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Differential scanning calorimetry of the irreversible denaturation of bovine superoxide dismutase

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Abstract

The thermal denaturation of bovine superoxide dismutase has been studied by means of differential scanning calorimetry (DSC). Analysis of the calorimetric profile by means of Freire and Biltonen's algorithm to determine the accessible states in the transition path did not succeed. At least three factors influenced this analysis negatively: (a) under the experimental conditions used, the denaturation of the enzyme was completely irreversible, as judged by the absence of any endotherm on re-scanning; (b) the denaturation process was followed by a change in molecularity, as the dimeric nature of the enzyme suggested; and (c) there was distortion at the high temperature side of the DSC profile, caused by the existence of an exothermic aggregation process.

Moreover, the difference between the van't Hoff and the calorimetric enthalpy on one hand, and the dependence of the specific heat C_p on scan rate on the other, allowed us to exclude a simple two-state reversible or irreversible transition for the denaturation of the enzyme. In the light of these results, we attempted to fit the experimental C_{pexc} curve using the SIMPLEX minimization algorithm by taking in account only three processes: two endothermic and one exothermic. The deconvolution of the calorimetric profile agreed very well with the experimental data and allowed us to suggest a tentative mechanism for the thermal denaturation of this dimeric enzyme.

Keywords: BSOD; Denaturation; DSC; Enzyme

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1. Introduction

Native bovine copper–zinc superoxide dismutase (Cu_2Zn_2BSOD) is an enzyme characterized by two identical sub-units, each of which contains one Cu^{2+} and one Zn^{2+} ion and has an approximate molecular mass of 16 000 Da. The sub-units are strongly associated with non-covalent interactions between residues that occur in predominantly hydrophobic regions of each sub-unit [1–4]. X-ray investigation [1] shows a globular protein with each sub-unit comprising eight anti-parallel β-strands, one disulfide linkage, and a metal-binding region in which the copper and zinc ions are bridged by a histidyl imidazolate which holds them 6.3 Å apart. Superoxide dismutases (SODs) are inherently stable enzymes, found mainly in eukaryotes, which catalyse the dismutation of two superoxide radicals to molecular oxygen and hydrogen peroxide, thus helping to protect cells against toxic by-products of aerobic metabolism.

BSOD is highly resistant to thermal unfolding (denaturation) [5,6]. Temperatures exceeding 70°C are needed for irreversible unfolding. Thermal resistance probably arises from a number of factors, including stabilization by Cu^{2+} and Zn^{2+} binding [7], the disulfide bridge, intramolecular hydrogen bonds, and the close packing of the hydrophobic interfaces, both between the sub-units and between the two halves of the flattened β-barrel [8].

These effects can be detected by micro differential scanning calorimetry (micro-DSC) measurements, in which samples of enzyme, in a suitable environment, are scanned over a range of temperatures. In the last few years X-ray scattering [9], NMR spectrometry [10–12], micro-DSC [6,13,14], circular dichroism (CD) and dynamic fluorescence [15–17] techniques have been used to determine the thermal stability and the role of ions in the stability of SODs and to investigate, through the study of mutated SODs, the contribution of certain amino acid residues in stabilizing the structure [13].

In order to evaluate the calorimetric profile, the choice of a hypothetical starting model is often critical. The mechanical statistical methods proposed by Freire and Biltonen [18] and modified by Filimonov et al. [19], when applied to the calorimetric profile of SOD, do not fit the curve particularly well, especially in the high temperature region. In the present paper we attempt to fit the experimental C_{pexc} curve using the SIMPLEX minimization algorithm taking into account only two endothermic processes and one exothermic process. The latter is due either to the irreversible process of aggregation or to the "intrinsic" asymmetry of the curve related to the occurrence of kinetic factors.

2. Experimental

Wild-type bovine Zn–Cu superoxide dismutase (BSOD) was obtained from Sigma Chemical Co. (St. Louis, MO, USA); potassium phosphate of analytical grade was obtained from Fluka Chemie AG (Buchs, Switzerland).

DSC analysis was carried out using a Setaram (Lyon, France) micro differential scanning calorimeter (micro-DSC) with stainless steel 0.7 ml sample cells, interfaced

to a Bull 200 Micral computer. The sampling rate was one data point s^{-1} over the measuring range. A solution of 1–2 mg ml^{-1} of enzyme was prepared in 100 mM phosphate buffer at pH 7.03. The ionic strength was adjusted to 0.1 M using sodium chloride. The same solution without protein was used in the reference cell. Both sample and reference were scanned from 30 to 100°C with a precision of ± 0.08°C at a heating rate of 0.5 K min^{-1}. The excess apparent specific heat C_{pexc} is the amount by which the apparent specific heat exceeds the baseline specific heat during a transition involving the solute. One problem in using the DSC technique was establishing an appropriate baseline, since its direct observation was not possible during the measurements. The baseline was obtained as follows: reference and sample cells were filled with the same volume of buffer solution used in the denaturation experiment and scanned at the same rate (see Fig. 1, upper panel).

The average noise was approximately ± 0.4 μW and the reproducibility in repeated runs was ≈ 0.1 mJ K^{-1} cm^{-3}. This baseline was subtracted from the experimental denaturation curve to obtain the $C_{ptot}(T)$. In order to obtain the C_{pexc} related to the denaturation process, a straight line from the start to the end of the peak of the $C_{ptot}(T)$, as given by Privalov and Potekhin [20,21], was used (see Fig. 1, lower panel). Calibration in energy was obtained by the supply of a definite power, generated by the Joule effect (EJ2 Joule calibrator from Setaram) in one of the cells.

3. Results and discussion

Fig. 2 shows the C_{pexc} as a function of temperature in the range 70–100°C corresponding to the denaturation of BSOD under the experimental conditions adopted above. As can be seen, the C_{pexc} profile is complex and is asymmetric on the high temperature side of the thermograms.

In principle, several factors may distort the DSC profiles of proteins and make the determination of the equilibrium thermodynamic parameters unreliable. In most studies concerning the DSC analysis of the thermal irreversible unfolding of proteins, the rapid decrease in C_p values at the end of the transition has been attributed to kinetic factors only [22], accompanied by negligible thermal effects. On the other hand it is known that, on heating protein solutions, the solubility of the protein decreases drastically over a narrow temperature range, resulting in intensive aggregation [21]. Although the decrease in solubility is one of the most characteristic features of protein denaturation, it is the least well studied quantitatively. Moreover, this decrease in solubility is the major complication in studying the denaturation of proteins, since aggregation produces great problems with the physical methods used.

In the present study, a second scanning run on a previously scanned sample showed no endotherm, and the entire process was clearly irreversible. Moreover, it is clear from the literature [6] and our unpublished observations that there exists a dependence of the C_{pexc} profile on scanning rate, which is again evidence for the irreversible nature of the process. In this case the dependence results from kinetic factors. On the other hand, the ratio of $\Delta H_d/\Delta H^{VH} = 17.9$ (calculated as suggested

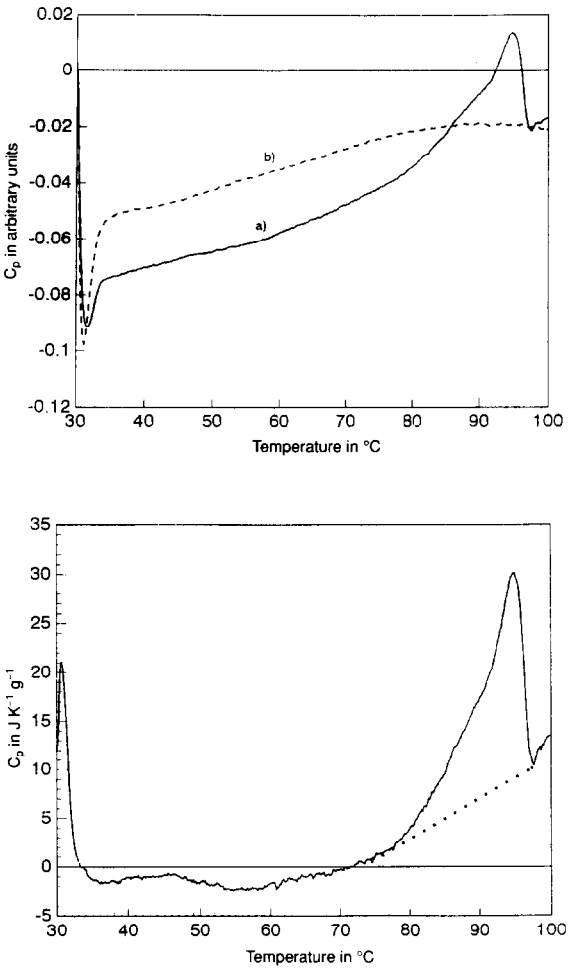

Fig. 1. Upper panel, DSC scans of (a) BSOD; pH = 7.03, ionic strength 0.1 M in NaCl, scan rate 0.5 K min^{-1}; (b) an instrumental zero line obtained under the same conditions as in (a). Lower panel, DSC scan expressed in C_p units (J K^{-1} g^{-1}). The straight line under the transition is the baseline that allows C_{pexc} to be obtained.

in Ref. 21) is further evidence of both the irreversibility and the complexity of the thermal denaturation process in BSOD.

Despite the irreversibility of the process, previous papers [13,14] have involved a thermodynamic analysis of the calorimetric profiles based on Freire and Biltonen's and on Filimonov's algorithms in order to obtain the populations of the accessible states of the system. The fits between the experimental and the calculated C_p curves were not good and the discrepancies became more and more marked at the high temperature side of the thermograms. Fig. 3 shows the deconvolution of the C_p profiles obtained via the two algorithms using our experimental data.

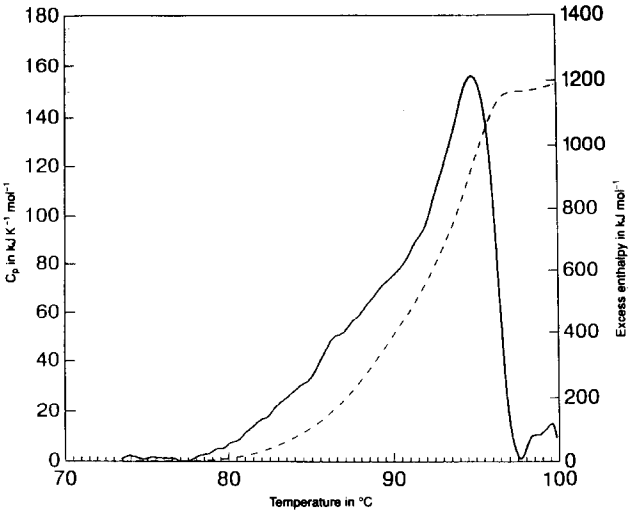

Fig. 2. Excess specific heat C_{pexc} (solid line) and average excess enthalpy function $\langle \Delta H \rangle$ (broken line).

The results obtained from the Filimonov algorithm (lower panel) show better agreement between calculated and experimental curves than those obtained by Freire and Biltonen analysis, but again the fit is not good at the high temperature side. In order to overcome this problem, some authors have assumed the presence of an endothermic reaction, and a kinetic approach assuming pseudo first-order kinetics and an Arrhenius dependence for all the transitions involved [13] has been adopted.

The data calculated agree well with the experimental curve but assume the denaturation model:

$$N_1 \xrightarrow{k_1} D$$

$$N_2 \xrightarrow{k_2} D$$

in which N_1 and N_2 are two different native states [13,14] and D is the unfolded state and k_1 and k_2 are rate constants. This approach also assumes negligible heat changes associated with the irreversible aggregation. This assumption cannot be upheld because in some cases the enthalpic effect accompanying the aggregation process is anything but negligible. For example, in the case of albumin [23] this exothermic effect ranges from 100 to 600 kJ mol^{-1} depending on concentration. This suggests that, even though albumin and BSOD are quite different systems, the thermal effect accompanying the aggregation phenomena of the BSOD cannot be neglected. (It may be noted here that BSOD and albumin have a similar percentage of hydrophobic amino acid residues — $\approx 35\%$). If we take into account the specific structure of this enzyme and some experimental evidence [17] which suggests a change in the molecularity in at least one step of the overall process, both the above reversible (thermodynamic) and irreversible (kinetic) analysis will fail.

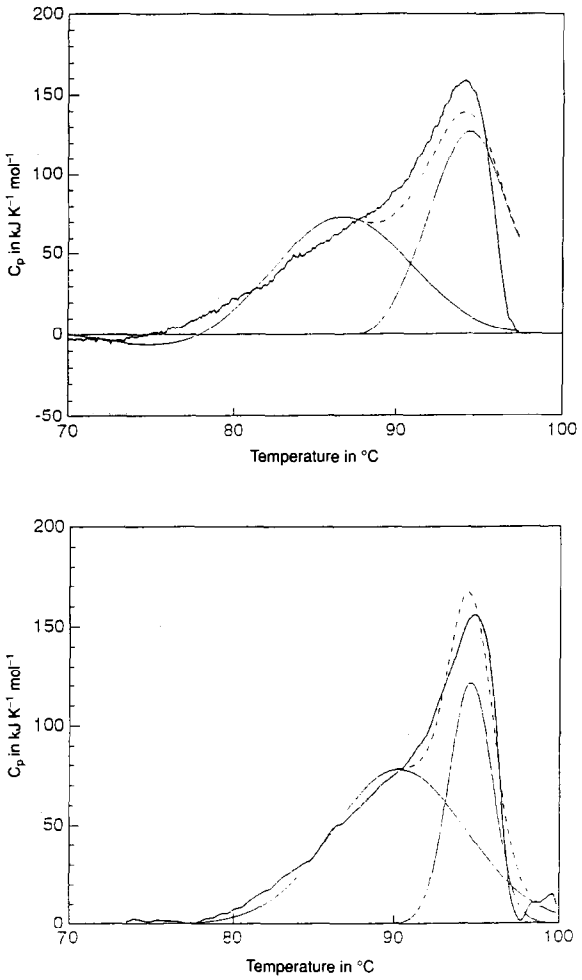

Fig. 3. Thermal deconvolution profile of BSOD. Upper panel, deconvolution by means of Freire and Biltonen algorithm. Lower panel, deconvolution by means of Freire and Biltonen algorithm refined by simplex minimization. Experimental profile (thick solid line) is reported with theoretical profile (broken line) calculated from the sum of components (thin solid line).

In the light of the above discussion, we decided to fit the experimental C_p curve applying the SIMPLEX minimization algorithm [24]. In particular, we have minimized the difference between the calculated and the experimental curve assuming the most probable number of states (three) on the basis of previous studies [13,14] and the shape of our C_{pexc} profile. Following the recommendations of Privalov and Potekhin [21] for the best fit procedure, we increased the number of states, assuming an exothermic process at the end of the transition. The position, the height and the breadth of all three peaks are automatically determined by the minimization procedure. In using the best fit procedure for this analysis of the

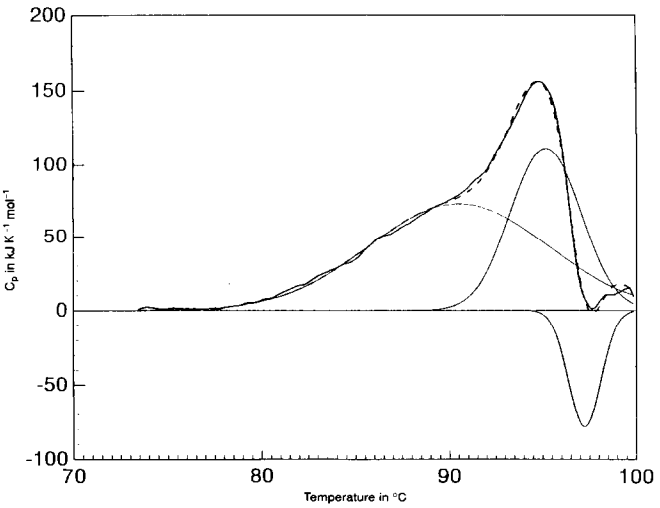

Fig. 4. Deconvolution of C_{pexc} profile of BSOD. The broken line is obtained using the SIMPLEX algorithm and also considering the exothermic aggregation occurring in the high temperature region.

calorimetric data, one should bear in mind that the approximation of the experimental heat capacity curve by the calculated one improves with an increase in the number of the considered states, but this improvement slows significantly after some definite number of states. In the present study, we chose the number of states in such a way that the experimental coincided with the calculated curve within the accuracy of the experiment. We found that only two endotherms and one exotherm satisfied this condition, as reported in Fig. 4.

The error function δ

$$\delta = \sum_{i=1}^{np} \left(\frac{C^i_{ptheor}}{C_{pmax}} - \frac{C^i_{pexp}}{C_{pmax}} \right)^2$$

of fitting was below 0.03, where np is the total number of points in the C_p profile, C_{ptheor} is the calculated excess specific heat and C_{pexp} is the experimental excess specific heat. The results obtained for BSOD are summarized in Table 1. The negative peak could be due either to a reversible process of aggregation (exothermic) and/or to the intrinsic asymmetry of the thermal C_p profile related to the occurrence of kinetic factors.

Our data, together with previous observations on the stability of the dimeric protein, allow us to propose two alternative models for the unfolding mechanism in BSOD:

$$N_2 \rightleftharpoons 2N \rightleftharpoons 2U \rightarrow A \quad \text{(a)}$$

$$N_2 \rightleftharpoons U_2 \rightleftharpoons 2U \rightarrow A \quad \text{(b)}$$

At present we have no way of discriminating between the two denaturation pathways. Moreover, we cannot exclude the possibility that the whole irreversible

Table 1
BSOD thermodynamic parameters, obtained by statistical thermodynamic deconvolutions using Freire and Biltonen algorithm (i), Filimonov algorithm (ii), Freire and Biltonen with minimization algorithm (iii), and our fit (iv) (two endotherms and one exotherm)

Method	T_1	ΔH_1	T_2	ΔH_2	T_{ag}	ΔH_{ag}	δ
(i)	86.5	564	94.2	677			50.52
(ii)	86.5	486	94.2	683			10.82
(iii)	90.3	792	94.6	399			0.78
(iv)	90.5	838	95.1	514	97.2	−170	0.003
Ref. 18	82.8	473	89.5	674			

Notes: T_i and T_{ag} (aggregation temperature) are given in degrees centigrade; ΔH_i and ΔH_{ag} (aggregation heat) are given in kJ mol^{-1}; the error function for each point of the scan is defined in the text. The ΔH_{tot} obtained by integration of the total DSC unfolding curve was 1182 kJ mol^{-1} (this paper) and 1146 kJ mol^{-1} (Ref. 18).

unfolding process could be a continuous phenomenon with single steps which are difficult to rationalize in a simple schematic model.

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