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THE TEMPERATURE-INDUCED DENATURATION OF SMALL GLOBULAR PROTEINS AS A FIRST-ORDER PHASE TRANSITION OF 'CRYSTAL MOLECULES'

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Abstract

A general feature of temperature-induced reversible denaturation of small globular proteins is its all-or-none character. This strong cooperativity leads to think that protein molecules, possessing only two accessible thermodynamic states, the native and the denatured one, resemble 'crystal molecules' that melt at raising temperature. An analysis, grounded on mean field theory, allows to conclude that the two-state transition is a first-order phase transition. The implication of this conclusion are briefly discussed.

Keywords: crystal molecules, phase transition, proteins

Introduction

The experimental investigations about the reversible denaturation process, with both spectroscopic and calorimetric techniques, have pointed out the strongly cooperative behaviour of the tertiary structure of globular proteins [1, 2]. The process happens in an all-or-none manner for each protein molecule and is not due to interactions between single macromolecules, neither to the disruption of aggregates present in solution. The cooperative behaviour that ultimately gives rise to the well-shaped DSC peak, is a peculiar feature of the tertiary structure of each molecule. The all-or-none character of the temperature-induced denaturation arises from the fact that only two states result thermodynamically stable, the native and the denatured one, the former being maintained by a dense three-dimensional pattern of specific and non-specific interactions that build up the tertiary structure. This analysis leads to think that a

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globular protein molecule can be regarded as a solid structure which, in the course of denaturation process, undergoes a phase transition from an ordered state, the native, to a disordered state, the denatured one. Often some authors have spoken of melting and melting temperature for the denaturation, in order to emphasize the analogy with the fusion process of a crystal [3, 4].

Really from the 1966 a fascinating hypothesis was proposed by A. M. Liquori [5]; this author suggested that a protein molecule can be considered as a 'crystal molecule', namely a crystal of molecular dimension [6–8]. This hypothesis has received numerous experimental validations. In fact a detailed analysis of X-ray crystal structures of globular proteins, accomplished independently by Klapper [9, 10], Richards [11, 12], Chothia [13–15] and Liquori [16], has evidenced that the packing density is about 0.70, comparable to that found in organic molecular crystals. The packing density is an adimensional number, corresponding to the ratio of van der Waals volume to the volume of really filled space by the macromolecule [17]. Furthermore measurements of the compressibility of globular protein molecules in solution have been performed and the obtained values are close to those of molecular solids [18, 19]. These results confirm that the three-dimensional tertiary structure can be assimilated to a crystalline solid, even if it lacks the characteristic translational symmetry. Further it has been suggested that the solid-like nature of globular proteins has selective advantage respect to a liquid-like character, allowing for the highly conservative core regions of homologous proteins and the strict specificity of protein–protein associations [4, 20]. Finally, the experimental DSC measurements, showing directly the heat capacity peak associated to the denaturation process, have lent the strongest support to the hypothesis that small globular proteins can be considered as 'crystal molecules' from the thermodynamic point of view [21, 22].

In this paper we show that the reversible denaturation of globular proteins which possess only two accessible thermodynamic states, the native and the denatured one, does not correspond to a second-order phase transition, but it must be regarded as a first-order phase transition, namely the melting of a 'crystal molecule'. In this manner the area of DSC peak corresponds to the latent heat associated to the transition, the native and denatured states being assimilated to two phases which differ in symmetry. To reach this conclusion we exploit the mean field theory approach to phase transitions in order to analyze the two-state transition that well represents the denaturation process of most small globular proteins.

The thermodynamic approach

It is interesting to determine the order of the two-state transition according to the mean field theory [23]. The two-state transition is the fundamental model

to treat the reversible denaturation process because the experimental investigations have shown that most small globular proteins unfold with a single step mechanism [24]. It is suitable to develop, in a concise and ordered form, a statistical-mechanical treatment of the two-state model [25]. Considering a physical system which possesses only two accessible thermodynamic states, N and D, each representative of a population of microstates, the canonical partition function (i.e. assuming a P, T statistical ensemble) results:

$$Q(T) = \exp(-G_N / RT) + \exp(-G_D / RT) \tag{1}$$

where G_N and G_D represent the standard free energies of native and denatured states, respectively. In the study of conformational transitions of globular proteins in solution, it is convenient to select the native state as reference. Thus, by dividing all for the term $\exp(-G_N/RT)$, the Eq. (1) becomes:

$$Q_N(T) = 1 + \exp(-\Delta_d G^\circ / RT) = 1 + K \tag{2}$$

where $\Delta_d G^\circ \equiv G_D - G_N$ is the denaturation free energy change, K is the thermodynamic constant of the equilibrium $N \leftrightarrow D$, and the well-known thermodynamic relation $\Delta G^\circ = -RT\ln K$ is used. Moreover, the equilibrium constant is temperature-dependent according to the following expression derived from the integration of the van't Hoff equation:

$$K(T) = \exp - \{[\Delta_d H^\circ(T_d) / R][1 / T - 1 / T_d] + (\Delta_d C_p^\circ / R) \cdot$$

$$\cdot[1 - T_d / T - \ln(T / T_d)]\} \tag{3}$$

where T_d is the transition temperature and corresponds to the maximum of DSC curve; $\Delta_d H^\circ(T_d) \equiv H_D(T_d) - H_N(T_d)$ is the denaturation enthalpy change referred to T_d and corresponds to the area delimited by the DSC curve and the reference baseline; $\Delta_d C_p^\circ \equiv C_{p,D} - C_{p,N}$ represents the heat capacity difference between denatured and native states, assumed temperature-independent. The excess enthalpy function is directly obtained from the partition function by means of a general relation of statistical mechanics:

$$< \Delta H(T) > = RT^2[\partial \ln Q_N(T) / \partial T] \tag{4}$$

from which it is derived:

$$< \Delta H(T) > = \Delta_d H^\circ(T)[(K / (1 + K)] = f_D \Delta_d H^\circ(T) \tag{5}$$

where f_D is the fraction of denatured molecules and corresponds also to the advancement degree of the process, θ, and $\Delta_d H^\circ(T) = \Delta_d H^\circ(T_d) + \Delta_d C_p^\circ \cdot (T - T_d)$.

The excess heat capacity function, being the temperature derivative of the excess enthalpy, results:

$$< \Delta C_p(T) > = (df_D / dT)\Delta_d H^\circ(T) + f_D \Delta_d C_p^\circ \quad (6)$$

where the first term gives rise to the calorimetric peak, while the second term represents the heat capacity change distributed along the temperature range where the process takes place, weighted for the advancement degree. The Eq. (6) is an analytical relation that allows to simulate two-state DSC curves, using as input parameters the values of T_d, $\Delta_d H^\circ(T_d)$ and $\Delta_d C_p^\circ$, and to fit the experimental DSC profiles shown by globular proteins.

The self-consistent or mean field theory of critical phenomena assumes that a system of interacting particles can be replaced by a system of non-interacting particles immersed in an external field, since the last physical situation can be exactly solved. With this approach the free energy of the system results an analytic function of thermodynamic variables and a set of universal critical exponents, and can be expressed as a power expansion of some order parameter [23]. Thus the mean field theory, despite the great variety of physical systems, has allowed to achieve a universal treatment of phase transitions, by simply identifying the right order parameter. This type of approach, though quantitatively wrong, offers very reliable qualitative results and is always a good starting point for more sophisticated approaches which take explicitly into account fluctuations. The analyze with the mean field theory the two-state transition, it is necessary to define an order parameter, η, that must reflect the structural order of macromolecule and must be built using the experimentally determined thermodynamic parameters of denaturation process. The order of a phase transition can be determined from the temperature dependence of the order parameter η. This, as usually defined [26, 27], can only assume values comprised in the range [−1, 1], with $\eta = -1$ for the totally disordered state, and $\eta = 1$ for the completely ordered one. The order parameter is zero in correspondence of transition temperature, that, for globular proteins, is equal to T_d, the temperature of maximum in DSC peaks.

According to the mean field theory, as developed by L. D. Landau [23], for a second-order phase transition, the temperature dependence of the order parameter for temperature values lower or equal to T_d (i.e. the temperature where $\eta = 0$), is described by an exponent β, called 'critical exponent'. This dependence assumes the following form:

$$\eta = c(T_d - T)^\beta \quad (7)$$

where c is a proportionality constant. The value of critical exponent depends on the universality class at which belongs the investigated system. The mean field theory predicts that, for a second-order phase transition, β must have a value equal to 1/2. Further, the modern theories of critical phenomena, based on the renormalization group, have shown that the 1/2 value represents an upper limit to the expected value of critical exponent for a second-order transition [28].

In the case of a first-order phase transition the order parameter changes with temperature in a discontinuous manner, because the two phases coexist at equilibrium. Strictly speaking the phase transitions only occur when the system dimension is infinite, namely in the thermodynamic limit (i.e. the number of particles in the system tends to infinity). In a system of finite dimension the discontinuity of the order parameter for a first-order transition is not sharp and its temperature variation resembles that of a second-order transition. It has been shown that to analyze the effects of finite dimension on a first-order phase transition and to directly establish the transition order, thermodynamic data, such as the heat capacity of the system, are necessary [29, 30].

In any case the value of the critical exponent β, obtained from a plot of the order parameter η *vs.* (T_d-T), or better from the slope of the plot of ln η *vs.* $\ln(T_d-T)$, allows to establish if the transition is second-order, because the 1/2 value is an upper limit for a truly second-order transition. When the transition is not second-order (i.e. $\beta > 1/2$), to state that the transition is first-order it is necessary a comparison with a system that is known to exhibit this kind of transition [31].

Discussion

From the thermodynamic point of view the native state is unique and represents the absolute minimum of the free energy function for the polypeptide chain in solution at room temperature. This assumption is confirmed by X-ray diffraction studies, which have shown that all the protein molecules in a crystal possess the same conformation, apart from some side-chains. The denatured state is not well-defined and there is still debate about its actual structural description [32]. According to the experimental studies, these are the only states significantly populated at changing the temperature for single domain globular proteins (i.e. there are not stable intermediate states in the unfolding process). To apply the mean field approach we have thought that the order parameter η for the two-state model can be defined by the following relation:

$$\eta \equiv 1 - 2\theta \equiv 1 - 2[K / (1 + K)] \tag{8}$$

where θ is the advancement degree of the process. Indeed, for $\theta=0$, there is the native state and $\eta=1$; for $\theta=0.5$, it is $K(T_d)=1$ and $\eta=0$; finally for $\theta=1$,

there is the denatured state and $\eta = -1$. To calculate the temperature dependence of η, it is necessary the knowledge of $\theta(T) = f_D(T)$, above defined. Thus, by experimentally determining the values of T_d, $\Delta_d H^\circ(T_d)$ and $\Delta_d C_p^\circ$ with DSC measurements, it is possible to calculate $K(T)$, $\theta(T)$ and then the temperature dependence of the order parameter, $\eta(T)$. At this point it is sufficient to report on a plot $\ln\eta$ *vs.* $\ln(T_d - T)$ to determine the value of critical exponent β. In drawing the plot it is important to recognize that only the values corresponding to $T < T_d$ can be reported because Eq. (7) is valid for $T \leq T_d$.

From the plot slope, calculated with a least squares regression on the points close to T_d, the value of β for a two-state transition is readily obtained. If the value of β is greater than 1/2, it can be stated that the process described by the two-state model is not a second-order phase transition, but corresponds to a first-order transition that shows the effects due to the finite dimension of thermodynamic system constituted by a single 'crystal molecule'. This statement is justified because it has been pointed out, with deep theoretical studies by various authors, that the denaturation process of 'model' globular proteins closely corresponds to a first-order phase transition [33–43].

We have performed this analysis on a series of two-state transitions characterized by different values of thermodynamic parameters T_d, $\Delta_d H^\circ(T_d)$ and $\Delta_d C_p^\circ$, and we have always obtained for β, the critical exponent, values close to 1.0, greater than the upper limit 1/2, expected for a second-order transition. In Fig. 1 are reported the two-state DSC curves simulated with Eq. (6), using the following values of thermodynamic parameters: $T_d = 330$ K; $\Delta_d H^\circ(T_d) = 300$, 400 and 500 kJ/mol; $\Delta_d C_p^\circ = 5.0$ kJ/K mol. By raising the value of denaturation enthalpy change, the sharpness of the heat capacity peak and the apparent cooperativity of the phenomenon increase. In Fig. 2 are reported the order parameters η as function of temperature for the same transitions of Fig. 1. Finally, in Fig. 3 is shown the plot of $\ln\eta$ *vs.* $\ln(T_d - T)$ for the two-state transition with $\Delta_d H^\circ(T_d) = 500$ kJ/mol; in this case the least squares regression gives a value of the critical exponent equal to 0.998. But, despite the apparent increase in cooperativity visible in $\langle\Delta C_p\rangle$ profiles of Fig. 1, there is not change in the values of β, that result all close to one.

The mean field approach allows to conclude that the temperature-induced denaturation process of small globular proteins is a first-order phase transition, namely the melting of a 'crystal molecule'. This fact seems very surprising because globular proteins are macromolecules constituted by twenty different monomers (i.e. they are heteropolymers), and have a very large number of accessible molecular conformations. Indeed, in the past, some authors, such as Poland and Scheraga [44], tried to confute the validity of two-state model on the basis of statistical-mechanical arguments, suggesting a more gradual and continuous transition. However, actually, both theoretical and experimental investi-

gations, allow to establish the first-order character of temperature-induced denaturation. This has very important consequences for the accomplishment of biological functions.

For instance, the cooperative character of the three-dimensional pattern of non-covalent interactions that give rise to all-or-none transition, results funda-

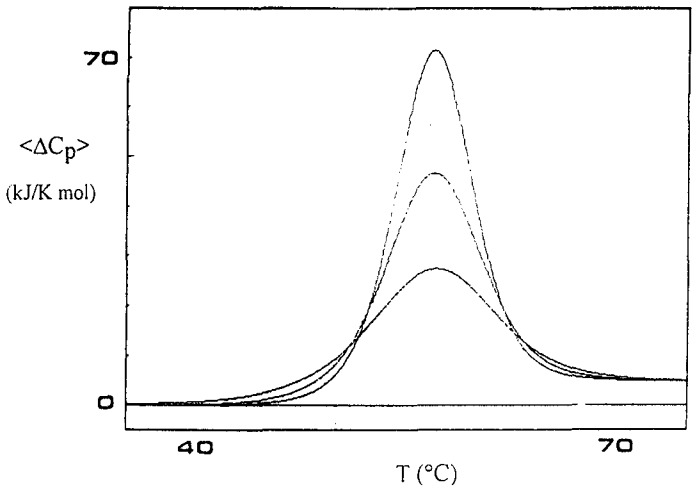

Fig. 1 Excess heat capacity profiles, obtained with Eq. (6) for two-state transitions characterized by the following thermodynamic parameters: T_d = 330 K; $\Delta_d H^o(T_d)$ = 300, 400 and 500 kJ/mol; $\Delta_d C_p^o$ = 5.0 kJ/K mol. The sharpness of DSC peak increases at raising the value of $\Delta_d H^o(T_d)$

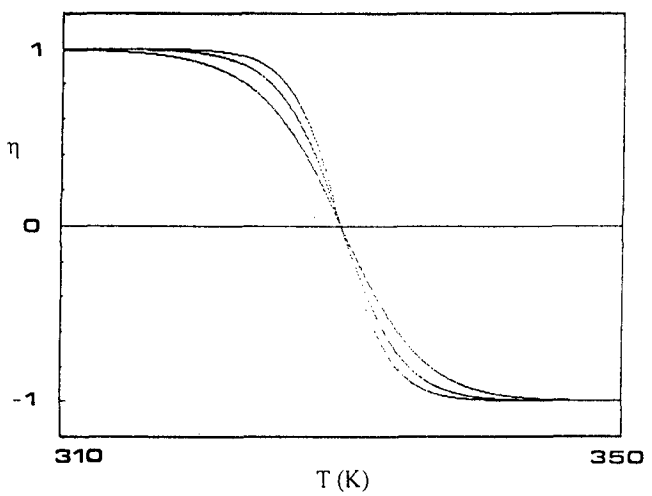

Fig. 2 Temperature-dependence of the order parameter $\eta \equiv 1-2 \cdot \theta$ for the two-state transitions reported in Fig. 1. See text for further details

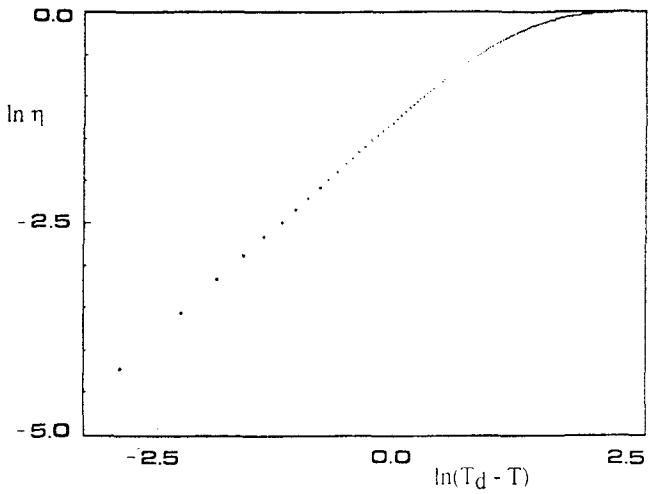

Fig. 3 Plot of ln η *vs.* $\ln(T_d-T)$ for the two-state transition of Fig. 1, characterized by $\Delta_d H^\circ(T_d) = 500$ kJ/mol. By performing a linear least squares regression on the points close to T_d, the value of critical exponent β is readily determined

mental for the protection of active sites against thermal fluctuations and to assure the stability of native structure. The experimental investigations have clearly shown that the native structure is always marginally stable, as $\Delta_d G^\circ(298\ K)$ amounts to about 40–50 kJ/mol, corresponding to 0.4–0.5 kJ/mol per amino acid residue for a protein comprised of 100 residues. If residues could fluctuate independently from each other, each of them would occupy for large periods of time non-native conformations, because random thermal energy *RT* amounts to about 2.5 kJ/mol at room temperature. As residues cannot independently move, due to the network of interactions which link them together, the tertiary structure behaves in a strongly cooperative manner and the native active conformation is preserved from random fluctuations. The denatured state is very flexible due to the destroy of the three-dimensional network of non-covalent interactions, so that it is populated by a great number of interconverting conformations. In other words, the gradual and continuous phase change characteristic of a second-order transition would be incompatible with the stability of native structure.

According to this result, the area of the excess heat capacity function corresponds to the latent heat associated to denaturation process, and, as consequence, the entropy change also assumes a finite value, as it must be for a first-order transition. The fact that the heat capacity does not go to infinity at the transition temperature (i.e., it is not a Dirac delta function), must be ascribed to the finite dimension of these thermodynamic systems. Indeed, because a single polypeptide chain is comprised of thousands of atoms, a 'crystal mole-

cule' results very small with respect to a macroscopic crystal. The finite dimension of thermodynamic system has strong influences on the heat capacity function and on the transition temperature. A first-order transition in a small system results rounded or smeared over a range of temperatures rather than perfectly sharp. In recent years an increasing number of theoretical studies, supported by Monte Carlo simulations, are trying to shed light into this interesting field [29, 30, 45–47].

It must be stressed that the whole developed analysis is grounded on the fundamental assumption that a single protein molecule can be considered as a macroscopic thermodynamic system. This assumption is practically equivalent to the hypothesis that a globular protein is a 'crystal molecule'. As just pointed out, a 'crystal molecule' is a too small thermodynamic system to have very sharp peaks for the distribution functions of physico-chemical observables. The finite and small number of particles makes possible thermal fluctuations of great amplitude around the average values. Indeed fast techniques, such as hydrogen exchange, fluorescence quenching and NMR relaxation measurements, being able to probe these fluctuations, lend support to a liquid-like view of globular proteins in solution. However, it must be stressed that is the general thermodynamics to require that in a small system the fluctuations around the equilibrium values of physico-chemical observables must be large [48, 49]. Thus, the proved existence of these fluctuations does result perfectly compatible with the solid-like nature of small globular proteins, emphasized throughout all this work.

In conclusion, a very large number of thermodynamic investigations of globular proteins in aqueous solution has clearly shown the all-or-none character of the temperature-induced reversible denaturation process. This fact has induced to visualize each protein molecule as a 'crystal molecule' that melts at raising the temperature. The mean field approach allows to state that the two-state denaturation process of small globular proteins can be assimilated to a first-order phase transition.

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Zusammenfassung — Ein allgemeines Merkmal thermisch induzierter reversibler Denaturierung kleiner Globularproteine ist deren "Alles-oder Nichts"-Charakter. Diese enge Beziehung führt zu der Annahme, daß Proteinmoleküle, die nur über zwei erreichbare thermodynamische Zustände verfügen, gleichen "Kristallmolekülen", die bei steigender Temperatur schmelzen. Eine auf der Durchschnittsfeldtheorie beruhende Analyse erlaubt den Schluß, daß die Zweistufenumwandlung eine Phasenumwandlung erster Ordnung ist. Die Bedeutung dieser Schlußfolgerung wird ausführlich erläutert.