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# ON THE COOPERATIVITY OF THE THERMAL DENATURATION OF MINI-PROTEINS

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It is well established that the reversible thermal denaturation of small globular proteins is a cooperative two-state transition, analogous to a first-order phase transition in a finite-size system. Finite-size effects on the cooperativity of the reversible thermal denaturation become more important when the polypeptide chain is very small, as in the case of some synthesized mini-proteins. The analysis of two specific examples of mini-proteins, by means of a statistical mechanical approach, leads to the conclusion that their thermal denaturation, in view of its broadness and energetics, cannot be considered a cooperative first-order phase transition.

***Keywords:*** *cooperativity, differential scanning calorimetry, finite-size effects, first-order phase transition, mini-proteins, two-state model*

## Introduction

The reversible thermal denaturation of globular proteins does appear to be a cooperative process and several theoretical approaches have been devised to explain and characterize such cooperativity [1–9]. In particular, Camacho and Thirumalai [10], by means of Monte Carlo simulations for a class of self-avoiding walk models on the square lattice, concluded that: (a) polypeptide chains undergo a coil-to-globule collapse transition at a temperature indicated by $T_\theta$, that is a second-order phase transition involving no discontinuity in the free energy; (b) polypeptide chains undergo a folding transition from coil conformations to a unique globular conformation possessing a well defined spatial architecture at a temperature indicated by $T_f$; the latter is a first-order phase transition, but, since $T_f \cong T_\theta$ in order to have an efficient two-state folding sequence, the discontinuity in the free energy at $T_f$ cannot be large; (c) polypeptide chains behave as homopolymers at $T>T_f$, assuming coil-like conformations, and in a sequence-specific manner at $T<T_f$, assuming a unique native structure.

On this basis, the reversible thermal denaturation of globular proteins, being the reverse of the folding transition, is expected to be a weakly first-order phase transition [3, 5, 7, 10]. This theoretical expectation is in line with a lot of experimental data for globular proteins accumulated over the years and is now widely accepted. In fact, since the pioneering studies of Brandts and co-workers [11, 12], the reversible thermal denaturation of small globular proteins is represented as a two-state N⇔D transition or an all-or-none process. The two-state nature of thermal denaturation was questioned by Poland and Scheraga [13], but supported and unequivocally demonstrated by differential scanning calorimetry, DSC, measurements [14–18]. The latter allow the direct evaluation of both the calorimetric enthalpy change $\Delta_d H(T_d)_{cal}$ (i.e., the area of the DSC peak), and the van't Hoff enthalpy change $\Delta_d H(T_d)_{vH}$ (i.e., a measure of the sharpness of the DSC peak); the ratio $\Delta_d H(T_d)_{cal}/\Delta_d H(T_d)_{vH}$ is called the cooperative unit CU, and is close to one for all the investigated small globular proteins [14, 15]. The finding that CU=1 is a necessary condition to state that the reversible thermal denaturation is a two-state N⇔D transition [19]. In addition, thermodynamic treatment of experimental data in the framework of the N⇔D transition has emphasized the marginal stability of the native conformation, N-state, with respect to denatured conformations, D-state [14, 20–22].

Actually, a globular protein is a small thermodynamic system (it does not reach the thermodynamic limit of a number of particles approaching infinity), and large fluctuations in average values of thermodynamic properties occur, as first noticed by Cooper [23]. In fact, the DSC peaks of globular proteins are not sharp, as for the melting of macroscopic crystals, but extend over a temperature range whose width decreases on increasing the $\Delta_d H(T_d)$ value [8]; note that in the two-state N⇔D transition model, $\Delta_d H(T_d)_{cal} = \Delta_d H(T_d)_{vH}$, and so the subscript can be omitted. More correctly, as claimed by Liquori [24], globular proteins should be considered 'crystal molecules' in which the surface-to-volume ratio is large, leading to significant fluctuations. Therefore, finite-size effects

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are always present in the reversible thermal denaturation of globular proteins, but they could give rise to unphysical-unreliable situations when the polypeptide chain is very small. An analysis of such situations is presented in this work.

## Finite-size effects and thermal denaturation cooperativity

It is necessary to study the dependence of the cooperativity of the reversible thermal denaturation on the number of residues in the polypeptide chains, $N_{res}$. In this respect Thirumalai and co-workers [25] proposed that a reliable measure of cooperativity could be given by the following dimensionless parameter:

$$\Omega_c=(T_f^2/\Delta T)\cdot|df_N/dT|_{T=T_f} \quad (1)$$

where $T_f$, the folding temperature, can be considered to correspond to the denaturation temperature $T_d$ (i.e., the maximum of the DSC peak), at which the N-state and D-state are in equilibrium, and Eq. (1) becomes:

$$\Omega_c=(T_d^2/\Delta T)\cdot|df_N/dT|_{T=T_d} \quad (2)$$

where $\Delta T$ is the full width at half maximum of the DSC peak, and $f_N$ is the fraction of protein molecules in the N-state. From the physical point of view, the cooperativity parameter $\Omega_c$ can be regarded as the convolution of the sharpness of the transition given by the ratio $T_d/\Delta T$, and the extent to which the N-state changes around $T_d$, as measured by the temperature derivative of $f_N$ [25]. By assuming that thermal denaturation is a reversible two-state N⇔D transition, the fraction of protein molecules in the N-state is $f_N=1/(1+K)$, where $K$ is the equilibrium constant of the reversible thermal denaturation, and its temperature derivative is given by:

$$|df_N/dT|_{T=T_d}=[\Delta_d H(T_d)/RT_d^2][K/(1+K)^2] \quad (3)$$

and, since in a two-state N⇔D transition $K=1$ at $T=T_d$, Eq. (3) becomes:

$$|df_N/dT|_{T=T_d}=\Delta_d H(T_d)/4RT_d^2 \quad (4)$$

By inserting Eq. (4) into Eq. (2), the expression of the cooperativity parameter $\Omega_c$ becomes:

$$\Omega_c=\Delta_d H(T_d)/4R\Delta T \quad (5)$$

This formula clarifies that $\Omega_c$ can be directly calculated from an analysis of DSC peaks; what is necessary, is a relationship between $\Omega_c$ and $N_{res}$. On the basis of scaling theories for finite-size systems undergoing regular first- and second-order phase transitions [26, 27], Thirumalai and co-workers proposed that [25]:

$$\ln\Omega_c\propto\zeta\ln N_{res} \quad (6)$$

where $\zeta=1+\gamma=2.2$, because $\gamma$ is the critical exponent that characterizes the divergence of the magnetic susceptibility at the critical point for a $n$-component ferromagnet in a self-avoiding walk, on the basis of the universality character of phase transitions [26, 27]. By analysing a lot of experimental thermal denaturation data, Thirumalai and co-workers found the following relationship:

$$\ln\Omega_c= -2.9+2.2\ln N_{res} \quad (7)$$

confirming the universal scaling law of Eq. (6). As a by-product, they found that:

$$\ln(\Delta T/T_d)=1.4-1.1\ln N_{res} \quad (8)$$

that means $\Delta T/T_d\propto 1/N_{res}$, the sharpness of the transition is inversely proportional to the number of residues. Equations (7) and (8) are used to construct the straight lines shown in Figs 1 and 2, respectively.

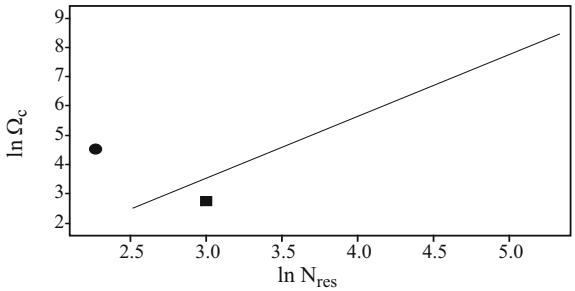


**Fig. 1** Plot of $\ln\Omega_c$ *vs.* $\ln N_{res}$ according to Eq. (7), that describes the behavior of real globular proteins [22]. The filled circle corresponds to trpzip4, and the filled square corresponds to trp-cage

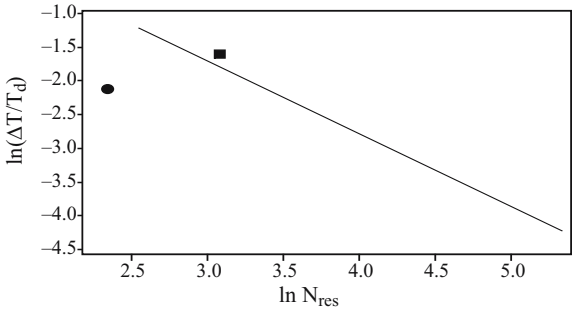


**Fig. 2** Plot of $\ln(\Delta T/T_d)$ *vs.* $\ln N_{res}$ according to Eq. (8), that describes the behavior of real globular proteins [22]. The filled circle corresponds to trpzip4, and the filled square corresponds to trp-cage

## The case of two mini-proteins

The tryptophan zipper (trpzip) family of peptides was designed by Cochran and colleagues [28], based on the β-hairpin of the GB1 protein (note that β-hairpins are simple β-sheet structures constituted by two anti-parallel β-strands connected by a reverse turn).

Trpzip peptides proved to be highly soluble in aqueous solutions, maintaining the monomer state, and showed reversible thermal denaturation when probed by circular dichroism measurements [28, 29]. Specifically trpzip4 consists of 16 residues, and is a triple variant of the protein GB1 β-hairpin where Trp5, Trp12 and Trp14 substitute Tyr, Phe and Val, respectively. Cochran and colleagues were able to show, by means of NMR measurements, that the four Trp residues in trpzip4 interdigitate, forming the so-called Trp-zipper motif in which the non-hydrogen bonded cross-strand pairs of Trp residues interact [28]. In order to clarify the two-state nature of the thermal unfolding of trpzip4, Streicher and Makhatadze performed DSC measurements at pH 7.0, 20 mM sodium phosphate buffer [30]. Analysis of DSC traces led to the following thermodynamics parameters: $T_d$=75°C, $\Delta_d H(T_d)_{cal}$=89 kJ mol$^{-1}$, $\Delta_d H(T_d)_{vH}$=94 kJ mol$^{-1}$, and $\Delta_d C_p$=1.4 kJ K$^{-1}$ mol$^{-1}$. The closeness of the two enthalpy values and the superimposition of the thermal transitions profiles obtained by monitoring the unfolding transition at different wavelengths in CD measurements, were used by Streicher and Makhatadze to conclude that the reversible thermal denaturation of trpzip4 is a cooperative two-state N⇔D transition [30].

The trp-cage is a synthetic 20-residue peptide based on the 39-residue peptide exendin-4 from the Gila monster saliva [31]. By truncating the N-terminal region of exendin-4 by 19 residues and further substituting 5 residues, Andersen and colleagues were able to obtain a monomer peptide which proved to be folded in aqueous solution [31]. The NMR structure of trp-cage indicates that the peptide possesses an α-helix (residues 2–8), a short $3_{10}$-helix (residues 11–14) and a polyproline II helix at the C-terminus [31]. The interactions between the side-chains of Tyr3, Trp6, Gly11, Pro12, Pro18 and Pro19 give rise to the trp-cage fold and so the latter does appear to be a mini-protein. Its thermal denaturation proved to be a rather cooperative process when investigated by means of different spectroscopic methods [31, 32]. In order to reach a more robust conclusion on the two-state cooperative nature of the thermal denaturation of the trp-cage, Streicher and Makhatadze performed DSC measurements at pH 7.0, 20 mM sodium phosphate buffer [33], obtaining: $T_d$=43.9°C, $\Delta_d H(T_d)_{cal}$=56 kJ mol$^{-1}$, $\Delta_d H(T_d)_{vH}$=56 kJ mol$^{-1}$, and $\Delta_d C_p$=0.3 kJ K$^{-1}$ mol$^{-1}$. On the basis of the equality of the two enthalpy values, Streicher and Makhatadze concluded that the reversible thermal denaturation of trp-cage can be correctly represented as a cooperative two-state N⇔D transition [33].

Simulated DSC curves can be calculated according to the two-state N⇔D transition model by means of the following analytical formula:

$$\Delta C_p=[\Delta_d H(T_d)^2/RT^2][K/(1+K)^2] \quad (9)$$

where $\Delta C_p$ is the heat capacity change in excess with respect to the N-state, $K$ is the equilibrium constant of the reversible thermal denaturation, and the contribution of $\Delta_d C_p$ has been deliberately neglected because it simply affects the post-transition baseline [8]. The DSC traces for the two selected mini-proteins, calculated in order to reproduce the experimental thermodynamic values determined by Streicher and Makhatadze [30, 33], are shown in Fig. 3. The broadness of these two DSC peaks is particularly impressive: the full width at half maximum $\Delta T$ amounts to ≈40°C for trpzip4, and ≈60°C for trp-cage; note that also the height of the DSC peaks is

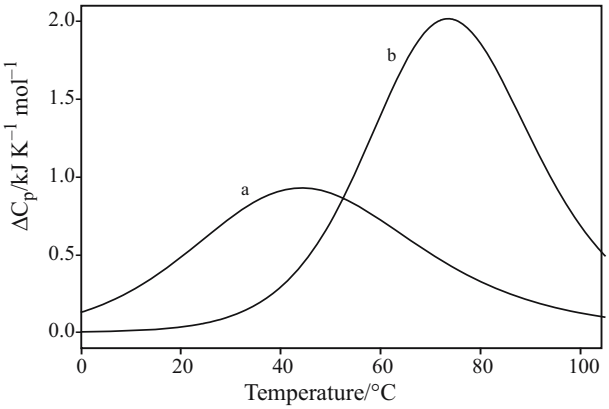


**Fig. 3** DSC traces calculated by means of Eq. (9) to reproduce the experimental ones for trp-cage (curve a) and trpzip4 (curve b) determined by Streicher and Makhatadze [27, 30]

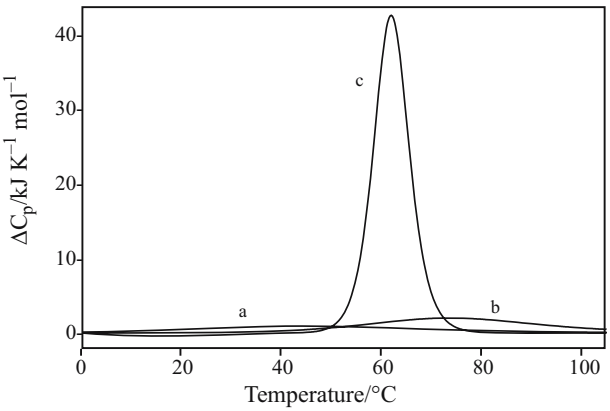


**Fig. 4** Comparison between the DSC traces calculated by means of Eq. (9) to reproduce the experimental ones for trp-cage (curve a) and trpzip4 (curve b) determined by Streicher and Makhatadze [27, 30], and the DSC peak (curve c) obtained by fixing in Eq. (9) $T_d$=62°C and $\Delta_d H(T_d)$=400 kJ mol$^{-1}$, which are typical values for a mesophilic globular protein of 100 residues [14]

very small. Such a broadness is not compatible with the usual practice, adopted also by Streicher and Makhatadze [30, 33], to assign the entire area of the DSC peak (i.e., the calorimetric enthalpy change) at the temperature of its maximum.

In order to gain perspective, these two DSC traces (curves a and b) are compared in Fig. 4 with the DSC profile (curve c) calculated by means of Eq. (9) fixing $T_d$=62°C and $\Delta_d H(T_d)$=400 kJ mol$^{-1}$, which are typical values for mesophilic globular proteins consisting of about 100 residues [14]. The difference is striking: the latter DSC peak is sharp (i.e., $\Delta T \approx 8$°C), as expected for a first-order phase transition affected by finite-size effects. Such a comparison unequivocally indicates that, when $\Delta_d H(T_d)$ is too small, even within the two-state N⇔D transition model, the process cannot be considered cooperative. Indeed, in the case of trp-cage, the DSC peak extends over more than 100°C, and so the N-state and D-state are both significantly populated over this temperature range (i.e., at 4°C the population of the D-state amounts to 5% of the total [28]). Such a situation is at odds with the notion of a cooperative all-or-none process: the protein molecules should populate the N-state up to the denaturation temperature, and then should sharply pass to populate the D-state; in other words, the energy distribution function should have a bimodal shape with a significant gap between the average energy values of the two states. This condition is very difficult to fulfil by a chain of about 20 residues because its folded structure possesses a too small core not accessible to water molecules [15, 22].

This is confirmed by the finding that the points corresponding to trpzip4 (closed circle) and trp-cage (closed square) do not fall on the straight lines defined by Eqs (7) and (8), as shown in Figs 1 and 2. In other words, the dimensionless cooperativity parameter $\Omega_c$ calculated according to Eq. (5) for the two mini-proteins does not have the value expected on the basis of the general relationship of Eq. (7), established by Thirumalai and co-workers on the basis of experimental thermal denaturation data [25].

It emerges that a polypeptide chain consisting of a very small number of residues, around 20, even though able to assume a folded structure so to be defined a mini-protein, does not behave as a 'crystal molecule' whose thermal denaturation is a first-order phase transition, resembling the melting of a macroscopic crystal.

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