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The effects of scan rate and protein concentration on DSC thermograms of bovine superoxide dismutase

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Abstract

DSC thermograms of bovine Cu–Zn superoxide dismutase (BSOD) have been recorded at different scan rates and protein concentrations in order to clarify the process of its unfolding. The lack of calorimetric reversibility made direct thermodynamic analysis of the thermograms impossible. The study of the effect of the scan rate on the shape of the heat capacity (C_p) profiles of BSOD has allowed the calculation of the apparent activation energy (E_{app}) for the whole irreversible process. Extrapolation of the excess heat capacity curves (C_{pexc}) to infinite scan rate has provided the scan-rate-independent part of the thermograms. The protein concentration effect is explained by a change in molecularity that takes place before the kinetically-controlled step. The data collected suggest that, on thermal denaturation, BSOD dissociates during the thermally-induced transitions of the protein; this is followed by a kinetically controlled, exothermic step which is responsible for the global irreversibility of the entire process.

Keywords: Bovine superoxide dimutase; DSC; Heat capacity; Protein; Scan rate

1. Introduction

Cu–Zn superoxide dismutases are a family of dimeric enzymes, composed of two equivalent subunits of molar mass $\cong 16000$ Daltons for each subunit, which catalyse the dismutation of the superoxide anion, O_2^-, into oxygen and hydrogen peroxide [1]. The three-dimensional structure of the bovine erythrocyte enzyme has been solved to 2 Å resolution using X-ray diffraction data [2], and shows a rigid β-barrel core

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supporting external loops in the active site, the core consisting of a cluster of adjacent Cu^{++} and Zn^{++} ions. The action mechanism, which has also been studied extensively for this enzyme, has been shown [3] to involve two half-cycles of reduction and oxidation of the catalytically active copper ions by two molecules of O_2^- to give O_2 and H_2O_2 respectively.

Over the last few years, the thermodynamic stability of the tertiary structure of this enzyme has been the focus of intense research. In particular, many previous works [4–9] have shown a structure which is highly resistant to thermal unfolding: temperatures exceeding 70°C are necessary for irreversible denaturation. This thermal resistance has been ascribed to a number of factors, including stabilization by Cu^{++} and Zn^{++} binding [4], disulphide bridges, intramolecular hydrogen bonds, and the close packing of the hydrophobic interfaces.

The whole of these effects can be detected by micro differential scanning calorimetry (DSC) measurements, when samples of enzyme are temperature-scanned in a suitable environment.

Literature data show a complex, irreversible, calorimetric profile with at least two components. The true nature of these two components has been the object of some controversy. Two suggestions have been put forward: a two-step denaturation path [7,8], and the existence of two distinct species of differing thermostability in equilibrium with each other during the thermal denaturation of the enzyme [9].

Unfortunately, application of the classical method of mechanical–statistical deconvolution is impossible in our case for at least two reasons: (a) the lack of calorimetric reversibility, and (b) the classical mechanical–statistical deconvolution algorithms do not take into account the eventual change in molecularity during the process, which is a realistic hypothesis in this case because BSOD is a dimer [10,11].

The remarkable asymmetry of the calorimetric profiles at the end of the transition is a further complication in the analysis of the curves. It is ascribable both to exothermic phenomena and to the occurrence of kinetic factors related to the whole denaturation process [12,13].

The effect of scan rate on DSC profiles provides a very useful tool with which to investigate the kinetically controlled steps of the thermally induced transitions. In particular, from a series of DSC curves obtained at different scan rates, and if we assume that the kinetically controlled step occurs with first-order kinetics, it is possible to calculate the apparent activation energy (E_{app}) of the entire process [10,13].

Moreover, application of an extrapolation procedure of the calorimetric scans to infinite scan rate has allowed us, as described in the following section, to isolate the part of the thermograms pertaining only to the populations (and their relative energies with respect to a reference state, generally the native state) that are in equilibrium with each other [11].

On the other hand, protein concentration effects on DSC curves can be due to a change in molecularity during the transition [10]. Since BSOD is a dimeric enzyme, an investigation of the protein concentration effect should elucidate a crucial point in the study of the thermal unfolding of BSOD: the dissociation of the protein during the unfolding.

As we will explain, our experimental data confirm the dissociation of the two subunits before the kinetically controlled step.

2. Experimental

2.1. Chemicals

The lyophylized powder of Zn–Cu superoxide dismutases from bovine erythrocytes (BSOD), containing 98% protein, were obtained from Sigma Chemical Co. (St. Louis, USA) and used without further purification. Potassium phosphate (analytical grade) was obtained from Fluka Chemie AG (Buchs, Switzerland).

2.2. Experimental procedures

DSC scans were carried out using a Setaram (Lyon, France) micro differential scanning calorimeter (microDSC) with 1-ml stainless steel sample cells, interfaced with a BULL 200 Micral computer. The sampling rate was 1 point per second in all the measuring ranges. The enzyme was dissolved in 100 mM phosphate buffer at pH 7.03. The ionic strength was adjusted to 0.1 M by sodium chloride. The same solution but without the protein was used in the reference cell. Both the sample and reference were scanned from 30 to 100°C at four different scan rates (0.3, 0.5, 0.7 and 1°C min^{-1}) with a precision of 0.08°C. The calorimetric experiments were carried out under an extra nitrogen pressure of 1.5 bar.

Buffer–buffer baselines were obtained at the same scanning rate and then subtracted from the sample curves (see Fig. 1). The excess apparent molar heat capacity (C_{exc}), is defined as the amount by which the apparent molar heat capacity exceeds the baseline heat capacity during a transition involving the solute. In order to obtain the C_{pexc} related to the denaturation process, a straight line from the start to the end of the peak, as suggested by Privalov and Potekhin [14], was used.

In the experimental conditions described, the average instrumental level of noise was about $\pm 0.4\,\mu W$ and the reproducibility at refilling was about 0.1 mJ K^{-1} ml^{-1}.

Calibration in energy was obtained by giving a definite power supply, electrically generated by an EJ2 Setram Joule calibrator, within the sample cell.

3. Results and discussion

In Fig. 1, curve a shows a typical apparent heat capacity (C_p) profile for the thermal denaturation of native BSOD in buffer solution in the 60–100°C temperature range. Curve b represents the instrumental baseline obtained as reported in the experimental section. The shape of curve a and its remarkable asymmetry suggests a complex denaturation path. Moreover, in the concentration range of all studied samples (2–13 mg ml^{-1}) and under the experimental conditions reported in the experimental section, the thermal denaturation of BSOD was completely irreversible, as no endothermic peak was observed by rescanning the sample after it had been cooled from the first run.

-Similar irreversible behaviour has been found for many other globular proteins, and in several cases equilibrium thermodynamics have been applied to the analysis of

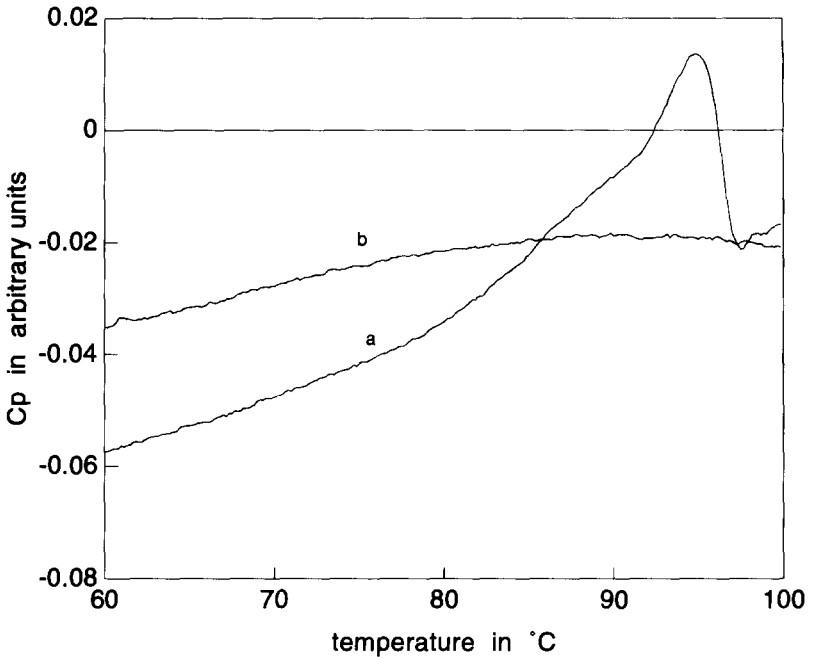

Fig. 1. Calorimetric scan of BSOD carried out at 0.5°C min^{-1}, pH 7.03, ionic strength 0.1 M, and protein concentration 2.5 mg ml^{-1} (curve a). Curve b represents the buffer–buffer baseline obtained as described in the text.

calorimetric curves [15–17]. However, it has been shown recently that justification for this procedure is critical, due to the occurrence of kinetically controlled steps [10–13]. Thus, when an irreversible process occurs, the kind of information that can be obtained from calorimetric experiments depends on each particular situation [11].

Moreover, the dimeric nature of BSOD would suggest the possible dissociation of the two subunits when the protein is temperature-scanned. This being so, the thermodynamic analysis of the calorimetric thermograms would appear to be much more complex, considering that mechanical–statistical deconvolution methods do not take into account the dissociation of the protein.

Sanchez-Ruiz [10] has recently proposed a set of mathematical equations that enable the change in the molecularity occurring during the thermal denaturation process to be calculated by studying the effect of protein concentration on the position of the peaks.

For both reasons mentioned above, we carried out two sets of measurements of the thermal denaturation of BSOD as a function of the scan rate and the enzyme concentration before evaluating the experimental data.

3.1. The effect of the scan rate

The thermal denaturation of BSOD (at a protein concentration of 2.5 mg ml^{-1}) was carried out in the 30–100°C temperature range, at four different scanning rates in the

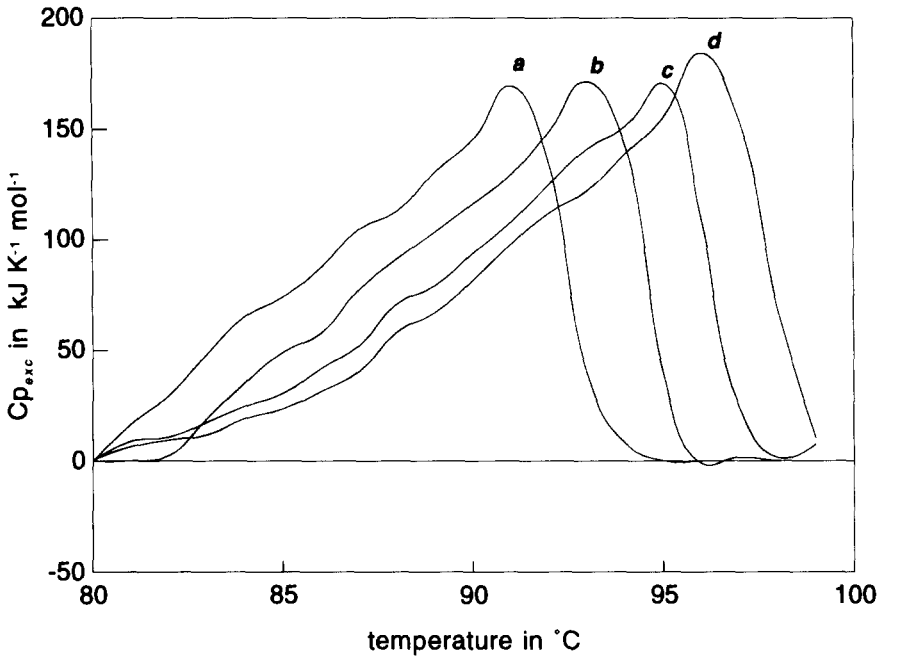

Fig. 2. C_{pexc} profiles relative to the thermal denaturation of BSOD obtained at four different scan rates: 0.3°C min^{-1} (curve a), 0.5°C min^{-1} (curve b), 0.7°C min^{-1} (curve c) and 1°C min^{-1} (curve d). Protein concentration = 2.5 mg ml^{-1}. All the experimental curves were smoothed using a computer program in order to decrease the noise level.

range 0.3–1°C min^{-1} (Fig. 2). The corresponding calorimetric enthalpies ΔH were calculated from the equation

$$\Delta H = \int_{T_i}^{T_f} C_{Pexc} dT \quad (1)$$

and T_m from the maximum value of C_{pexc} in all the calorimetric profiles.

In Eq. (1), C_{pexc} represents the excess molar heat capacity function obtained as described in the experimental section, T_i is the temperature at which all molecules are in the native state, and T_f is the temperature at which all molecules are in the final state.

These values are collected in Table 1 and show that the whole denaturation process (in particular the temperature of the maximum heat capacity, T_m) is scan-rate-dependent. These results indicate that denaturation, taken as a whole, occurs as a kinetically controlled process [10, 11, 13].

Recently, Freire et al. [11] developed equations to account for the effect of irreversibility on calorimetric data, based on a complex model which consists of a series of sequential equilibrium transitions connecting the native state to a number of intermediate species followed by a final irreversible step.

Table 1
Scan rate effect on T_m and the calorimetric enthalpy ΔH at constant protein concentration for the folding-unfolding transition of BSOD; the pH and ionic strength were 7.03 and 0.1 M in NaCl, respectively; protein concentration was 2.5 mg ml^{-1}

β/°C min^{-1}	T_m/°C [a]	ΔH/kJ mol^{-1} [b]
0.3	91.4	1128 ± 60
0.5	93.1	1181 ± 52
0.7	94.8	1238 ± 42
1.0	96.3	1459 ± 38
∞ [c]	95.1	1490 ± 70

[a] The estimated uncertainty of these values is ±0.1°C.
[b] Values expressed as mean ± standard deviation.
[c] Values obtained for extrapolation as described in the text.

In particular, it has been shown that an increase in scan rate causes a decrease in the relative populations of the final, irreversible state.

Unfortunately, an a priori application of their equations to the case of BSOD is not possible, because the dimeric nature of BSOD would suggest that this protein dissociates during denaturation (see next subsection).

However, if we assume that there is equilibrium between the intermediate states, the extrapolation of experimental data to infinite scanning rate should enable us to separate the part pertaining to the species in thermodynamic equilibrium from the kinetically controlled part of the process [10, 18].

In the paper by Freire et al. [11], $\langle \Delta H \rangle$ is defined as an average excess enthalpy calculated over all the states and $\langle \Delta H \rangle_e$ is defined as an average excess enthalpy calculated over the states in thermodynamic equilibrium only. It is the excess enthalpy that one would obtain from DSC data if the irreversible process did not exist, and this contains the thermodynamic information associated with the transition. In the above-mentioned paper, it was shown that there is a mathematical relationship between $\langle \Delta H \rangle$, $\langle \Delta H \rangle_e$, ΔH, and T:

$$\Delta H - \langle \Delta H \rangle = (\Delta H - \langle \Delta H \rangle_e) \times e^{-F(T)/\beta} \tag{2}$$

where $$F(T) = \int_{T_0}^{T} k_{app} dT,$$

k_{app} is the apparent kinetic constant of the whole denaturation process and β is the scan rate.

According to Eq. (2), for any given temperature, the experimentally observed $\langle \Delta H \rangle$ can be considered an exponential function of $1/\beta$ [18]. Hence, the equilibrium value $\langle \Delta H \rangle_e$ can be obtained by extrapolating the exponential to $1/\beta = 0$. In general, this extrapolation procedure, obviously, runs well if the equilibrium contribution to the overall process is not too small; otherwise the intercept values of the exponential will be very close to 0.

In Fig. 3, we report the $\langle \Delta H \rangle$ functions obtained at different scan rates. The dotted curve represents the $\langle \Delta H \rangle_e$ function obtained by connecting all the points in an $\langle \Delta H \rangle$ vs. T plot obtained from the exponential plots of Fig. 4, in which for the sake of clarity we have reported only the fits for $T = 91°C$ (curve a) and $T = 92°C$ (curve b).

Finally, in Fig. 5 we report the $\langle \Delta H \rangle_e$ function (dotted line) and its first derivative with respect to temperature $C_{pexc,e}$ (solid line).

In general, if a C_{pexc} curve reflects the energetic variations associated with differently populated states in thermodynamic equilibrium with each other, the number of intermediate states and the thermodynamic variables associated with the transitions can be calculated by using an appropriate mechanical–statistical deconvolution algorithm [19]. In our case, as we have pointed out, the dimeric nature of BSOD and the consequent possibility of a change in molecularity during the transition, make this kind of analysis unreliable. So, a complete and detailed elucidation of the mechanism of unfolding of BSOD, would need a theoretical equation simulating the C_{pexc} function taking into account the eventual dissociation of the enzyme and the exothermic effects associated with the irreversible step of the process. Unfortunately, this theoretical study is not available at present.

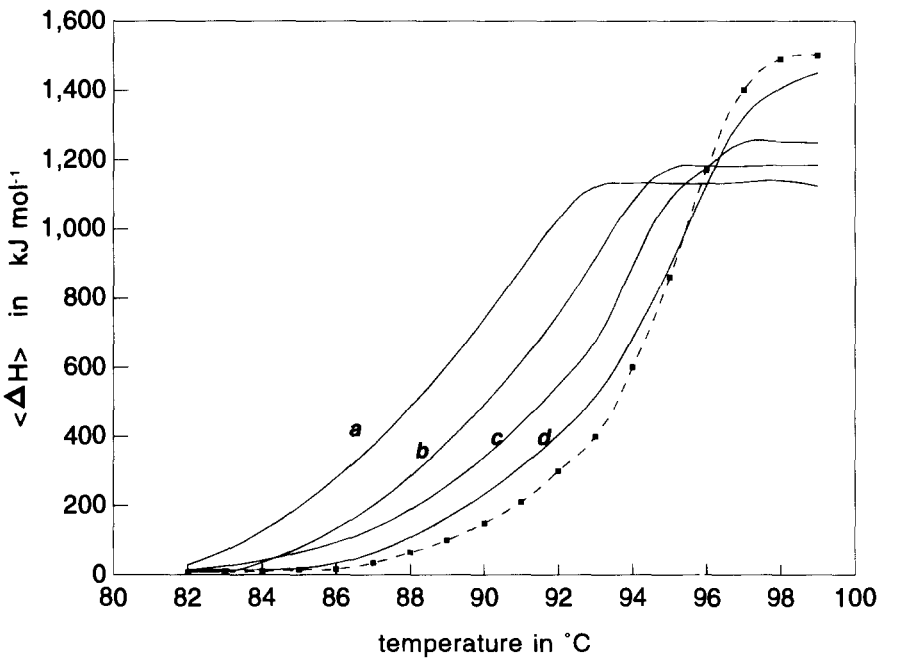

Fig. 3. Experimental average excess enthalpy $\langle \Delta H \rangle$ obtained at different scan rates: $0.3°C\,min^{-1}$ (curve a), $0.5°C\,min^{-1}$ (curve b), $0.7°C\,min^{-1}$ (curve c) and $1°C\,min^{-1}$ (curve d). The dotted curve represents the average thermodynamic excess enthalpy $\langle \Delta H \rangle_e$.

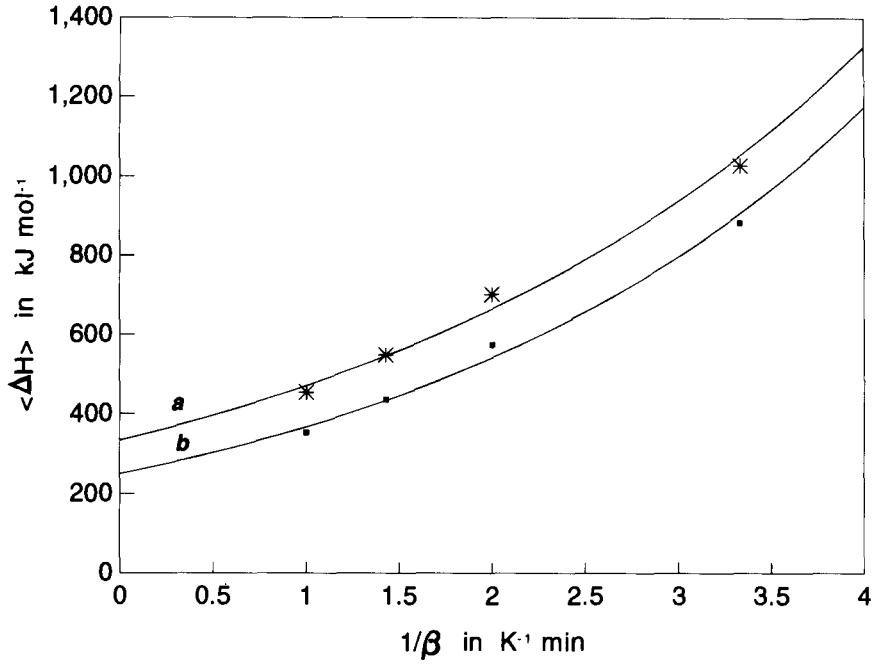

Fig. 4. Exponential plots of $\langle \Delta H \rangle$ as a function of $1/\beta$. The intercept values with the y-axis represent the $\langle \Delta H \rangle_e$ values for the chosen temperatures. For the sake of clarity, the figure reports only the fits for $T = 92°C$ (curve a) and $T = 91°C$ (curve b) (correlation coefficients ranging from 0.9891 to 0.9912 for all fits).

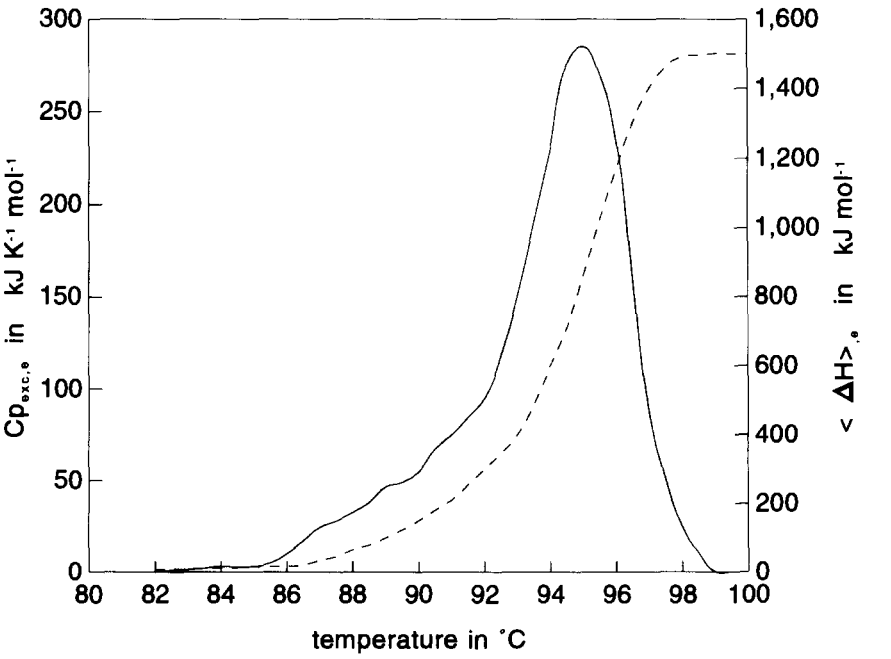

Fig. 5. Thermodynamic average excess enthalpy $\langle \Delta H \rangle_e$ relative to the thermal unfolding of BSOD (broken line) and its first derivative, $C_{pexc,e}$ (solid line), calculated over the entire denaturation range.

Moreover, the effect of the scan rate on the C_{pexc} curves has allowed us to calculate the apparent activation energy E_{app} of the irreversible step, using the equation [13]:

$$\ln\left(\frac{\beta}{T_m^2}\right) = C - \frac{E_{app}}{RT_m} \tag{3}$$

A value of 240 kJ mol^{-1} was calculated from the slope of the linear plot of $\ln(\beta/T_m^2)$ vs. $1/T_m$ shown in Fig. 6.

3.2. The effect of the protein concentration

Denaturation scans of BSOD samples have been carried out with enzyme concentrations ranging from 2.6 to 13 mg ml^{-1} using a scanning rate of 0.5°C min^{-1} in all cases and have been reported in Fig. 7. The T_m increase with protein concentration (see Table 2) indicates that some dissociation of the dimeric enzyme takes place during the denaturation process [10,18]. In order to calculate the change in molecularity μ accompanying the denaturation process, we used the following equation derived by Sanchez-Ruiz [10]

$$\frac{E_{app}}{RT_m} - 2\ln T_m + \frac{\mu - 1}{\mu}\ln C_t = \text{const.} \tag{4}$$

where C_t is the protein concentration in mg ml^{-1}, and the other symbols have their usual significance.

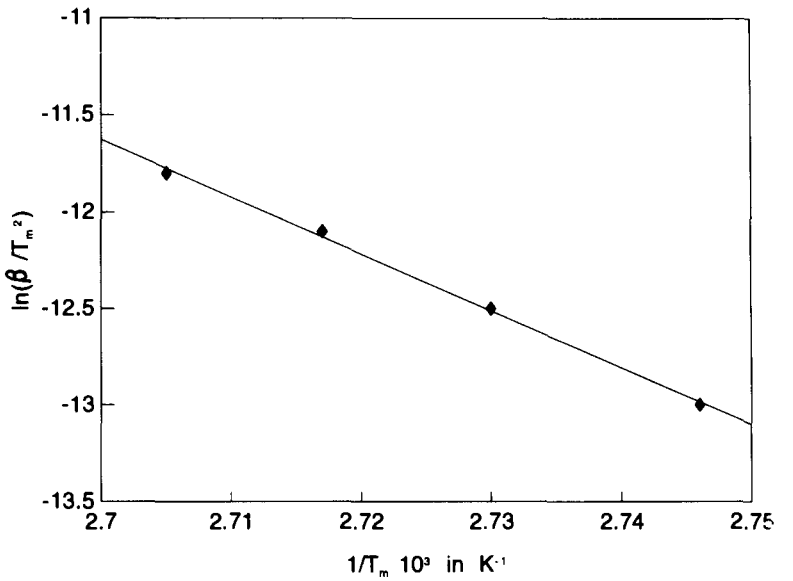

Fig. 6. Plot of $\ln(\beta/T_m^2)$ vs. $1/T_m$ obtained from different scanning rate runs at constant concentration of BSOD (2.5 mg ml^{-1}). The correlation coefficient was −0.9988.

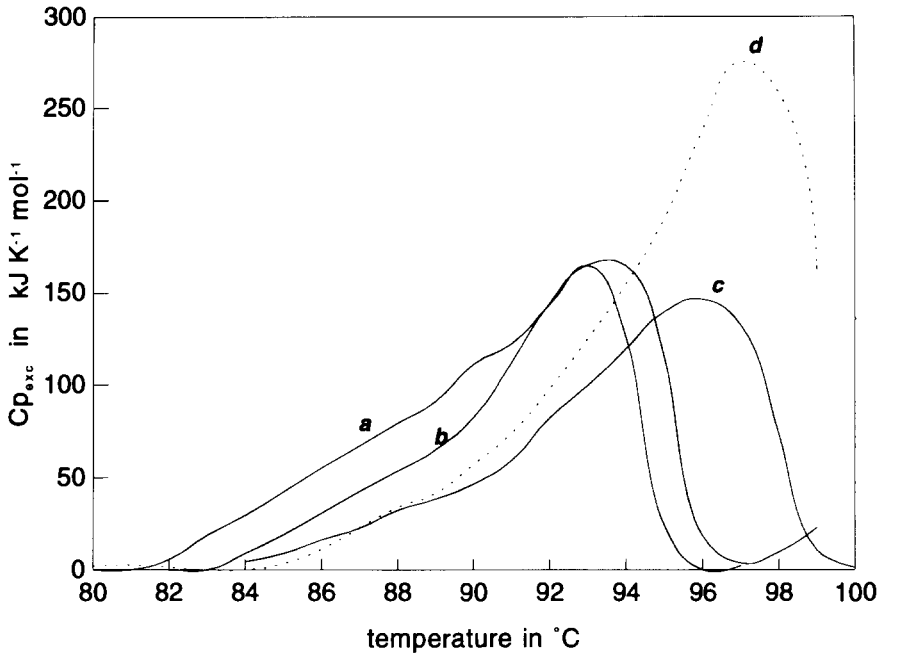

Fig. 7. Effect of protein concentration (C_t) on the C_{pexc} profile of BSOD: $C_t = 2.60\,mg\,ml^{-1}$ (curve a), $C_t = 5.08\,mg\,ml^{-1}$ (curve b), $C_t = 8.00\,mg\,ml^{-1}$ (curve c), $C_t = 13.00\,mg\,ml^{-1}$ (curve d). In the case of protein concentration $C_t = 13\,mg\,ml^{-1}$ (dotted curve), the offset temperature falls beyond 100°C, so it was not possible to calculate the C_{Pexc} function or ΔH. The scan rate was $0.5°C\,min^{-1}$ in all cases.

For E_{app} values ranging between 100 and 400 kJ mol^{-1}, the term $2\ln T_m$ changes much more slowly with T_m than the term E_{app}/RT_m [10]; this equation thus indicates that, in the case of BSOD, a plot of $\ln C_t$ vs. $1/T_m$ should be linear with a slope equal to

$$-\frac{\mu E_{app}}{(\mu - 1)R}$$

In our case, the linear relationship obtained (correlation coefficient, −0.9306), as shown in Fig. 8, has allowed the calculation of μ. The value of μ calculated with this procedure for BSOD was 1.8.

This probably means that BSOD does not totally dissociate during the denaturation process, or that the unfolding partially overlaps the dissociation during heating.

The excess enthalpy related to the whole process also depends on the protein concentration as shown in Table 2. In particular, the value of ΔH decreases with increasing protein concentration . This behaviour suggests the occurrence of exothermic effects, depending on protein concentration, ascribable to the irreversible aggregation of the unfolded polypeptide chains [12]. This effect could partially explain the remarkable asymmetry on the high temperature side of the denaturation peak.

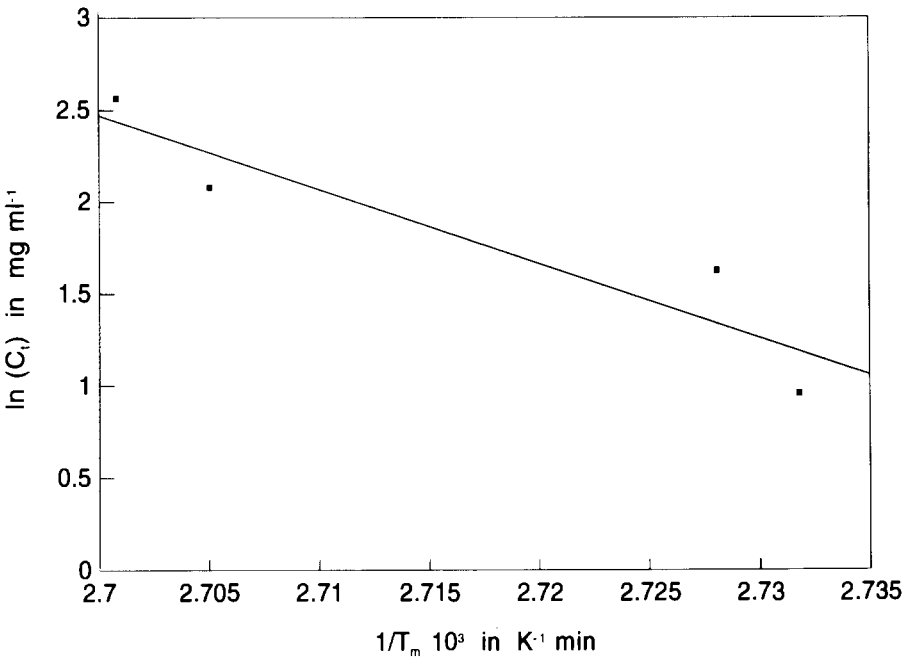

Fig. 8. Linear plot of $\ln C_t$ vs. $1/T_m$ obtained from the data reported in Table 2. The protein concentration ranged from 2 to 13 mg ml^{-1}, and the correlation coefficient of the linear fit was -0.9306.

Table 2
Protein concentration (C_t) effect on T_m and ΔH for the folding-unfolding transition of BSOD; the pH and ionic strength were 7.03 and 0.1 M in NaCl, respectively; the scanning rate was 0.5 °C min^{-1}.

C_t/mg ml^{-1}	T_m/°C[a]	ΔH/kJ mol^{-1}[b]
2.60	92.9	1148 ± 66
5.08	93.4	1107 ± 52
8.00	96.5	1047 ± 42
13.00	97.1	–

[a] The estimated uncertainty of these values is ± 0.1 °C.
[b] Values expressed as mean ± standard deviation.

4. Conclusions

In this work we have addressed the problem of elucidating the mechanism of the thermal unfolding of BSOD. Considering the lack of calorimetric reversibility in all the adopted experimental conditions, a detailed analysis of the effect of scan rate on the shape and on the position of the calorimetric curves has been carried out.

This analysis has a two-fold purpose: it allows the calculation of the apparent activation energy related to the whole process of denaturation, and it makes possible the extrapolation of the calorimetric curves to infinite scan rate.

The extrapolated, scan-rate-independent $C_{pexc,e}$ curve, contains all the thermodynamic information relative to the species that are in thermodynamic equilibrium only. This curve is asymmetrical and has at least two components, confirming previous literature data, but the separation of this curve into its components appears extremely difficult for the reasons explained in the preceding sections. The values of ΔH calculated for the curves obtained at different scan rates demonstrate that when the scan rate increases, the value of ΔH decreases, i.e. there is an exothermic, scan-rate-dependent effect convoluted in the C_{pexc} profile of BSOD.

However, the effect of the protein concentration has a considerable influence on the ΔH values: when the protein concentration increases, the values of ΔH decrease again. This means that this exothermic effect depends on intermolecular forces and can be attributed to the aggregation of the unfolded polypeptide chain.

Moreover, the analysis of the effect of protein concentration on the position of the maximum heat capacity allows us to affirm that a dissociation of the two subunits occurs before the irreversible step occurs.

Acknowledgements

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