 1. DSC curves observed during the thermal unfolding of soybean trypsin inhibitor (STI) at pH 7.0 and at various heating rates. The protein concentration was 2.0 mg ml^{-1}. The heating rates employed were: A, 1.0; B, 0.5; C, 0.25; D, 0.125; and E, 0.05 k min^{-1}. The inset shows the plot of the apparent peak temperature, t_p, versus the temperature scanning rate.

rate of 0.25 K min^{-1} or lower. This is a very important finding when one considers the thermodynamic properties of the thermally induced conformational transition in the STI molecule more quantitatively.

The thermal unfolding of most globular proteins studied so far has been discussed on the basis of DSC data taken at a heating rate of 1 K min^{-1} [7, 8, 19, 20]. This fact indicates that the unfolding of most globular proteins is thermodynamically in an equilibrium state at each step in the heating when they were scanned at a heating rate of 1 K min^{-1}. In constrast, the present result shows that the unfolding process of STI does not thermodynamically reflect the equilibrium state even when STI is heated at a scan rate as low as 0.5 K min^{-1}. Thus it is most likely that, unlike most globular proteins, STI has a rather rigid structure and that although the thermodynamically defined unfolding temperature is rather low ($t_m = 59°C$), the structural fluctuation is so small that the molecule does not readily unfold even at t_m.

3.2. *The net enthalpy change of unfolding*

To obtain the net enthalpy change associated with the protein unfolding alone, the DSC measurements were conducted with the samples in six different buffers, and the observed enthalpy changes, ΔH, were corrected for the contribution of the enthalpy term due to the possible proton uptake or release on the part of the buffer, according to the relation [23, 24]

$$\Delta H = \Delta H_0 + n\Delta H_i \tag{1}$$

where ΔH_0 is the net enthalpy change of unfolding, ΔH_i the net enthalpy change for deprotonation of each buffer, and n the number of protons released from the buffers and taken up by the protein under study during its unfolding. In Fig. 2, the observed enthalpy changes, ΔH, are plotted against ΔH_i values (A in Fig. 2) which are measured separately [25] or taken from the literature [26]. The buffer ionization heats at 59°C, except for phosphate buffer, were calculated on the basis of the following relations [25]; $\Delta H_i/\text{kJ mol}^{-1} = 4.02 - 0.200\theta + 4.18 \times 10^{-4}\,\theta^2$ (glycerol 2-phosphate), $10.44 - 0.238\theta + 1.01 \times 10^{-3}\,\theta^2$ (phosphate), $19.81 + 0.0480\,\theta$ (HEPES), $20.85 + 0.0389\,\theta$ (MOPS), $32.09 - 0.0270\,\theta$ (ACES), and $32.44 + 0.0467\,\theta$ (triethanolamine), where θ is the temperature (°C). The line was drawn by least-squaring, and the value of the net enthalpy change was found to be $\Delta H_0 = 429 \pm 6\,\text{kJ mol}^{-1}$ (uncertainty in standard error) or $\Delta H_0 = 20.0 \pm 0.3\,\text{J g}^{-1}$ at $t_m = 59$°C and at pH 7.0. The corresponding heat capacity change upon unfolding was $\Delta C_p^d = 11.0 \pm 0.5\,\text{kJ K}^{-1}\,\text{mol}^{-1}$ or $\Delta C_p^d = 0.50 \pm 0.04\,\text{J K}^{-1}\,\text{g}^{-1}$. These values seem to agree with those reported for many other globular proteins [7, 8, 20, 27]. From the slope of the plot, the number of protons taken up by the protein was determined as $n = -0.18 \pm 0.21$ (a total of 0.18 mol of H^+ was

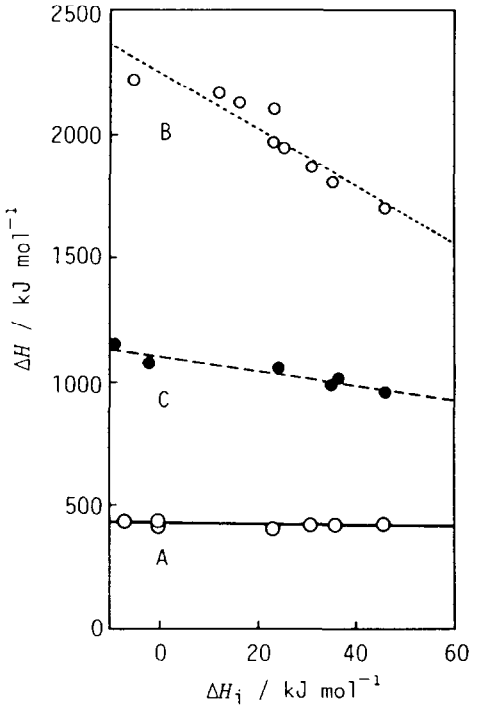

Fig. 2. The observed enthalpy change, ΔH, of the thermal unfolding of soybean trypsin inhibitor as a function of the buffer ionization heat, ΔH_i, at pH 7.0 (——). The buffers used were glycerol 2-phosphate, phosphate, HEPES, MOPS, ACES, triethanolamine, Tris. The data obtained for Taka-amylase A (···) [12] and phaseolamine (– – –) [15] are included in the figure for comparison.

released from the protein). For comparison, the plots obtained for the thermal unfoldings of Taka-amylase A(B) [12] and of phaseolamine, pancreatic α-amylase inhibitor from kidney bean (*Phaseolus vulgaris*) (C) [15] are shown in the same figure. In the former, the slope gave a value of of $n = -13$ and the latter $n = -3.5$. The dependence of ΔH on buffer composition is thus much less with the STI protein and the number of protons released from the protein during unfolding is very small compared to that found for the other proteins. The fact that the value of n is approximately zero means that STI is most stable at around pH 7.

3.3. *Curve fitting analysis*

The average ratio of the van't Hoff to calorimetric enthalpies [7] determined for the DSC tracings observed at a heating rate of 0.25 K min^{-1} was found to be 1.10 ± 0.04. This means that the STI unfolding is approximately regarded as a two-state transition. The fact that the ratio actually exceeds unity seems to indicate that there might be intermolecular interactions involved in the process to a certain extent.

In order to characterize the mechanistic process of the unfolding more quantitatively, a curve-fitting technique [20, 22] was employed for the analysis of DSC tracings observed at a heating rate of 0.25 K min^{-1} and slower. An example of the results for the DSC curve observed at the heating rate of 0.25 K min^{-1} is shown in Fig. 3. In the figure, the experimentally observed excess heat capacity values are shown by open circles, and the calculated curve fitted to the experimental values, obtained on the basis of a simple two-state process, is shown by a solid line. From the figure, the fitting is obviously very good, indicating that the thermal unfolding of STI in solution at pH 7.0 is well characterized by a simple two-state process. Thus the protein, as far as the molecular cooperativity is concerned, is reasonably considered to belong to the same family of

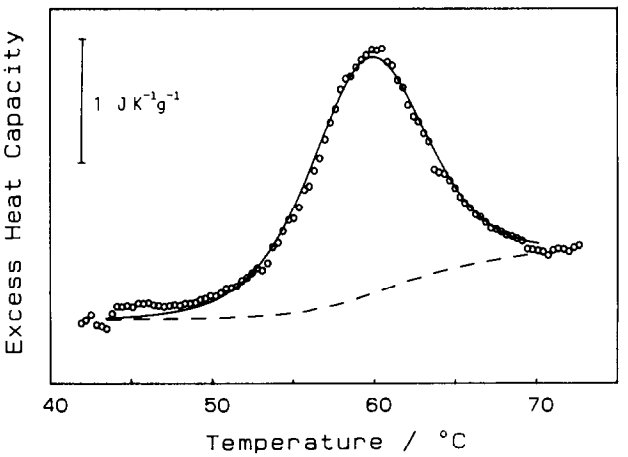

Fig. 3. Curve fitting based on the excess heat capacity values observed for the thermal unfolding of soybean trypsin inhibitor in phosphate buffer at pH 7.0: ○, experimentally observed excess heat capacity values; —, calculated curve; and – – –, calculated baseline.

compact globular proteins as lysozyme, cytochrome *c*, and ribonuclease, which were summarized by Privalov [7].

We reported in our earlier paper on the DSC studies of some globular proteins [28] that, in the thermally induced transition of STI where the DSC signals give assymmetric peaks, there may be a possibility that another intermediate state is involved between the initial native (N) and the final unfolded (D) states and that the process might be characterized by a three-state nature rather than a two-state. However, this consideration which we introduced previously on the mechanistic process of STI unfolding seems to be incorrect, because it was based on the excess heat capacity curves observed at a heating rate of 1.0 K min^{-1} where the signals are found to be kinetically limited and do not reflect the heat capacity of the protein solution at each step of the heating. The present result seems to imply that an erroneous conclusion might be drawn if one studies the thermal denaturation of proteins by DSC at only one heating rate and that researchers should carefully check the dependence of DSC curves on the temperature scanning rate before they quantitatively discuss the thermodynamic properties of protein unfolding.

Acknowledgements

Part of the present study was conducted at the Yale University Chemistry Department, New Haven, CT, USA, with the kind permission of Prof. J.M. Sturtevant to use the DASM-1M calorimeter. The cost of the experiments was partly supported by Travel-Cost-Aid from the Ministry of Education, Science and Culture, Japan.

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