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ON THE NATURE OF THE TEMPERATURE-INDUCED TRANSITION FROM THE MOLTEN GLOBULE TO THE UNFOLDED STATE OF GLOBULAR PROTEINS

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Abstract

In this paper we try to perform a thermodynamic analysis of the temperature-induced transition from the molten globule to the unfolded state of globular proteins. A series of calorimetric investigations showed that this process is not associated with an excess heat capacity absorption peak, and cannot be regarded as a first-order phase transition. This result contrasts with the well-established conclusion that the thermal unfolding of the native tertiary structure of globular proteins is a first-order phase transition. First, the theoretical approach developed by Ikegami is outlined to emphasize that a second-order or gradual transition induced by temperature is expected for globular proteins when the various secondary structure elements do not interact cooperatively. Secondly, a simple thermodynamic model is presented which, taking into account the independence of the secondary structure elements among each other, is able to rationalize the shape of the experimental DSC profiles.

Keywords: differential scanning calorimetry, globular proteins, molten globule state, phase transition

Introduction

In recent years several studies revealed the existence, under specified conditions, of stable partially unfolded states between the native and unfolded conformations of several globular proteins, as excellently reviewed by Kuwajima and Ptitsyn [1–4]. Such partially unfolded states have common properties: a) the molecule is compact relative to the unfolded form, as measured by intrinsic viscosity and rotational relaxation (i.e., the hydrodynamic radius is only 10–20% larger than that of the native molecule); b) it contains a significant amount of secondary structure, as indicated by far-UV CD spectra; c) it does not possess a unique tertiary structure because the side-chains are largely flexible, as indicated by NMR chemical shifts, and by near-UV CD spectra of aromatic side-chains. These partially unfolded states are collectively called the molten globule state [5]. Investigations by means of NMR tech-

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niques and H/D exchange methods have clarified the location of secondary structures and their dynamics in the molten globule state of several proteins [6, 7]. Solution X-ray scattering afforded complementary information on the size and shape of the molten globule state [8]. In addition, kinetic studies revealed that the molten globule is an important kinetic intermediate in the process of protein folding [9]. In particular, Kuwajima [1] showed that the acid form of bovine α-lactalbumin, also known nowadays as the equilibrium molten globule state, appears to be the same as, or very similar to, the kinetic refolding intermediate that is rapidly formed in folding at neutral pH. It is important to remark that for some globular proteins the molten globule is the equilibrium stable state at room temperature and acid pH or in mild denaturing conditions, and, according to Ptitsyn [3], should be regarded as a general thermodynamic state of globular proteins.

However, despite its relevance, the thermodynamic mechanism determining the conformational stability of the molten globule has not been clarified and remains a controversial subject. Thermodynamic measurements, especially differential scanning calorimetry, DSC studies, are vital for understanding the molten globule energetics, but their interpretation is not straightforward. The temperature-induced transition of the molten globule state of α-lactalbumin, investigated by means of careful DSC measurements, proved to be a gradual process, with no latent heat associated, which cannot be regarded as a first-order phase transition. This conclusion was reached independently by Pfeil *et al.* [10] for human α-lactalbumin at pH 2.0, 50 mM KCl–HCl buffer, and by Kuwajima and colleagues [11] for bovine α-lactalbumin at pH 7.6, 10 mM borate buffer containing 1 mM EGTA (i.e., in both these experimental conditions α-lactalbumin is in the molten globule state). On the other hand, Freire and colleagues [12, 13] claimed that the destruction of the molten globule state of α-lactalbumin occurs over a wide temperature range in a rather non-cooperative fashion. According to the X-ray structure, α-lactalbumin is a two-domain protein. The α-helical domain contains all four α-helices in α-lactalbumin, whereas the β-sheet domain contains a small antiparallel β-sheet and several looplike structures. In order to clarify the matter, Peng and Kim [14] dissected human α-lactalbumin to produce a molecule that consists only of the α-helical domain, called α-Domain, and should contain two partially intact α-helices in the molten globule state, according to NMR data on the intact α-lactalbumin [15]. As expected, α-Domain resembled the molten globule state of α-lactalbumin and did not show a cooperative thermal transition [14].

A gradual and non-cooperative thermal transition is also shown by the molten globules of the F2-fragment of E.coli tryptophan synthase [16], bovine carbonic anhydrase B [17] and serum retinol binding protein [18], because the transition enthalpy amounts to few dozens of kJ per mole of protein.

In addition, Griko and Privalov [19] found that the DSC profiles associated with the thermal unfolding of the molten globule state of sperm whale apomyoglobin at pH 3.5 and 4.0 did not show an excess heat capacity absorption peak, but had a sigmoidal shape. On this basis, the authors proposed that the temperature-induced unfolding of the apomyoglobin molten globule has to be regarded as a second-order phase transition, according to the classical Ehrenfest's classification. In contrast,

Goto and co-workers [20] found that the molten globule state of apomyoglobin, stabilized by high ionic strength, showed an excess heat capacity peak, resembling the classical two-state transition. Privalov [21], however, suggested the existence of a heterogeneous mixture of apo-myoglobin molecules in native and molten globule states in the presence of salts at acidic pH (i.e., the experimental conditions investigated by Goto and co-workers), to explain the occurrence of a defined DSC peak.

The situation is further complicated by the circumstance that any non-native conformation tends to be considered a molten globule state, as well evidenced by the work of Privalov and colleagues [22] on a derivative of staphylococcal nuclease lacking thirteen residues at the C-terminal end. Finally, it is to be remembered that, to evaluate the order of a phase transition, direct thermodynamic measurements of heat capacity are needed, because the sigmoidal shape of some physical observable cannot be taken as the proof of a two-state transition [23, 24], especially in a system of finite dimension (i.e., the number of atoms in a globular protein is far from infinity).

In order to proceed our investigations on the thermodynamics of globular protein conformational transitions [25–29], we develop an analysis of the temperature-induced transition from the molten globule to the unfolded state. The choice of models is largely dictated by the nature of the problem to be addressed. Theoretical models, based on statistical mechanics, should provide useful and reliable results, but it is necessary to use simplified representations of the polypeptide chain to adequately sample the huge conformational space accessible to the system. First of all, it is pointed out that, according to the mean-field statistical thermodynamic model of Ikegami [30, 31], a second-order thermal transition is expected for globular proteins when the cooperative interactions among the different secondary structure elements are largely reduced. The reliability of Ikegami's model in describing the stability of globular proteins has recently been supported by us in relation to the existence and physical origin of cold denaturation [29]. Secondly, it is shown that a simple thermodynamic model, inspired to the hierarchical cooperative model developed by Freire [13], can qualitatively account for the shape of the DSC profiles recorded for the thermal transition from the molten globule to the unfolded state in the experimentally studied cases.

The statistical thermodynamic model of Ikegami

Ikegami [30, 31] developed a mean-field statistical thermodynamic model of protein structure and stability, according to which the canonical partition function of the system is given by:

$$Q(N_b,T) = (N_o!\omega^{N_u}/N_u!N_b!)\cdot\exp([-N_u\varepsilon - (N_bN_uZJ/N_o)]/RT) \quad (1)$$

where N_o represents the number of non-covalent bonds which connect the system and are uniformly arranged on a lattice that is topologically similar to their distribution in the native structure; N_b is the number of intact bonds and N_u is the number of broken bonds. The ε parameter represents the energy required to break one bond; J

is the energy loss when two nearest neighbour bonds are not in the same state, assuming that each bond is surrounded by a number of nearest neighbours equal to Z (i.e., this term accounts for the characteristic cooperativity of the native structure of globular proteins). The ω parameter represents the increase in conformational degrees of freedom associated with the breaking of one bond. Finally, the term $(N_o!/N_u!N_b!)$ accounts for the number of modes to distribute over a lattice of N_o sites, N_b intact bonds and N_u broken bonds. Clearly, the partition function is based on the assumption that the state of the system with all bonds intact is the reference state with zero energy and a statistical weight equal to 1. The Gibbs energy of the system can be calculated with a general relationship of statistical mechanics, by considering that for a condensed phase at ordinary pressure the term PV is negligible and the Gibbs energy practically corresponds to the Helmholtz energy of the system:

$$G(N_b,T) \approx A(N_b,T) = -RT\ln Q(N_b,T) \tag{2}$$

which gives:

$$G(N_b,T) = N_u\varepsilon + (N_bN_uZJ/N_o) - N_u\alpha T - RT\ln(N_o!/N_u!N_b!) \tag{3}$$

where $\alpha \equiv R\ln\omega$ represents the conformational entropy gain associated with the breaking of one bond. In order to render Eq. (3) more amenable to direct calculation, it is possible to introduce a quasi-continuous variable $X=2(N_b/N_o)-1$, corresponding to an 'order parameter' of the structure: X is equal to -1 when all bonds are broken, and +1 when all bonds are intact. Accordingly, the Gibbs energy becomes:

$$G(X,T) = N_o\varepsilon[(1-X)/2] + N_oZJ[(1-X^2)/4] - N_o\alpha T[(1-X)/2] + N_oRT\,F \tag{4}$$

where $F=\{[(1+X)/2]\ln[(1+X)/2]+[(1-X)/2]\ln[(1-X)/2]\}$. Equation (4) corresponds to the Gibbs energy function expressed according to Landau's theory of phase transitions [32]. In order to select the stable states of the system, it is necessary to evaluate the derivative of G with respect to X, the order parameter of the system:

$$(\partial G/\partial X)_T = -(N_o\varepsilon/2) - (N_oZJX/2) + (N_o\alpha T/2) + N_oRT(\mathrm{d}F/\mathrm{d}X) = 0 \tag{5}$$

By eliminating the common factor $(N_o/2)$, dividing all the expression for ZJ and defining $A \equiv \varepsilon/ZJ$, $B \equiv 2R/ZJ$ and $C \equiv \alpha/2R$, Eq. (5) becomes:

$$-A-X+CBT+BT\,(\mathrm{d}F/\mathrm{d}X)=0 \tag{6}$$

which gives:

$$[(A+X)/BT]-C=(\mathrm{d}F/\mathrm{d}X)=(1/2)\ln[(1+X)/(1-X)]=\tanh^{-1}X \tag{7}$$

The solutions of this equation are determined from the intersection of the straight line $X=BT\kappa+(CBT-A)$ and the hyperbolic tangent curve $X=\tanh\kappa$, where κ represents a dummy variable for the order parameter. Ikegami showed that the transition temperature is given by $T_{tr}=A/CB$ and that the order of the transition is determined

by the ratio A/C [30, 31]. It results that when $A<C$ the straight line intersects $\tanh\kappa$ in three distinct points: near the transition temperature the Gibbs energy has two minima corresponding to the ordered and disordered states in equilibrium, separated by an energy barrier, and the transition is first-order. On the other hand, when $A>C$ the straight line intersects $\tanh\kappa$ in only one point: the Gibbs energy has only one minimum shifting gradually on increasing temperature from ordered states to disordered ones and the transition is second-order or gradual (i.e., there are not two states coexisting at equilibrium, separated by an energy barrier). In such cases, when the temperature increases, the order parameter X changes in a continuous manner from positive values (ordered states) to negative ones (disordered states).

By keeping fixed the values of ε and α, it results that $A<C$ when ZJ is large, whereas $A>C$ when ZJ is small. In other words, within Ikegami's model, the phase transition changes 'qualitatively' from first-order to second-order when the cooperative interactions among the non-covalent bonds connecting the system decrease, while the energy and entropy of a single bond remain constant. Specifically, the parameter ZJ has to pass from a large value to a small one. This situation corresponds to the current idea of the molten globule state; in fact, the residual secondary structure elements do not give rise to a unique folding pattern. They are arranged in a globular shape, but the cooperative long-range interactions among each other, which stabilize the native structure, are largely lost. The loosening of cooperative interactions causes a marked modification of the behavior of the system: the phase transition from first-order becomes second-order. Therefore, Ikegami's model suggests that the temperature-induced transition from the molten globule to the unfolded state should resemble a second-order phase transition.

This conclusion is in line with the results of a general and careful statistical thermodynamic analysis performed by Lifshitz *et al.* [33]. These authors showed that the collapse of a homopolymer from the random coil state into a compact globule without a unique structure (i.e., what may be called a molten globule), is expected to be a second-order phase transition in the thermodynamic limit (i.e., when the number of particles in the system tends to infinity), due to the absence of specific long-range interactions. In addition, Shakhnovich and colleagues [34, 35] demonstrated, by both a mean-field heteropolymer theory and Monte Carlo simulations on lattice models of globular proteins, that a first-order transition occurs only when non-local contacts predominate.

A simple thermodynamic model

Experimental data suggest that the molten globule can be considered representative of a large number of compact denatured conformations populated by protein molecules. These states can be characterized by the presence of several secondary structure elements still in native conformation, whereas the remaining parts of the polypeptide chain are already unfolded. In order to develop a simple thermodynamic model, suitable to describe the temperature-induced transition from the molten globule to the unfolded state, we assume that each secondary structure element

possesses a local cooperativity: it can exist only in the native or unfolded conformations. This assumption is not strictly correct because it is well established that, for example, the unfolding of an α-helix is not a two-state process [36], but it is a very useful working hypothesis to simplify the mathematical treatment. Moreover, we consider that the Gibbs energy difference between the native and unfolded conformations is equal for all the secondary structure elements, and, since the latter ones have small dimensions (i.e., at most 10–20 residues), it is indicated by δG. The canonical partition function for each secondary structure element, considering the native conformation as reference, is given by [25]:

$$q(T) = 1 + \exp(-\delta G/RT) = 1 + \exp[-(\delta H/R)(1/T - 1/T_o)] \quad (8)$$

where δH is the enthalpy difference between the unfolded and native conformations and T_o is the temperature at which the two conformations are equally populated.

As the molten globule state does not possess a rigid tertiary structure, it is reliable to assume that each element of secondary structure behaves independently from the others. Therefore, if the molten globule has N secondary structure elements, the overall unfolding process from molten globule to the completely unfolded state, indicated as a random coil, can be represented as:

$$MG(N) \leftrightarrow N\text{–}1 \leftrightarrow N\text{–}2 \leftrightarrow N\text{–}3 \leftrightarrow \qquad \leftrightarrow 1 \leftrightarrow RC \quad (9)$$

where MG stands for molten globule with N elements of secondary structure intact, and RC stands for random coil, the statistical ensemble of completely unfolded conformations with no residual secondary structure. Increasing the temperature, the distribution of protein molecules progressively shifts toward conformations having fewer elements of residual structure until a temperature, in which the random coil is the predominant thermodynamic state, is reached. It is worth noting that this model is independent of any folding mechanism and does not imply that secondary structure elements are the initial structures in the folding process. The corresponding overall partition function is given by:

$$Q_{MG}(T) = q^N = [1 + \exp(-\delta G/RT)]^N \quad (10)$$

It is not necessary to divide by $N!$ because the secondary structure elements are distinguishable in a polypeptide chain. The molten globule with all the N secondary structure elements intact is the reference state and has a statistical weight equal to 1. On the other hand, a state with i secondary structure elements unfolded has a Gibbs energy $i\delta G$ times higher than the reference state and its statistical weight is:

$$[N!/i!(N\text{–}i)!] \exp(-i\delta G/RT) \quad (11)$$

to correctly account for the number of modes in which it is possible to have i secondary structure elements unfolded over a total of N. It is clear that Eq. (10) is the closed form of the binomial expansion:

$$Q_{MG}(T) = \sum [N!/i!(N-i)!] \exp(-i\delta G/RT) \quad (12)$$

The summation extends from i=0 to i=N, where N is the total number of secondary structure elements which maintain their native conformation in the molten globule. It is to be noted that, when N is large, the assumption of an equal δG value for all the secondary structure elements, is the most conservative and less arbitrary, since the transition from *MG* to *RC* is a very broad process with no indication of dominant states [10–19]. The excess enthalpy function is given by the following relation of statistical mechanics:

$$<\Delta H(T)>_{MG} = RT^2(\mathrm{d}\ln Q_{MG}/\mathrm{d}T) \quad (13)$$

which results in:

$$<\Delta H(T)>_{MG} = N\delta H \exp(-\delta G/RT)/[1 + \exp(-\delta G/RT)] \quad (14)$$

The excess heat capacity function, which is the physical observable in DSC measurements, is the temperature derivative of the excess enthalpy function:

$$<\Delta C_p(T)>_{MG} = (\mathrm{d}<\Delta H_{MG}/\mathrm{d}T) \quad (15)$$

which gives:

$$<\Delta C_p(T)>_{MG} = N(\delta H^2/RT^2)(\exp(-\delta G/RT)/[1 + \exp(-\delta G/RT)]^2) \quad (16)$$

The model, according to Eq. (16), allows the calculation of DSC curves modeling the temperature-induced transition from molten globule to random coil using as input parameters the values of T_o, δH and N. It may be useful to remind that T_o is the temperature at which the two conformations of each secondary structure element are equally populated, δH is the enthalpy difference between these two conformations and N is the number of secondary structure elements. A DSC curve calculated with T_o=66.9°C, δH=40 kJ mol^{-1} and N=10 is reported in Fig. 1 (curve a), together with a curve calculated by fixing T_o=66.9°C, δH=400 kJ mol^{-1} and N=1(see curve b of

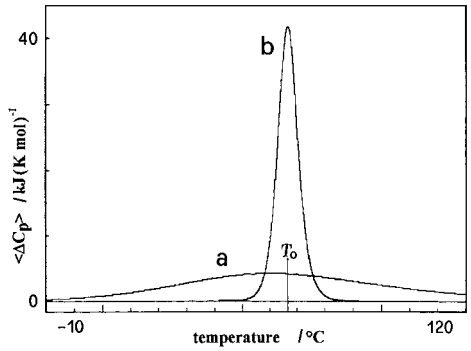

Fig. 1 DSC profiles calculated according to Eq. (16) with the following parameters: N=10, T_o=66.9°C, and δH=40 kJ mol^{-1} (curve a); N=l, T_o=66.9°C, and δH=400 kJ mol^{-1}, which correspond to a simple two-state N ↔ D transition with T_d=66.9°C, and $\Delta_d H(T_d)$=400 kJ mol^{-1} (curve b)

Fig. 1). The latter curve corresponds to a simple two-state N ↔ D transition, and the selected parameters are average values for the transition from the native to the unfolded state of small globular proteins [37, 38]. Clearly, Eq. (16) when N=1 corresponds to the well-known equation valid for a two-state N ↔ D transition [25]. It is clear that curve a represents a non-cooperative process, which occurs over a very large temperature range (i.e., more than 100°C) even though the total enthalpy change is equal for both curves a and b. Moreover, curve a shows a maximum of about 5.0 kJ $K^{-1}mol^{-1}$, a very low figure with respect to the values associated with the two-state transition from the native structure to the completely unfolded one of small globular proteins, as exemplified in curve b.

In addition, the complete unfolding of the molten globule state is accompanied by the exposure to water of nonpolar groups buried in the interior of the globule and this gives rise to a positive contribution to the heat capacity, indicated in its totality by δC_p. The expression of the excess heat capacity function becomes:

$$<\Delta C_p(T)>_{MG} = N(\delta H^2/RT^2)(\exp(-\delta G/RT) /[1 + \exp(-\delta G/RT)]^2) + \delta C_p[(q - 1)/q] \quad (17)$$

where the ratio $(q-1)/q$ represents the advancement degree of the overall process, described by Eq. (9), as a function of temperature. A DSC curve calculated by means of Eq. (17) with T_o=66.9°C, δH=40 kJ mol^{-1}, δC_p =1.5 kJ $K^{-1}mol^{-1}$ and N=10 is reported in Fig. 2 (curve a), together with a two-state transition curve calculated with T_d=66.9°C, $\Delta_d H(T_d)$= 400 kJ mol^{-1} and $\Delta_d C_p$=6.0 kJ $K^{-1}mol^{-1}$ (see curve b of Fig. 2).

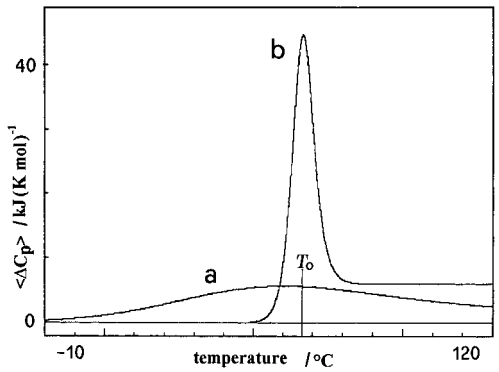

Fig. 2 DSC profiles calculated according to Eq. (17) with the following parameters: N=10, T_o=66.9°C, δH=40 kJ mol^{-1}, and δC_p=1.5 kJ $K^{-1}mol^{-1}$ (curve a); N=1, T_o=66.9°C, δH=400 kJ mol^{-1}, and δC_p=6.0 kJ $K^{-1}mol^{-1}$, which correspond to a simple two-state N ↔ D transition with T_d=66.9°C, $\Delta_d H(T_d)$=400 kJ mol^{-1}, and $\Delta_d C_p$=6.0 kJ $K^{-1}mol^{-1}$ (curve b)

The difference between curves a and b in both Figs 1 and 2 is striking and has important consequences. The temperature range covered by DSC instruments is not large enough to record completely such non-cooperative processes: the experimental DSC profile will contain only part of the process, rendering the analysis very prob-

lematic. In addition, even in favorable cases, the procedure usually employed to analyze raw DSC data [39] does not allow the calculation of reliable thermodynamic parameters from curve a of both Figs 1 and 2. In fact, it is very difficult, if even possible, to define correct pre- and post-transition baselines and so to calculate the area under the curve. Such area would be the transition enthalpy, but, in view of the large temperature range involved, its physical meaning is not well defined. Actually, a large number of conformational states separated by small differences in Gibbs energy exist between molten globule and random coil, and, on raising the temperature, the excess enthalpy function increases in a continuous manner, without abrupt jumps.

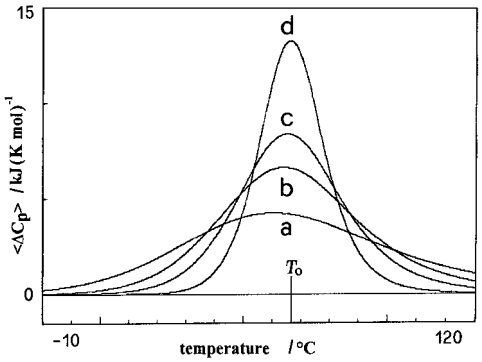

Fig. 3 A series of DSC profiles calculated according to Eq. (16) with the following parameters: N=10, T_0=66.9°C and δH=40 kJ mol^{-1} (curve a); N=7, T_0=66.9°C and δH=60 kJ mol^{-1} (curve b); N=5, T_0=66.9°C and δH=80 kJ mol^{-1} (curve c); N=3, T_0=66.9°C and δH=130 kJ mol^{-1} (curve d)

However, it has to be noted that, by lowering N and increasing the value of δH, a visible DSC peak occurs. In Fig. 3 is reported a series of DSC profiles calculated according to Eq. (16) with the following parameters: N=10, T_0=66.9°C and δH=40 kJ mol^{-1} (curve a); N=7, T_0=66.9°C and δH=60 kJ mol^{-1} (curve b); N=5, T_0=66.9°C and δH=80 kJ mol^{-1} (curve c); N=3, T_0=66.9°C and δH=130 kJ mol^{-1} (curve d). Clearly, the height of the peak increases on raising the value of δH, whereas the maximum occurs at temperatures lower than T_0 when δH is very small (i.e., these are peculiar features of the two-state model). In any case, also for N=3 and δH=130 kJ mol^{-1} the process is poorly cooperative (i.e., the heat absorption peak extends over about 80°C), and does not represent a first-order phase transition. As different proteins will have a different number of residual secondary structure elements in the molten globule state, it is possible to obtain experimentally DSC profiles corresponding to curve a (i.e., α-lactalbumin, apo-myoglobin at very low ionic strength), or to curve d (i.e., carbonic anhydrase B, apo-myoglobin at high ionic strength), or intermediate.

In general, the absence of a cooperative all-or-none transition is due to the absence of cooperative non-local interactions between the secondary structure elements constituting the molten globule. Their independence affects the nature of the transition, as already evidenced in the framework of Ikegami's model. Results from compressibility measurements support the independence of the secondary structure

elements in the molten globule. Kharakoz and Bychkova [40] found that the molten globule interior of human α-lactalbumin is filled up with about 270 water molecules, a figure close to the number of internal polar atoms in the protein. They suggested that probably the interior of all the molten globules is highly hydrated and a large part of structural fluctuations may be determined by the process of water exchange between the molten globule and the bulk solvent.

The high content of water inside the molten globule is compatible with the hypothesis of a 'non-uniform' expansion of native structure advanced by Ptitsyn [2], and supported by the finding that the packing density of protein interior is far from uniform. It implies that those secondary structure regions that carry nonpolar side-chains constituting the protein hydrophobic core are more or less preserved in the molten globule, whereas the rest of the molecule is unfolded and accessible to water molecules. In such a situation the packing interactions are largely lost or strongly reduced as a consequence of the distance increase. The globular shape is stabilized by loose hydrophobic interactions, similar to those existing in micelles. Furthermore, the loss of packing interactions causes an increase in the conformational degrees of freedom of both side-chains and backbone, and the overall structure of the molten globule is largely fluctuating. Thus, being small the difference between the energy levels of the various conformational states involved, the heat effect associated with the transition from molten globule to random coil is very low. From the physicochemical point of view, it can be stated that the energy required to break down the residual independent secondary structure elements and the loose hydrophobic interactions in the globule's interior is almost counterbalanced by the energy gained, mainly by polar peptide groups, in contacting water.

The pattern of the excess heat capacity function obtained with Eqs (16) and (17) well resembles the profiles obtained in the experimentally investigated cases. Therefore, the simple thermodynamic model developed seems to account for the main features of the DSC profiles experimentally obtained for the temperature-induced transition from the compact denatured molten globule to the completely unfolded state.

Discussion

Accurate calculations by Chothia's group [41] of packing densities for residues buried in the interior of native proteins showed that their mean volume is equal or even smaller than that in crystals of their amino acid. The packing density of native globular proteins is similar or even higher than that of organic solids because the different side-chains fit together like pieces of a three-dimensional jigsaw puzzle [42, 43]. This validates Liquori's suggestion [44] that globular proteins can be considered as 'crystal molecules'. The solid-like nature of protein interior is also confirmed by calculations of the Gibbs energy cost to create a cavity in the native protein core, performed by Lee and colleagues [45]. As a consequence, their internal design is dominated by close packing which allows the establishment of a three-dimensional network of van der Waals interactions among residues far apart in the sequence but close in space. In addition, each segment of the polypeptide chain usually folds to form α-helices or a strand in a β-sheet, and tertiary structures are mainly defined by the interactions among the component secondary structures. Chothia [46] showed

that close packing is the dominant factor also in determining the interactions of α-helices and β-sheets. Indeed simple geometrical models, such as the 'ridges into grooves' model for helix-helix interaction, and the 'complementary twist' model for helix-sheet interaction, are able to describe the packings observed in native protein structures. Clearly, when such close packing is strongly reduced, as in the molten globule, the three-dimensional network of van der Waals interactions is lost. The residual secondary structure elements are assembled together by loose hydrophobic interactions, useful to preserve a compact globular shape, but unable to stabilize a unique conformation, as evidenced by NMR measurements [6, 7]. In such conditions the protein molecule is not a cooperative domain and its thermal unfolding is a gradual process, resembling a second-order phase transition.

According to the theoretical investigations of Finkelstein, Shakhnovich and Karplus [47–49], the main physical reason for the first-order transition from the native structure to the unfolded state is the unfreezing of side-chains due to the breakage of the specific network of non-covalent interactions in the close packed core of globular proteins. Furthermore, these authors concluded that the transition from the native structure to the molten globule has to be a first-order process, whereas the transitions among denatured states with different compactness have to be second-order. Loosely speaking, the destruction of native structure corresponds to a 'melting' process, while the unfolding of molten globules corresponds to a 'swelling' process (i.e., molten globules are normal polymers whose dimensions and compactness vary significantly when the external conditions change from those characteristic of a 'poor' solvent to those of a 'good' solvent).

Actually, Uversky and Ptitsyn [50] have recently claimed that the denaturant-induced transition from molten globule to completely unfolded state at constant temperature corresponds to a first-order phase transition, because the molecule undergoes the transition as a whole. However, the reliability of the data used by Uversky and Ptitsyn is unclear; they used the number of denaturant molecules bound to the unfolded state in excess with respect to the molten globule, a very difficult quantity to calculate unambiguously [51]. Therefore, their conclusion cannot be taken as a convincing proof. Furthermore, their analysis refers to processes at constant temperature, whereas the present work tries to characterize the transition induced by temperature. In this respect the following point assumes great importance.

Attempts have been made to design *de novo* proteins for a number of typical fold topologies [52, 53]. The synthesized proteins proved to be stable and globular, with pronounced secondary structures. However, these artificial globules did not have unique tertiary structures, as the close packing of side-chains was lacking. Therefore, designed proteins form globules with features similar to natural proteins in their molten globule states. Cooperative transitions at constant temperature induced by urea or guanidine chloride have been observed for *de novo* designed proteins, whereas cooperative thermal transitions have never been observed, with only one exception. Nakamura and co-workers [54] designed a β / α-barrel protein by taking advantage of the protocol devised by Finkelstein and Nakamura [55] to improve close packing in the protein core. Such *de novo* protein showed a cooperative thermal transition in DSC measurements, even though the enthalpy change per residue was only 1/3 of that observed for natural proteins [37, 38].

In conclusion, only the native tertiary structure of natural proteins behaves in a strongly cooperative manner, due to the existence of a complete and intricate three-dimensional network of non-covalent interactions. This property is closely related to the uniqueness of native protein structure, firmly demonstrated by numerous X-ray and NMR studies, although the physical reason of this general fact is still rather elusive.

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