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Protein heat capacity: inconsistencies in the current view of cold denaturation

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Abstract

The present study shows on the basis of the thermodynamic stability criterion $(\partial S/\partial T)_p > 0$ that partitioning of the entropy of cold-unfolding of a protein into independent positive conformational and negative hydrational contributions is incorrect. Furthermore it provides a new microscopic interpretation of protein heat capacity that takes into account the significant fluctuations in energy and entropy which result from the small size of these macromolecules.

Keywords: DSC; Cold denaturation; Hydration entropy; Second law; Independent and coupled processes; Fluctuations; Enthalpy partitioning

1. Introduction

Classical equilibrium thermodynamics has been applied with great success to the description of protein stability and to its modulation by intrinsic mutational changes and extrinsic solution conditions such as pH, temperature, specific ligands and unspecific stabilizing and denaturing agents [1–18].

These studies have led to an increasingly sophisticated understanding of the molecular nature of the stabilizing interactions that maintain the native structure of proteins. Pivotal in the quantification of the thermodynamic properties of proteins, and biopolymers in general, was the advent of highly sensitive scanning microcalorimeters that have permitted accurate determination of the heat capacity of dilute solutions of these macromolecules. The thermodynamic parameters such as transition enthalpies, entropies and Gibbs energies derived from these studies have been interpreted intuitively in structural terms on the basis of X-ray data. This means that it has been tacitly assumed that any changes in the macroscopic, thermodynamic properties reflect also changes in the intrinsic interactions within the individual single macromolecule. Such an assumption is by no means trivial. All thermodynamic quantities are essentially ensemble averages and the step from the ensemble property to

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the molecular property requires some fundamental considerations of statistical thermodynamics. Recently, we performed a study that provides a rigorous statistical mechanical link between the average experimental heat capacity and the corresponding property of the single protein moiety [19].

This has made it possible now to discuss changes in the macroscopic thermodynamic properties in terms of their relation to changes in the individual protein molecules. A particularly intriguing question in this context is the molecular interpretation of the observed increase in protein heat capacity on cold denaturation. The phenomenon has been interpreted frequently in terms of the assumption that denaturation of the native structure of the protein results in a positive conformational entropy change of the solvent-free polypeptide chain and a negative entropy change due to hydration of the unfolded chain. The increasing degree of water structuring around the unfolded chain with a decrease in temperature, coupled with a release of entropy was held responsible for the positive heat capacity change on unfolding at low temperature. This mechanism is just the reverse of the situation postulated for the processes occurring during high temperature unfolding. The negative hydrational entropy contribution had to be assumed to be larger in magnitude than the positive conformational contribution of the chain in order to be compatible with the experimental result which clearly shows that cold denaturation is associated with an overall negative entropy change and a positive heat capacity change.

In the following sections we will challenge the view that the observed entropy change can be partitioned in this manner by demonstrating that this leads to a conflict with the second law of thermodynamics. Instead we will suggest a new approach based on the idea of equilibrium fluctuations, that provides a 'natural' explanation of the heat capacity changes observed in both cold and heat denaturation.

2. Experimental basis of cold unfolding

Fig. 1a shows the variation of the heat capacity of myoglobin as a function of temperature. One

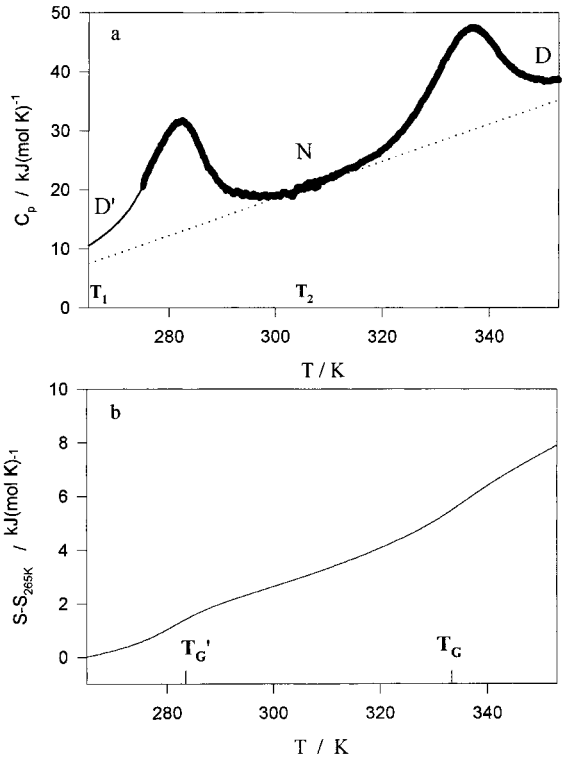

Fig. 1. Variation with temperature of the heat capacity and entropy of myoglobin. (a) Temperature dependence of the heat capacity of myoglobin. Experimental conditions: 8.5 mg/ml; pH = 4.35; buffer: 0.1 NaAc; heating rate: 0.2 K/min (cold-den.); 0.5 K/min (heat-den.). The bold curve shows the experimental heat capacity curve. The extension of the experimental curve below 274 K has been calculated by using a two-state approximation for the conformational transition, employing the following parameters: $T_G = 333.3$ K, $T_G' = 283.5$ K, $\Delta H(T_G) = 212$ kJ/mol, $\Delta c_p\,(T_G) = 6.8$ kJ/mol. The dotted line refers to the heat capacity of the native state. D′, N and D refer to the cold-denatured, native and heat-denatured state, respevtively. (b) Temperature dependence of the entropy of myoglobin. The entropy curve was calculated from the experimental heat capacity data shown under (a) by numerical integration. The entropy at 265 K, was set equal to zero. T_G' and T_G indicate the midpoint temperatures of cold and heat denaturation, respectively.

observes two heat capacity peaks at $T_G' = 283.5$ K and $T_G = 333.3$ K, respectively, which are characteristic of the so-called cold- and heat-unfolding of the protein. Numerical integration of the heat capacity curve according to the equation $S(T) - S(265) = \int_{265}^{T} c_p \mathrm{d}\ln T$ results in the variation with temperature of the entropy of the protein shown

in Fig. 1b. It is important to note that the entropy is a monotonically increasing function of temperature. This is equivalent to saying that the slope of the curve, given by dS/dT, is always positive. The rate of increase is largest in the transition ranges. The midpoints of these ranges are marked by the transition temperatures T_G' and T_G. The symbols D′ and D refer to the low and high temperature-unfolded states of the protein, respectively, and N denotes the folded native state. The entropy of the protein relative to the native state, $S - S_N$, is shown in Fig. 2 as a function of temperature. This diagram illustrates particularly clearly that the entropy of the cold-unfolded state of the protein is lower than that of the native state, while that of the heat-unfolded state is higher than that of the native state. The molar transition entropy, $\Delta_N^D S(T_G')$, which refers to the hypothetical isothermal cold unfolding of the protein at T_G', is marked by the arrow. The direction of the arrow indicates that the entropy of the protein must decrease on cold unfolding. It is important to emphasise at this point, that $\Delta_N^D S(T_G')$, or an equivalent transition enthalpy $\Delta_N^D H(T_G')$, are the thermodynamic quantities that correspond to a hypothetical, synchronous transition of 1 mol of protein molecules from the native to the unfolded state at T_G'. As long as the environmental variables such as solution composition, pH, pressure, etc., are kept constant, such an isothermal transition can not occur under reversible conditions. Reversible cold denaturation becomes measurable by decreasing the temperature and, concomitantly, removing entropy from the system. To avoid any misunderstanding, it should be noted at this point that the entropy change of the protein (as a thermodynamic system) can, of course, be determined isothermally under irreversible conditions. A possible experimental scenario could be the following. A protein solution could be mixed with a cold buffer solution so that the final temperature of the mixture, T_F, would assume a value well below T_G'. This would guarantee completion of cold denaturation and permit the determination of the cold unfolding entropy at T_F via the observed enthalpy, if proper controls are performed. It is important to realize, however, that the entropy value obtained in this manner has no direct relation to the actual entropy and heat capacity curves of the protein,

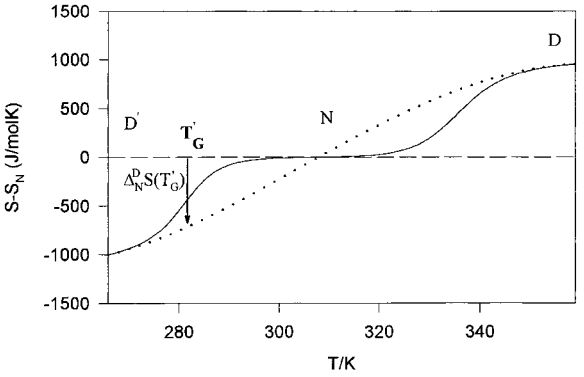

Fig. 2. Variation with temperature of the entropy difference $(S - S_N)$ of myoglobin. The solid curve shows the entropy, $(S - S_N)$, of myoglobin relative to the native state as a function of temperature. In this graph the dashed horizontal line represents the entropy of the native state, and the dotted line refers to the variation with temperature of the entropy of the denatured state relative to the native state $(S_D - S_N)$. It has been calculated using a second and first order polynomial respectively for the temperature dependence of the heat capacity of the denatured and native state. $c_{p,den}(T_G)$ and $c_{p,nat}(T_G)$ are the heat capacity values of the denatured and native state at the transition temperature $T_G = 333.3$ K. $\dot{c}_{p,den}(T_G)$ and $\ddot{c}_{p,den}(T_G)$ are the first and second derivatives of the heat capacity function at T_G. The equations are given below.

$$c_{p,den}(T) = c_{p,den}(T_G) + \dot{c}_{p,den}(T_G)(T - T_G) + \frac{1}{2}\ddot{c}_{p,den}(T_G)(T - T_G)^2;$$

$$c_{p,nat}(T) = c_{p,nat}(T_G) + \dot{c}_{p,nat}(T_G)(T - T_G).$$

The fit parameter have the following values at T_G:

$$c_{p,den}(T_G) = 35.9 \text{ kJ / mol K}; \; c_{p,nat}(T_G) = 29.0 \text{ kJ / mol K}, \; \dot{c}_{p,den}(T_G) = 159.5 \text{ J / mol K}^2;$$

$$\dot{c}_{p,nat}(T_G) = 314.9 \text{ J / mol K}^2; \; \ddot{c}_{p,den}(T_G) = -6.24 \text{ J / mol K}^3$$

The entropy difference $S_D - S_N$ has been calculated by integration

$$S_{den}(T) - S(T)_{nat} = \int_{310}^{T} \frac{c_{p,den}(T) - c_{p,nat}(T)}{T} dT$$

The arrow in the graph indicates the negative isothermal entropy difference $\Delta_N^D S(T_G')$ at T_G' between the cold-unfolded and native state. $\Delta_N^D S$ varies with temperature and is always given by the difference between the dotted and dashed curve.

shown in Fig. 1. It is the slope dS/dT of curve 1b that determines the heat capacity of the protein at any temperature point and not the temperature dependence of the transition entropy, $d\Delta_N^D S/dT$.

The numerical value of the transition entropy, $\Delta_N^D S(T_G')$, is obtained by integration of the heat capacity curve shown in Fig. 1 between T_1 and T_2, i.e. over the transition range with the use of a proper baseline. Alternately, it can be read directly from Fig. 2 at T_G'. The quantity $\Delta_N^D S(T_G')$ is, therefore, an entropy change that is determined by integration over the temperature range $T_2 - T_1$ but assigned to a single temperature T_G'. This procedure is perfectly legitimate for the calculation of stability parameters, such as standard Gibbs energies of unfolding $\Delta_N^D G^0$. It can be very hazardous, however, if used to dissect $\Delta_N^D S(T_G')$ into entropy contributions from diverse molecular processes, with the implication that these entropies and their signs have physical meaning. Such hypothetical contributions could be, for example, the pure conformational entropy change of the polypeptide chain, $\Delta_N^D S_{conf}$, and the hydration contribution, $\Delta_N^D S_{hydr}$. It has been common practice to assume that the cold unfolding entropy, $\Delta_N^D S$, is given by the equation

$$\Delta_N^D S = \Delta_N^D S_{conf} + \Delta_N^D S_{hydr} \quad (1)$$

The assumption of additivity of the entropies implies that the two processes, namely the conformational entropy change and the hydration reaction, are completely uncoupled or, in other words, that no cross-term exists in the entropy expression of Eq. (1). Such analyses have led to the deduction that, for cold denaturation, the overall transition entropy, $\Delta_N^D S$, is composed of a positive conformational entropy change, $\Delta_N^D S_{conf}$, resulting from the loss of the native structure and an overcompensating negative entropy change, $\Delta_N^D S_{hydr}$, due to hydration of the unfolded chain [20,21]. This view is illustrated schematically in Fig. 3, where the arrows pointing down refer to negative, hydration-related entropy changes and the arrow pointing up indicates a positive conformational entropy change.

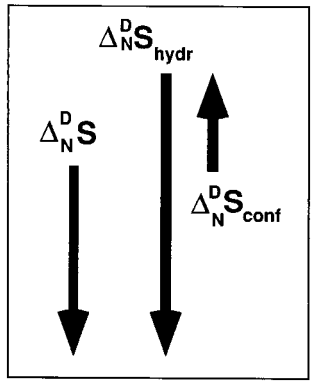

Fig. 3. Illustration of the current interpretation of the entropy changes involved in cold denaturationl. The hypothetical entropy changes, $\Delta_N^D S$, imply an isothermal phase change.

The questions arise now: is such a splitting of the measured overall entropy change, $\Delta_N^D S$, physically meaningful? and are the results consistent with the laws of thermodynamics?

It will be shown in what follows, that the assumption of a positive conformational entropy change of the polypeptide chain on cold denaturation accompanied by an independent negative entropy change resulting from hydration leads to conflicts with the second law of thermodynamics. This is easily understood if the physical basis of the heat capacity measurements is reexamined.

3. Thermodynamic implications of the temperature dependence of the entropy of the protein

Inspection of Fig. 2 shows that the folded native state, N, of myoglobin is flanked by two denatured states, D′ and D, on the low and high temperature sides, respectively. They differ from the native state by having a lower (cold-denatured) and a higher (heat-denatured) entropy. This shows that the native state can be transformed into a denatured state by either decreasing or increasing the entropy of the protein. Both processes are associated with increases in protein heat capacity in the transition region, and in both cases the heat capacity of the denatured state is higher than that of the native state. However, in order to achieve cold denaturation the entropy of the protein has to be decreased, while for heat

denaturation it has to be increased. Let us consider the process of cold denaturation more closely and ask the question: which entropy changes determine the heat capacity curve? According to the thermodynamic relationship

$$c_p = T\left(\frac{\partial S}{\partial T}\right)_P \tag{2}$$

the c_p signal at each temperature is given by the product of the absolute temperature, T, and the slope of the entropy curve at that temperature. Therefore, it is evident that the hypothetical, isothermal entropy change $\Delta_N^D S(T_G')$, represented by the arrow at T_G', has no bearing on the magnitude of the heat capacity signal.

In order to decrease the entropy of the system which, as mentioned before, we identify with a single hydrated protein molecule, we have to transfer heat from the protein via the heat bath into the surroundings. This is done experimentally in a reversible manner, so that, in the process of the temperature decrease in the DSC instrument, the protein solution is at equilibrium at every temperature. This situation is illustrated in Fig. 4 for cold denaturation. The figure shows schematically the unfolding of a single protein molecule by the withdrawal of entropy by means of decreasing the temperature. It is necessary to stress the illustrative nature of Fig. 4. It is meant to show only a few replicas of the 10^{17} protein molecules that form the canonical ensemble in the DSC cell. Native proteins are shown as circles surrounded by water and denatured proteins are represented as rectangles with shells of hydration different from those of the native protein. At any given temperature, native and denatured proteins coexist in a well defined proportion. For the moment we neglect the dynamic aspects of the equilibrium. We shall discuss them later. Here we shall concentrate only on the population changes that result from the decrease in temperature. We recognize that the entropy removed from the protein system as the temperature is decreased is the genesis of the observed positive heat capacity signal, according to Eq. (2).

It is only in the limiting case of a reversible process at temperature T that the entropy change,

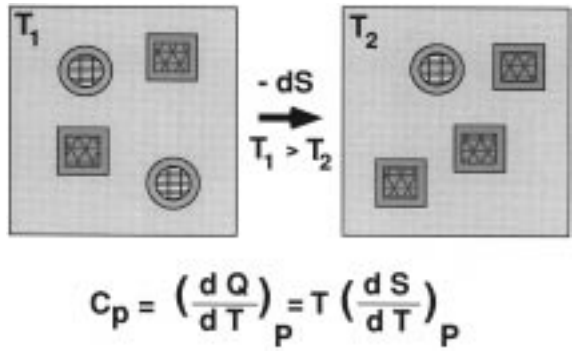

Fig. 4. Schematic illustration of the microscopic events associated with cold denaturation. The heat capacity signal arises from the withdrawal of entropy from the ensemble consisting of the hydrated native proteins (circular) and denatured proteins (rectangular) (For details, see text.)

dS, is equal to dQ/T. This is what makes possible to obtain entropy changes from heat changes. The necessary conditions are realised to good approximation in a DSC experiment.

To answer the question of whether it is permissible to partition the entropy change involved in cold denaturation into independent positive conformational and negative hydrational contributions, we have to concentrate on the infinitesimal entropy changes, dS, that have to be removed when the system is cooled reversibly along the curve shown in Fig. 1b.

In general, each infinitesimal entropy change, dS, of a system can be considered to be composed of a transfer term, $\delta S_{\text{transfer}}$, that is given by $\delta q/T$ and describes the heat exchange with the surroundings and an irreversible entropy production term, $\delta S_{\text{irreversible}}$, which represents the contributions from irreversible processes within the system [22]:

$$dS = \delta S_{\text{transfer}} + \delta S_{\text{irreversible}} \tag{3}$$

The symbol 'd' implies that dS is a property of the system, while the symbol 'δ' reminds us that both the exchanged and the produced entropies are, in general, path-dependent.

According to the second law of thermodynamics, $\delta S_{\text{irreversible}}$ is always positive. It is only in the case of reversible processes that the entropy production is equal to zero.

This is the situation which applies to the reversible cold denaturation of proteins.

Thus, for the purpose of the discussion of the reversible unfolding reactions seen in the calorimeter, Eq. (3) reduces to

$$dS = dS_{transfer} \quad (4)$$

We must conclude, therefore, that the reduction of the entropy of the protein in the process of cold denaturation is exclusively and quantitatively due to the transfer of entropy from the system into the surroundings. This is a significant conclusion since it enables us to decide whether it is possible to partition the observed entropy decrease, dS, into a positive contribution due to the unfolding of the solvent-free polypeptide chain and a negative contribution that overcompensates this positive term to such an extent that the experimental observation is the negative dS.

This scenario is illustrated in Fig. 3 for the molar transitional entropy changes. It is easy to demonstrate now that, on the basis of Eq. (4), such a splitting of the entropy is in conflict with the second law of thermodynamics. What the separation of conformational and hydrational contributions implies is the following: although the entropy of the hydrated protein is reduced by heat transfer to the surroundings, the system separates spontaneously into a high entropy part which is the unfolded chain and a low entropy part which consists of highly ordered water molecules. Since the assumption of the additivity of entropies, which is the foundation of Eq. (1), implies negligible coupling, such a splitting is a clear violation of the second law.

Another way of looking at this problem is as follows. Let us write the observed entropy change, dS, as the sum of the conformational and hydrational contributions, dS_{conf} and dS_{hydr}, respectively, and differentiate with respect to temperature. Then, we obtain the equation

$$\frac{dS}{dT} = \frac{dS_{conf}}{dT} + \frac{dS_{hydr}}{dT} \quad (5)$$

Partitioning of the observed negative entropy, dS, into positive conformational and negative hydrational terms leads to the following signs of the derivatives for the process of cold-denaturation:

The term on the left hand side of Eq. (5) is positive ($dS < 0$, $dT < 0$). The first term on the right hand side is negative ($dS_{conf} > 0$, $dT < 0$) and the second term on the right hand side is positive ($dS_{hydr} < 0$, $dT < 0$). The very fact that (dS_{conf} / dT) turns out to be negative implies that a decrease in temperature must lead to an increase in the conformational entropy of the chain, which is tantamount to saying that the polypeptide chain has a negative heat capacity $c_{p\,(chain)} = T(dS_{conf}/dT)$. A polypeptide chain with a negative heat capacity is physically impossible. This is easily understood when the definition of heat capacity is recalled. The heat capacity of a system determines the temperature increment, δT, with which the system reacts to a heat supply, δQ. A negative heat capacity would imply a decrease in temperature as the consequence of the addition of heat. This is clearly a physically unrealistic situation. We must conclude, therefore, that the starting hypothesis is incorrect. The observed negative entropy cannot be partitioned into independent positive conformational and overcompensating negative hydrational contributions.

The overall entropy decrease, which is required as a necessary condition for reversible cold denaturation by a temperature decrease, can result only from the sum of two negative entropy contributions, if they are mutually independent. Both the conformational entropy change and the hydrational entropy change must have the same sign with their sum adding up to the observed experimental value. This conclusion is illustrated graphically by the unidirectional arrows given in Fig. 5.

The present considerations based on thermodynamic reasoning are challenging the intuitive feeling that destruction of native ordered protein structure must lead to greater ‘disorder’ and, consequently, to an increase in entropy. This notion is evidently not tenable in the process of cold denaturation. Our conclusion has been based on the assumption that coupling between hydration and conformational changes is very weak, just sufficient to allow for thermal equilibration. This is the usual postulate for the treatment of independent systems in statistical thermodynamics. It

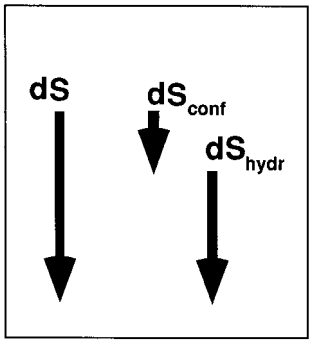

Fig. 5. Arrow diagram of the reversible independent entropy changes involved in cold denaturation. These entropy changes are in agreement with the postulates of the second law of thermodynamics. We use the differential symbols because the entropy changes dS in going through the transition along the entropy curve in Fig. 1b are determined by the slope of the tangent to the curve at the point (T,S) times dT. The length of the arrows makes no implication concerning the magnitude of the various effects, it is only the direction which is of significance in this plot.

is implied in the assumption of additivity of hydrational and conformational entropies, made above. On the other hand, if there existed strict coupling, no partitioning of the combined system of protein and hydration sphere would be possible [23]. In this latter case, the entropy change of the hydrated protein associated with cold denaturation could not be split into dS_{conf} and dS_{hydr}, as shown in Fig. 5, but would have to be represented by a single arrow identical in magnitude with dS.

In summary, we can conclude that partitioning of the observed negative entropy change associated with cold denaturation into a positive conformational and an overcompensating negative hydration contribution is incorrect. The observed protein heat capacity curve should be viewed as the consequence of the reversible entropy transfer between the hydrated protein and the surroundings which can be realized experimentally in a DSC experiment.

4. Statistical thermodynamic interpretation

Now, let us examine whether this phenomenological interpretation, based on classical equilibrium thermodynamics, can be extended by using fundamental relations from statistical physics. Such a treatment provides a deeper insight into the molecular mechanism that underlies protein heat capacity and places more emphasis on the dynamic nature of the unfolding processes. What concepts of statistical physics have to be applied to achieve this goal? A key consideration is the realization that a protein solution of approximately 1 ml, such as that employed in measuring protein heat capacity by DSC, contains approximately 10^{17} identical protein molecules. Such a solution can be considered, in the Gibbsian sense, as a canonical ensemble of small, yet macroscopic, systems immersed in a heat bath. The macroscopic nature of the single protein molecule can be derived from the Heisenberg uncertainty principle, as has been shown recently by Cooper [24,25]. The canonical ensemble of protein molecules is characterized by a definite energy distribution at any given temperature that comprises, in the simplest case, folded and unfolded protein molecules. Thus, a single protein molecule can assume, at each temperature, enthalpy values that fluctuate about the ensemble average, $\langle H_i \rangle$. Because of the small size of protein molecules, these individual enthalpy fluctuations are in the order of or even exceed the average stability of the macromolecule [24,26]. The fundamental question is: how can these stochastic fluctuations of single protein molecules be related to the observable average ensemble properties?

On the basis of the ergodic assumption, which postulates for equilibrium that the fluctuation distribution of a single system and the ensemble distribution are identical, we have shown recently [19] that the experimental thermodynamic ensemble averages provide information on the thermodynamics of the single protein molecule, as well. In particular, the isobaric heat capacity, c_p, is related to the instantaneous average rate of fluctuation of a single protein molecule via the relationship

$$c_p = \frac{\langle (\delta H)^2 \rangle}{k_B T^2} = \frac{\langle \dot{H}_S(t) \rangle}{\dot{T}} \tag{6}$$

$\dot{T}$ is the heating rate of the DSC instrument, $\langle H_S(t) \rangle$ is the time averaged enthalpy of the

single protein and $\langle \dot{H}_S(t) \rangle$ is its time derivative, i.e. the enthalpy flux. $\langle (\delta H)^2 \rangle$ is the average fluctuation in the enthalpy and is given by $\langle (\delta H)^2 \rangle = \langle (H - \langle H \rangle)^2 \rangle = \langle H^2 \rangle - \langle H \rangle^2$ where k_B is the Boltzmann constant and T is the absolute Kelvin temperature. An analogous relation holds for entropy fluctuations. Written in terms of entropy fluctuations, Eq. (6) becomes

$$c_p = \frac{\langle (\delta S)^2 \rangle}{k_B} = \frac{\langle \dot{S}_S(t) \rangle}{\dot{T}} \cdot T \tag{7}$$

with the terms having the following meaning: $\langle (\delta S)^2 \rangle$ is the variance of the entropy distribution defined by $\sigma_S^2 = \langle S^2 \rangle - \langle S \rangle^2$ and $\langle \dot{S}_S(t) \rangle$ is the time averaged entropy flux between the single protein and the heat bath, k_B is Boltzmann's constant, T is the Kelvin temperature and $\dot{T}$ is the heating rate of the DSC instrument. This equation can be interpreted in the following manner. Provided the heating rate, $\dot{T}$, is constant and slow relative to the rate of entropy fluctuations, the observed heat capacity is a measure of both the variance of the ensemble entropy distribution and the time averaged entropy flux which characterizes the entropy exchange between the individual protein and the heat bath. Thus, the faster this exchange proceeds at equilibrium, the larger will be the heat capacity. This perception provides us with a completely new understanding of the origin of protein heat capacity. From a statistical physics point of view, the heat capacity signal at temperature T arises from the rate of isothermal entropy (or enthalpy) fluctuations at equilibrium which persist also in the absence of any measuring device such as the calorimeter. However, in order to render these equilibrium fluctuations observable, for example for the cold denaturation process, entropy has to be withdrawn from the protein solution at a rate that is significantly smaller than the intrinsic rate of fluctuations. Only if the fluctuations are faster than the perturbation of the system by the temperature change, do they contribute to c_p [27].

This view emphasises the dynamic nature of the unfolding equilibrium. Unfolding and refolding occur at any temperature, but the rate at which these processes proceed is highest in the transition range both at low and high temperature as is reflected in the increased corresponding slopes of the entropy curve shown in Fig. 1b. The temperature determines the mean ensemble average energy and entropy of the protein and the heat capacity is a measure of the fluctuations about the average. Depending on temperature, these fluctuations occur preferentially in the native state or, at low and high temperature, preferentially in the unfolded state.

The present interpretation of protein heat capacity is based on the fact that a small system, such as a protein molecule, undergoes significant fluctuations in its thermodynamic properties. Both at low and high temperature for some proteins, such as myoglobin, staphylococcal nuclease, β-lactoglobin or barstar [28–30], the restoring forces which consist mainly of non-covalent short-range interactions appear to become too small to drive the unfolded form of the protein back to the native state. In this sense one could say that the entropy withdrawn from the protein in the process of cold denaturation is not the direct cause of the unfolding reaction, but only the means to decrease the temperature of the system towards a new ensemble average entropy with a new distribution function of the fluctuations.

5. Conclusions

We have shown on the basis of the general stability criterion that additive partitioning of the entropy changes measured for isothermal cold unfolding into positive conformational and negative hydrational contributions is incorrect. The implicit assumption of independent processes forbids spontaneous entropy changes of opposite signs in the same system as the result of a temperature decrease (cold-denaturation) or, equivalently, a temperature increase (heat-denaturation).

We have presented a new view of the origin of the heat capacity of proteins observed in a DSC experiment which is based on a rigorous statistical thermodynamic treatment of the protein solution as a canonical ensemble. Due to the small size of the proteins, fluctuations become signifi-

cant and their presence provides a rationale of the experimental heat capacity changes observed for both cold and heat unfolding.

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