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Extended theoretical analysis of irreversible protein thermal unfolding

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Abstract

The theoretical analysis of the protein denaturation model which includes an irreversible, exothermic and rate-limited step has been improved and applied to the DSC profile of Azurin. The two-step nature of the irreversible denaturation of globular proteins is usually depicted in the following simplified scheme: $N \Leftrightarrow U \Rightarrow F$, which is known as the Lumry and Eyring model. In most of the works concerning the thermal unfolding of proteins, it is usually assumed that the irreversible step of the process does not take place significantly during the short time the protein spends in the temperature range of the DSC transition, or if this is not the case, that this irreversible step occurs with a negligible thermal effect. As we will show, this last assumption cannot be accepted acritically; in fact we have found that in the case of Azurin an evident exothermic effect occurs at the end of the transition. In order to fit the experimental Cp_{exc} profile of Azurin, we have analyzed a model in which the exothermic effects of the irreversible step and the variations of ΔH with temperature are taken into account. Our model was first tested simulating a series of profiles and considering the effects of the variation of the parameters on the shape of the curves, and successfully used to fit the experimental calorimetric profile of Azurin.

Keywords: Proteins; Thermal unfolding; Azurin

1. Introduction

Over the last few years the development of high-sensitivity differential scanning calorimetry [1,2] has allowed the protein unfolding induced by temperature to be studied extensively [2–4] for the processes that occur at thermodynamic equilibrium. The direct calculation of all the unfolding thermodynamic functions is therefore possible [5]. The equilibrium criterion usually applied is the reproducibility of the trace in a second heating of the sample, the so-called calorimetric reversibility [6].

Moreover, it is possible to check the validity of the two-state model for the unfolding process by comparing both the calorimetric and van't Hoff enthalpies of denaturation [7]. In addition, the experimental heat capacity profile of a protein permits the calculation of its molecular partition function and, through the Freire and Biltonen algorithm [8], the deconvolution of complex unfolding thermal profiles into two-steps-type processes [9–11]. Many proteins, however, denature in an irreversible way, and thus the thermodynamic functions of the process cannot be extracted from the calorimetric curve. Nevertheless, several authors, assuming that the irreversible alteration of the unfolded state takes place with a

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negligible thermal effect, have interpreted the DSC thermograms of these proteins in terms of a reversible model in spite of calorimetric irreversibility [6].

It has recently been shown that DSC traces corresponding to the irreversible protein unfolding are scan-rate dependent [12,13].

This behaviour was initially explained by a simple irreversible two-state model $N-^{k}-D$ where N and D are the native and denatured form of the protein respectively, and k is the kinetic constant of this first-order process. Subsequently, the analysis of the irreversible unfolding of proteins was referred to a more complex model, reported below, which assumes a two-step process (for protein denaturation): (a) reversible unfolding of the native protein (N); (b) irreversible alteration of the unfolded protein (U) to yield a final state (F) which is unable to fold back to the native one,

$$N \Leftrightarrow U \Rightarrow F.$$

As a consequence of the irreversible step denaturation, the DSC profiles are distorted and the asymmetry increases remarkably with the decrease of scan rate. Changes of the molecularity concomitant with the unfolding are also expected to distort the DSC trace [13]. Moreover, it is foreseeable that the asymmetricity increases if the irreversible step is accompanied by exothermic effects.

In each case the Cp profiles corresponding to a thermal denaturation generally show a significant ΔCp, i.e. the heat capacity of the protein in the denatured state is different from the native state [14].

It has also been reported that the denaturational heat capacity is composed of a large positive contribution from the exposure of apolar groups and a significant negative contribution from the exposure of polar groups to water [15].

Recently, we started a DSC study on the thermal denaturation of Azurin [16], a blue copper protein, and we found that in the experimental conditions chosen, the whole irreversible process did not fit with any of the theoretical models so far proposed. In particular, an exothermal peak at the end of the transition was evident. Although experimental irreversible DSC transitions of many proteins have been studied, in which the irreversible step follows the unfolding at somewhat higher temperature, no theoretical studies taking into account the exothermal effect of the irreversible step are available in literature. In this work we have developed a theoretical analysis of the irreversible denaturation of proteins which takes this exothermal effect into account.

The results of this analysis were used to fit the DSC profile of the Azurin.

2. Materials and methods

Chemicals. Azurin from *Pseudomonas Aeruginosa* was purchased from SIGMA Chemical Co. (St. Louis, MO, USA) and used without further purification. The lyophilized powder contained 72.2% protein. The impurities were principally ammonium acetate. Protein concentration was determined by the procedure of Lowry [17]. Potassium phosphate analytical grade were obtained from FLUKA Chemie AG (Buchs, Switzerland).

Experimentals. DSC scans were carried out by a SETARAM (Lyon, France) micro differential scanning calorimeter (microDSC) with stainless steel 1 ml sample cells, interfaced to a BULL 200 Micral Computer. The sampling rate was 1 point/s in all measuring ranges. The calorimetric experiments were conducted under an extra nitrogen pressure of 1.5 bar. Buffer–buffer base lines were obtained under the same experimental and scanning conditions and subtracted from the sample curves.

About 2 mg of lyophilized powder were dissolved in 1 ml of phosphate buffer 50 mM at pH = 7.03 and scanned from 30 to 100°C with a precision of ± 0.08°C at a scan rate of 0.5°C/min. The average noise was ± 0.4 μW, and the reproducibility at refilling was 0.1 mJ K^{-1} ml^{-1}.

In order to obtain the Cp_{exc} function related to the denaturation process of Azurin, a 4th-order polynomial fit simulating the trend of the Cp of the native and denatured state was used.

Electrical calibration was carried out with a SETARAM EJ2 Joule calibrator.

3. Results and discussion

The Cp thermal denaturation profile of Azurin in buffer solution is reported in Fig. 1. Together with the lack of calorimetric reversibility, and the high scan-rate dependence, the curve shows some peculiarities: (a) a decrease of the Cp of the denatured form with increase of temperature; (b) the occurrence of an exothermic effect after the main peak corresponding to the unfolding process.

This evidence is further proof of how the exothermic effects following the irreversible step must be taken into account. In the case of Azurin, this exothermic peak would be partially covered by the main endothermal peak corresponding to the unfolding.

In order to explain this unusual thermal behaviour, we re-elaborated the Lumry–Eyring theoretical model [13] considering two new effects: (a) a not negligible thermal effect related to the irreversible step; (b) the temperature dependence of ΔH_U. The model can be represented by the following scheme:

$$N \underset{\Delta H_U}{\overset{K}{\rightleftharpoons}} U \xrightarrow[\Delta H_i]{k} F,$$

where N, U, and F are respectively the native, the unfolded, and final state of the protein; ΔH_U and ΔH_i are the enthalpies of the states U and F (taking N as reference state). It is to be assumed that the chemical equilibrium between N and U is always established.

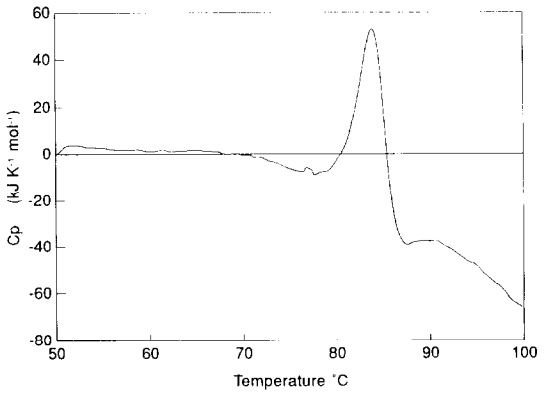

Fig. 1. Calorimetric scan of Azurin obtained in 50 mM potassium phosphate buffer pH = 7.03 at a scan rate of 0.5 K/m. Protein concentration is 1.4 mg/ml.

The apparent excess enthalpy, $\langle \Delta H \rangle$ is given by

$$\langle \Delta H \rangle = X_U \Delta H_U + X_F \Delta H,$$

where the calorimetric enthalpy, taking N as the reference state, is

$$\Delta H = \Delta H_U + \Delta H_i$$

Starting from this and following the procedure described in the Appendix, we obtain the final expression for the theoretical Cp_{exc}:

$$Cp_{exc} = \left[\frac{K \Delta H_U}{(K+1)^2} \left(\frac{k}{v} + \frac{\Delta H_U}{RT^2} \right) + \Delta H_i \frac{1}{v} \frac{kK}{K+1} \right] \times \exp\left(-\frac{1}{v} \int_{T_0}^{T} \frac{kK}{K+1} \, dT \right), \quad (1)$$

where v is the scanning rate expressed in K/min.

The above equation obviously reduces to the one proposed by Sanchez–Ruiz [13] when ΔH_i is zero, and to the following equation corresponding to a two-state reversible unfolding, either by setting $k = 0$ at any temperature or by setting $1/v = 0$,

$$Cp_{exc} = \frac{\Delta H}{RT^2} \frac{K}{(K+1)^2}. \quad (2)$$

The equation (1) contains several parameters, some of these being temperature dependent, so that the simulation of an experimental curve could be somewhat arbitrary.

In this contest the availability of experimental data obtained independently through different approaches is of extreme importance. In the following paragraphs we point out the effect of some parameters on the calculated Cp_{exc} profile.

Effect of the scan rate. The theoretical Cp_{exc} curves calculated by Eq. (1) at different scan rate are reported in Fig. 2. The scan rate ranges from 0.01 to 16 K/min, while for the other parameters the values reported in the legend for the same figure were adopted, and named 'standard values'. The figure shows that when v is 0.5 K/min or more, the left side of the main peak and T_m is maintained while the right side changes in a symmetrical way and a broad negative peak ap-

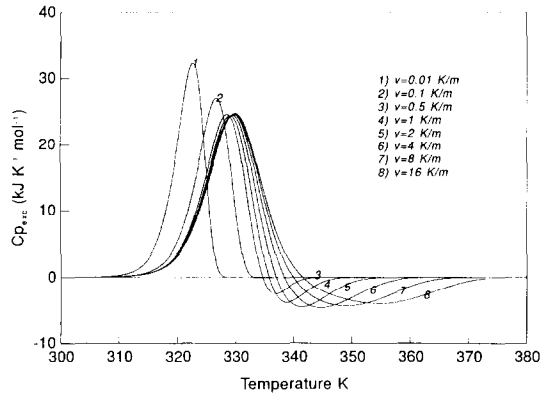

Fig. 2. Effect of scan rate on the calculated Cp_{exc} profile. The values used in the calculation are: $\Delta H_U = 300$ kJ/mol, $\Delta H_i = -100$ kJ/mol, $T_{1/2} = 330$ K, $T^* = 350$ K, $E_{app} = 100$ kJ/mol. ΔH_U = is assumed to be constant.

pears, with a minimum shifting towards the high temperature side as the scan rate is increased. Surprisingly the curves obtained at lower scan rates (1) and (2) do not show the negative peak, as we would expect from a superficial analysis of the kinetic model.

In order to clarify this behaviour we followed the evolution of both endothermic and exothermic peaks against scan rate. The results are reported in Fig. 3. It is evident that the scan rate effect influences the two thermal phenomena differently. Even if both peaks become sharper and shift towards the low-temperature side when v decreases, this effect is much greater for the exotermic peak than for the endothermic one. As a consequence of this, at a low scan rate (0.1–0.01 K/min) the two peaks are practically opposed and their sum gives the profiles reported in Fig. 2 (curve (1) and (2)).

Effect of activation energy. The effect of the activation energy of the kinetic step on the theoretical Cp_{exc} profiles is reported in Fig. 4. The parameters used are the 'standard values' and the scan rate is 0.5 K/min. As expected, an increase in the activation energy slackens the irreversible reaction and the corresponding exothermal peak shifts toward higher temperatures.

Effect of temperature dependence of ΔH_U. The influence on the theoretical Cp_{exc} profile of the dependence of ΔH_U as a function of temperature is reported in Fig. 5.

The function was calculated from ΔCp values through the classical Kirchoff equation. For the other parameters standard values were used. The figure shows that the influence of these parameters modifies the form of Cp_{exc} profiles remarkably. It is for this reason that the temperature effect on the enthalpy cannot be neglected when thermal denaturation of protein is characterized by a valuable ΔCp.

The above reported theoretical model was then applied in order to fit the experimental Cp_{exc} profile of Azurin.

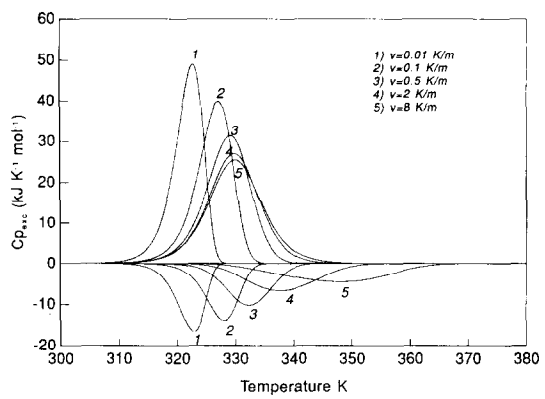

Fig. 3. Effect of scan rate on the two components (endo- and exo-thermic) of the calculated Cp_{exc} profile. The some parameters reported in the legend of Fig. 2 were used.

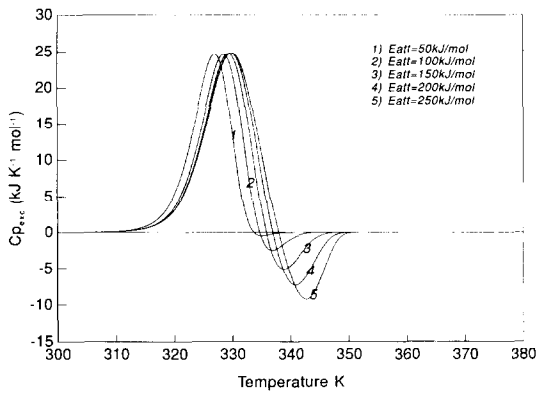

Fig. 4. Effect of the apparent activation energy (E_{app}) on calculated Cp_{exc} profile. Scan rate was fixed at 0.5 K/m. All the other parameters were not changed.

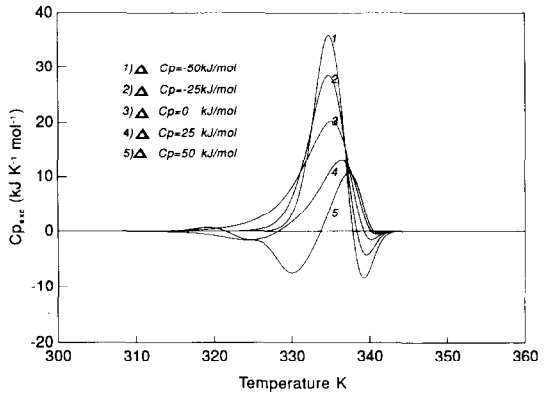

Fig. 5. Influence of ΔCp on the shape of the Cp_{exc} curve. These curves were calculated at a scan rate of 0.5 K/m.

In the case of Azurin some of the parameters appearing in Eq. (1) are known. In particular, from the analysis of the scan rate dependence of the shape of the curve (data not published), for Azurin we have calculated an apparent activation energy of 356 kJ/mol. Moreover, the difference between Cp^D calculated at the offset temperature and Cp^N calculated at onset temperature was extracted from the calorimetric trace, its value being −39 kJ/mol.

In different maps, Fig. 6 reports the comparison between the experimental curve and the theoretical ones calculated in different ways: by considering the simplest reversible two state model by Eq. (2) (map a); by Eq. (13) of ref. [13] considering the irreversible model of Lumry and Eyring (map b); by Eq. (1) considering ΔH_U constant (map c); and by Eq. (1) considering the temperature dependence of ΔH_U (map d). In this latter case the value of −39 kJ/mol for ΔCp, measured experimentally, was used in the calculations.

At this level of approximation, we supposed that ΔCp was constant, so that the variation of ΔH_U with temperature could be considered linear.

Strictly speaking, this assumption is not correct, but in the narrow temperature range of denaturation it can be considered acceptable.

The set of parameters corresponding to the calculated curve is: $\Delta H_U = 550$ kJ/mol, $\Delta H_i = -250$ kJ/mol, $T_{1/2} = 358.15$ K, $E_{app} = 356$ kJ/mol, $T^* = 360$ kJ/mol, $\Delta H_i = -250$ kJ/mol.

As anticipated in the introduction, we founded many other sets of parameters that fitted the curve, but this particular combination of parameters fitted the experimental curve best when the experimental value of ΔCp was considered in the calculation.

The successful fit between experimental and

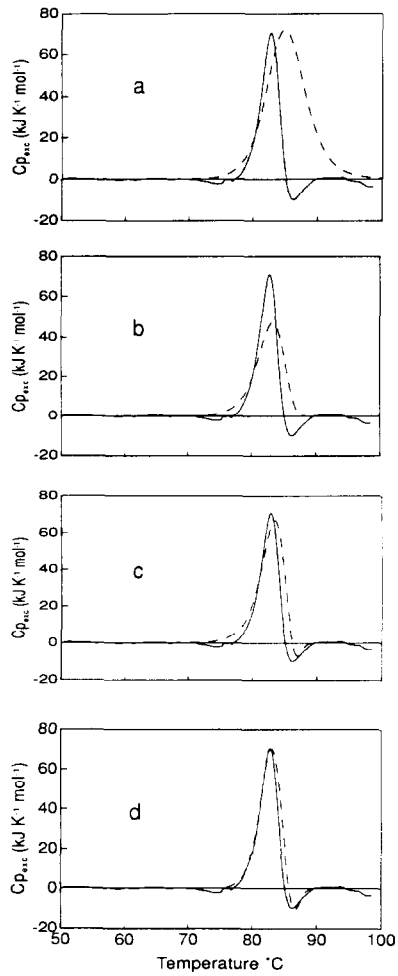

Fig. 6. The excess heat capacity function of Azurin (solid lines) fitted with different equations (dashed lines): (a) the reversible two-state model, (b) the irreversible model of Lumry and Eyring, (c) the irreversible model with a not negligible exothermic effect, (d) the further improvement of the fit by considering the temperature dependence of ΔH_U.

calculated curve shown in Fig. 6d, proves the correctness of the model used to derive the theoretical Cp_{exc} curve and suggests its potential application in the analysis of Cp profiles of the irreversible thermal denaturation of globular proteins. To conclude, we think that to assume the thermal effect of the irreversible step following the unfolding as negligible can be arbitrary and that in each case this assumption requires great care and consideration.

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Appendix

We supposed that the irreversible step occurs with a not negligible thermal effect.

In particular:

$$\Delta H = \Delta H_U + \Delta H_i$$

The average excess enthalpy can therefore be expressed as

$$\langle \Delta H \rangle = X_U \Delta H_U + X_F \Delta H,$$

or, remembering that $X_U + X_F + X_N = 1$

$$\langle \Delta H \rangle = X_U \Delta H_U + X_F(\Delta H_U + \Delta H_i),$$

$$\langle \Delta H \rangle = \Delta H_U(X_U + X_F) + X_F \Delta H_i,$$

$$\langle \Delta H \rangle = \Delta H_U(1 - X_N) + X_F \Delta H_i.$$

The shape of the curve is given by

$$Cp_{exc} = \frac{d\langle \Delta H \rangle}{dT},$$

$$Cp_{exc} = \frac{d}{dT}[\Delta H_U(T)(1 - X_N)] + \Delta H_i \frac{dX_F}{dT}$$

Where the temperature dependence of ΔH_U is given by the classical Kirchoff equation,

$$\Delta H_U(T) = \Delta H_U(T_d) + \int_{T_d}^{T} \Delta Cp \, dT$$

and, for a constant value of ΔCp,

$$\Delta H_U(T) = \Delta H_U(T_d) + \Delta Cp(T - T_d).$$

Thus,

$$Cp_{exc} = -\Delta H_U(T) \frac{dX_N}{dT} + \Delta H_{ag} \frac{dX_F}{dT}, \qquad (A.1)$$

and consequently, considering the equations derived in ref. [13] for X_U, X_N, X_F,

$$\frac{dX_N}{dT} = -\frac{K}{(K+1)^2}\left(\frac{k}{v} + \frac{\Delta H_U(T)}{RT^2}\right) \times \exp\left(-\frac{1}{v}\int_{T_0}^{T} \frac{kK}{K+1} \, dT\right), \qquad (A.2)$$

$$\frac{dX_F}{dT} = \frac{1}{v}\frac{kK}{K+1} \exp\left(-\frac{1}{v}\int_{T_0}^{T} \frac{kK}{K+1} \, dT\right), \qquad (A.3)$$

and substituting Eqs. (A.2) and (A.3) into Eq. (A.1), we obtaine the final expression of the shape of the curve,

$$Cp_{exc} = \left[\frac{K\Delta H_U(T)}{(K+1)^2}\left(\frac{k}{v} + \frac{\Delta H_U(T)}{RT^2}\right) + \Delta H_i \frac{1}{v}\frac{kK}{K+1}\right] \exp\left(-\frac{1}{v}\int_{T_0}^{T} \frac{kK}{K+1} \, dT\right)$$

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