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Biophysical Chemistry 74 (1998) 153–161

Biophysical Chemistry

The 'Janus' nature of proteins: systems at the verge of the microscopic and macroscopic world

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Received 6 April 1998; received in revised form 8 June 1998; accepted 15 June 1998

Abstract

Direct measurement of the heat capacity of proteins by microcalorimetry has had a decisive impact on understanding the behaviour of these biopolymers. Statistical mechanics allow a straightforward calculation and prediction of the enthalpy and heat capacity curves from the partition function. We show that these predictions can differ from the more intuitive models used so far for the description of the thermodynamic behaviour of proteins if the transition involves a stoichiometry other than 1:1. Furthermore, we delineate that the characteristics of protein unfolding are governed by the fluctuations associated with the small size of these molecules. Therefore it may be necessary to modify the picture of the unfolding of small proteins in the light of statistical physics, while for very large proteins the current view may be maintained as a useful limiting approximation. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Enthalpy fluctuations; Heat capacity; DSC; Small systems; Enthalpy shifts and population changes; Unfolding stoichiometries

1. Introduction

Proteins are not only fascinating biological objects, they represent also physically intriguing systems. By their very nature they can participate in reactions, such as association, dissociation and unfolding, which can be treated conventionally as chemical reactions. However, each protein molecule is also a small macroscopic system in the strict statistical thermodynamic sense that undergoes permanently significant fluctuations. The combination of these two apparently alternative views of the physical nature of proteins reveals new aspects that lead to a radically new interpretation of heat capacity transition curves. The most powerful tool for the characterisation of the thermodynamic behaviour of proteins is the direct determination of the heat capacity of protein solutions by differential scanning microcalorimetry (DSC).

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PII S0301-4622(98)00182-3

Pioneering work has shown convincingly that proteins and some synthetic polypeptides can exist in only a limited number of physically different states [1–5]. For small proteins, these are the native state, N, and the unfolded state, D. At physiological temperature the protein molecules populate preferentially the biologically functional N-state. In the temperature range of the peak of the heat capacity curve the population is cooperatively shifted towards the unfolded state, D. At properly adjusted experimental conditions the transition is reversible. This means that each point of the C_p-curve represents the heat capacity of an equilibrium population of native and denatured states of the protein, which can be described by a two-state reaction in the usual way:

$$N \rightleftharpoons D, K = \frac{[D]}{[N]},\ \alpha_D = \frac{[D]}{[N]+[D]} = \frac{K}{1+K},$$
$$\alpha_N = 1 - \alpha_D = \frac{[N]}{[N]+[D]} = \frac{1}{1+K} \quad (1)$$

where K is the equilibrium constant, $[N]$ and $[D]$ are the concentrations of native and unfolded proteins, respectively, and α_N and α_D are the fractions of unfolded and native molecules.

Although this description is perfectly correct, a more general characterisation of the 'system protein' can be achieved by the introduction of the partition function. Since the partition function, Z, contains all thermodynamic information on the system, a deeper physical understanding of the thermodynamic nature of proteins can be expected from such an approach. That this is indeed the case will become evident in the following considerations. Particularly significant thermodynamic properties for the present discussion are the mean enthalpy of the system, $\langle H \rangle$, which is given by the expression [6]

$$\langle H \rangle = k_B T^2 \frac{\partial \ln Z}{\partial T} = -\frac{\partial \ln Z}{\partial \beta} \quad (2)$$

and the variance, σ_H^2, of the enthalpy distribution which is represented by the equations

$$\sigma_H^2 = \langle H^2 \rangle - \langle H \rangle^2 = \frac{\partial^2 \ln Z}{\partial \beta^2} = k_B T^2 C_p \quad (3)$$

in which β has been equated to $1/k_B T$, with k_B being Boltzmann's constant. It is worth emphasizing that the experimentally accessible isobaric heat capacity directly reflects the fluctuations in enthalpy. It has been extensively discussed in Wyman and Gill [7] that for complex systems such as proteins a relative partition function Q defined by the following equation

$$Q = \frac{Z}{Z_0} \quad (4)$$

is more useful. Z_0 is the partition function of the reference state. By making this transformation and using the native state as reference state we can relate directly the observable molar enthalpy change, $\Delta H = H - H^N$ to the partition function Q.

$$H - H^N = RT^2 \frac{\partial \ln Q}{\partial T} \quad (5)$$

H is the enthalpy at temperature T and H^N is the enthalpy of the native state at the same temperature.

The molar heat capacity function is then represented by the temperature derivative of the enthalpy function

$$C_p - C_p^N = \frac{\partial (H - H^N)}{\partial T} = \frac{1}{RT^2} \frac{\partial^2 \ln Q}{\partial (1/RT)^2} \quad (6)$$

This résumé of well known relationships has been presented here to render the new ideas discussed in the following more easily understandable.

2. Two-state model, 1:1 stoichiometry

The conventional two-state model for a protein exhibiting 1:1 stoichiometry in its unfolding reaction can be expressed in terms of the partition function in the following manner

$$Q = \frac{[N]+[D]}{[N]} = 1 + K \quad (7)$$

where $[N]$ has been taken as reference state. K can be readily calculated from the standard Gibbs

energy change ΔG^0 (the Gibbs energy difference between the pure D- and N-state) according to the relation $K = \exp[-\Delta G^0/RT]$. The enthalpy relative to the native state is obtained by differentiation of the partition function according to Eq. (5)

$$H - H^N = RT^2 \frac{\partial \ln Q}{\mathrm{d}T} = \frac{K}{1+K}\Delta H^0 = \alpha_D \Delta H^o \tag{8}$$

where α_D is the fractional population of the denatured state as defined in Eq. (1). The equation states that the instantaneous enthalpy change at temperature T, $\Delta H(T) = H(T) - H^N(T)$, is directly proportional to the fraction of unfolded proteins α_D. The heat capacity is obtained from the second derivative according to

$$\begin{aligned} C_p &= C_p^N + \frac{\partial (H - H^N)}{\partial T} \\ &= C_p^N + \Delta C_p \frac{K}{1+K} + \frac{(\Delta H^0)^2}{RT^2}\frac{K}{1+K}\frac{1}{1+K} \\ &= C_p^N + \Delta C_p \alpha_D + \frac{(\Delta H^0)^2}{RT^2}\alpha_D \alpha_N \end{aligned} \tag{9}$$

where $\partial \ln K/\partial T = \Delta H^o/RT^2$ was used.

3. Two-state model, 1:2 stoichiometry

The reaction scheme for this model is

$$N_2 \rightleftharpoons 2D; \qquad K = \frac{[D]^2}{[N_2]};$$

$$\alpha_D = \frac{[D]}{2[N_2] + D} = 1 - \frac{1}{Q}; \quad \alpha_N = \frac{1}{Q} \tag{10}$$

The total concentration of monomers, $c = 2[N_2] + [D]$, remains constant in the transition. This conservation equation allows one to express the native state dimer concentration in terms of the concentration $[D]$ of unfolded monomers. With the equilibrium expression the concentration $[D]$ of unfolded proteins can be calculated. The result is

$$[D] = (K/4)\left(\sqrt{1 + \frac{8c}{K}} - 1\right) \tag{11}$$

Now the partition function is

$$\begin{aligned} Q &= \frac{[N] + [D]}{[N]} = \frac{2[N_2] + [D]}{2[N_2]} \\ &= 1 + \frac{2}{\sqrt{1 + \frac{8c}{K}} - 1} \end{aligned} \tag{12}$$

From this partition function we immediately get the relations

$$\sqrt{1 + \frac{8c}{K}} - 1 = \frac{2}{Q-1} \quad \text{and} \quad \frac{8c}{K} = \frac{4Q}{(Q-1)^2} \tag{13}$$

which are needed for the following calculations.

Differentiation of Q with respect to T yields the expression

$$\begin{aligned} \frac{\partial Q}{\partial T} &= \frac{-2}{\left(\sqrt{1 + \frac{8c}{K}} - 1\right)^2} \frac{1}{2\sqrt{1 + \frac{8c}{K}}} \frac{-8c\Delta H^0}{KRT^2} \\ &= \frac{8c\Delta H^0}{KRT^2\left(\frac{2}{Q-1}\right)^2\left(\frac{2}{Q-1} + 1\right)} \\ &= \frac{\Delta H^0}{RT^2}\frac{Q(Q-1)}{Q+1} \end{aligned} \tag{14}$$

from which the enthalpy change can be calculated. We obtain:

$$H - H^N = RT^2 \frac{\partial \ln Q}{\partial T} = \Delta H^0 \frac{Q-1}{Q+1} = \Delta H^0 \frac{\alpha_D}{1 + \alpha_N} \tag{15}$$

This result is extraordinary and deserves some thought. In contrast to the result derived for the $N \rightleftharpoons D$ transition in Eq. (8), Eq. (15), which is valid for the $N_2 \rightleftharpoons 2D$ equilibrium, unequivocally shows that the direct proportionality between the population shift, given by α_D, and the instantaneous enthalpy signal $(H - H^N)$ is lost. Rather,

the enthalpy is weighted by the ratio of $\alpha_D/(1+\alpha_N) = \alpha_D/(2-\alpha_D)$. This insight resulting from straightforward application of statistical thermodynamics is of fundamental importance for the interpretation of heat capacity curves of proteins. The essence of the new picture is illustrated in Fig. 1a which shows the enthalpy change $\Delta H = H - H^N$ of a protein solution as a function of temperature. The solid sigmoidal curve represents the enthalpy as a function of temperature calculated according to Eq. (15) whereas the dotted curve illustrates the enthalpy calculated in the conventional way according to $H - H^N = \alpha_D \Delta H^0$. It should be kept in mind that both enthalpy curves in Fig. 1a are equilibrium curves, thus their relative shift on the temperature axis has nothing to do with kinetic effects. Therefore the only interpretation of the fundamental discrepancy between the two curves is the following: it is evident from Fig. 1a that on increasing temperature the shift in fractional population of unfolded states, given by α_D, occurs prior to the shift in enthalpy. Therefore it is not permissable to derive the degree of unfolding α_D from the ratio $\Delta H(T)/\Delta H^0_{\mathrm{cal}}$, i.e. the instantaneous enthalpy at temperature T and the total standard transition enthalpy, determined calorimetrically in cases where stoichiometries other than 1:1 are involved in the unfolding transition.

Let us recapitulate: strict application of statistical thermodynamics challenges the intuitive view that the enthalpy change is directly coupled to the fractional degree of unfolding or, in other words, to the shift in population of the unfolded state. This difference carries, of course, further to the heat capacity function, which is again obtained from Eq. (15) by performing the derivative with regard to temperature. The resulting equation is:

$$C_p = C_p^N + \Delta C_p \frac{Q-1}{Q+1} + (\Delta H^0)^2 \frac{\frac{\partial Q}{\partial T}(Q+1) - \frac{\partial Q}{\partial T}(Q-1)}{(Q+1)^2}$$

$$= C_p^N + \Delta C_p \frac{Q-1}{Q+1} + \frac{(\Delta H^0)^2}{RT} \frac{2Q(Q-1)}{(Q+1)^3}$$

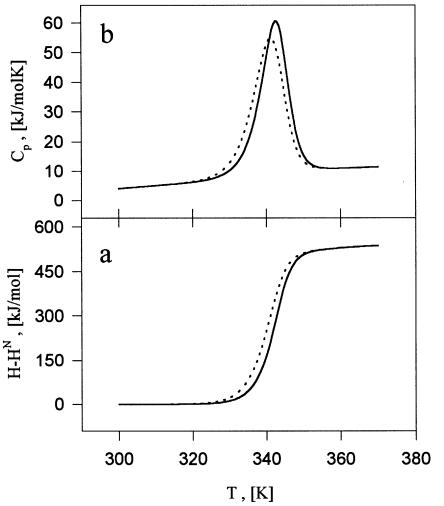

Fig. 1. Variation with temperature of enthalpy (a) and heat capacity (b) of a protein according to models I and II for a 1:2 transition. The solid lines were calculated from the proper partition function according to model II, the dotted lines illustrate the variation with temperature of ΔH and C_p if direct proportionality between enthalpy and population shift is assumed in the conventional manner. The following parameters were used for the calculations: $\Delta H^0(T_{1/2}) = 500$ kJ/mol, $T_{1/2} = 340$ K, $\Delta C_p(T_{1/2}) = 2$ kJ/molK, $\Delta C_p'(T_{1/2}) = -50$ J/molK^2, $\Delta C_p''(T_{1/2}) = -0.2$ J/molK^3, $c = 1$ mM. These parameters are chosen similar to those observed for ROP unfolding [8].

$$= C_p^N + \Delta C_p \frac{\alpha_D}{1+\alpha_N} + \frac{(\Delta H^0)^2}{RT} \frac{2\alpha_N \alpha_D}{(1+\alpha_N)^3} \tag{16}$$

The corresponding graph of the heat capacity function is illustrated in Fig. 1b. Compared to the conventional model [Eq. (9), dotted line in Fig. 1b] the C_p-curve calculated using Eq. (16) (solid line in Fig. 1b) is obviously sharper, which corresponds to higher cooperativity.

An excellent proof for the correctness of the present model resulting from the statistical mechanical approach is furnished by its application to unfolding of the ROP dimer, whose unfolding mechanism follows our model II. The experimental heat capacity curve of the homodimer ROP is shown in Fig. 2. The open circles represent the curve published previously by Steif et al. [8]. In that study only the peak had been fitted. Further-

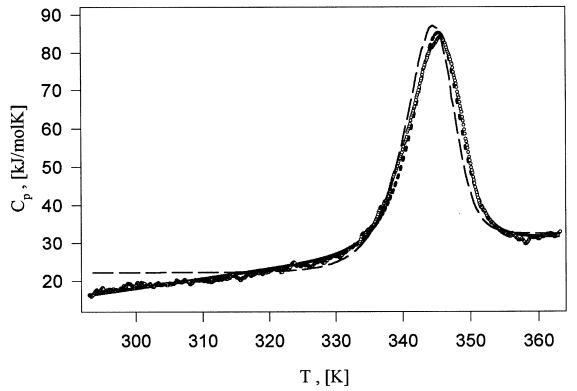

Fig. 2. Experimental DSC-curve of a 1 mg/ml ROPwt solution according to Steif et al. [8] (open circles). The dashed line represents the fits given by Steif et al. [8], the dotted line shows the best fit using Eq. (16) (fit parameters: $\Delta H^0(T_{1/2}) =$ 518 kJ/mol, $T_{1/2} = 342.7$ K, $\Delta C_p(T_{1/2}) = 2.9$ kJ/molK, $\Delta C_p'(T_{1/2}) = -0.25$ kJ/molK^2).

more, it had been assumed that the enthalpy derived from the heat capacity curve varied with temperature in proportion to the fractional degree of unfolded species, α_D, i.e. in accordance with the commonly held view.

Fig. 2 shows also the fit using Eq. (16) of the present study. It is evident that the fit achieved with the new model is significantly better than with the conventional model. The fundamental difference between the models is also reflected in the values of the heat capacity changes that result from the fitting procedures. As a consequence of assuming the proportionality between the enthalpy change and the population shift, the first model cannot cope satisfactorily with the higher cooperativity predicted by the second model. However, in an attempt to optimize the fit the algorithm overcame this problem partly by increasing the value of the heat capacity change ΔC_p (see Appendix). Therefore the larger heat capacity change of $\Delta C_p = 10$ kJ/mol$*K$ had been obtained previously, while the proper model provides now the smaller value of $\Delta C_p = 3$ kJ/mol$*K$. The excellent agreement between the predictions of model II and the experimental heat capacity curve observed for ROPwt unfolding is a good operational test for the validity of the theory outlined in this study.

It should be mentioned here that the present treatment is completely general and does not depend on the physical nature of the system. Therefore the above formulae are also valid for nucleic acid melting processes. An excellent example is given in the article of Marky et al. [9] on the melting behaviour of a DNA junction structure. The authors observe systematically larger values for the van't Hoff enthalpies than for the calorimetric enthalpies. This is exactly what is predicted by our derivations. The experimentally observed width of the transition peak at half height is smaller than the width expected on the basis of proportionality between enthalpy and shift of population.

It can be expected that the serious intrinsic differences that have been revealed to exist in the way population shifts and transition enthalpies develop with temperature change are of more fundamental physical origin. In the following we attempt to provide a rationalization of the intriguing results.

4. Physical origin of the enthalpy and heat capacity changes of proteins

From a strictly statistical thermodynamical point of view heat capacity is a response function monitoring the variance of the energy distribution as seen in Eq. (3). What this actually means and which consequences this statistical thermodynamical principle has is illustrated in Fig. 3. The solid curve in Fig. 3 displays the variation with temperature of the molecular enthalpy, $(\Delta H/N_A)$, obtained from the molar ΔH value plotted in Fig. 1a by division by Avogadro's number N_A. The dotted curves illustrate the fluctuations in molecular enthalpy calculated using Eq. (3). The molecular heat capacity values used for these calculations are obtained from the molar values shown in Fig. 1 by division by N_A. This usage of a molecular heat capacity is in agreement with Hill's treatment on the thermodynamics of small systems [10]. Briefly, the procedure is justified since the protein solution can be considered a macroscopic ensemble of equivalent, distinguishable and independent systems consisting of single protein moieties. In such a system heat capacity can be defined just as in macroscopic thermodynamics.

Now what does Fig. 3 teach us? It provides the astounding insight that the equilibrium fluctuations of the enthalpy at each temperature T inside the transition range are actually larger than the differential enthalpy $\mathrm{d}H = C_p\,\mathrm{d}T$ required to increase the temperature of the protein in a quasistatic fashion by $\mathrm{d}T$. This is an impressive manifestation of the fact that microscopically for small systems, such as proteins, fluctuations become large [11]. This finding can be stated in an even more pointed manner: what Fig. 3 actually demonstrates is the fact that the protein is not unfolded by the external heat supply required to increase the temperature of the sample cell in the microcalorimeter. Rather unfolding and refolding takes place permanently and is an intrinsic property of the protein and buffer fluctuations. Increase of temperature serves simply as a probe to sense these equilibrium fluctuations in enthalpy and heat capacity. At the same time it allows to quantitate the equilibrium distribution of unfolded and native protein molecules, provided the current model is chosen, since at each temperature in the transition range, equilibrium is established, when a small enough heating rate is employed. It is to be expected that fluctuations are largest near the phase transition temperature [12].

As pointed out above, for proteins that exhibit unfolding stoichiometries different from 1:1, apparently different transition temperatures can be defined. One, which we call $T_{1/2}$, in agreement with the conventional definition, can be assigned to the temperature at which 50% of the population is unfolded. Therefore this temperature is observed by definition at $\alpha_D = 0.5$. The other significant temperature is associated with the disappearance of the standard Gibbs energy change ΔG^0. This occurs at T_G. We use here the nomenclature introduced by Becktel and Schellman in their pivotal paper on protein stability [13]. We can interpret $T_{1/2}$ as the temperature at which the protein experiences the largest shift in population. T_G is then the temperature at which a macroscopic phase transition would occur. Now it can be shown (Appendix) that for an unfolding transition having a stoichiometry of 1:1 $T_{1/2}$ and T_G coincide. However, for 1:2 or other stoichiometries $T_{1/2}$ is usually lower than T_G. In such cases we should find a temperature of maximal fluctuations and therefore of maximal heat capacity at a temperature, $T_{\max}$, lying between these two temperatures. We therefore have the general relation between the so-defined transition temperatures.

$$T_{1/2} < T_{\max} < T_G$$

Only in the case of 1:1 stoichiometry are these three temperatures identical. Fig. 4 illustrates the relation for a transition having 1:2 stoichiometry. $\Delta G^0(T)$ has been calculated using Eq. (A5).

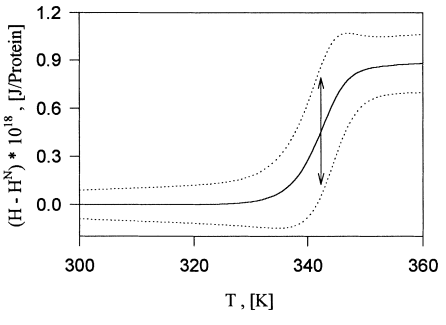

Fig. 3. Enthalpy relative to the native state $\Delta H = H - H^N$ calculated for a single protein molecule plus/minus standard deviation σ_H according to Eq. (3). The temperature of maximal energy fluctuations is indicated by a double-arrow.

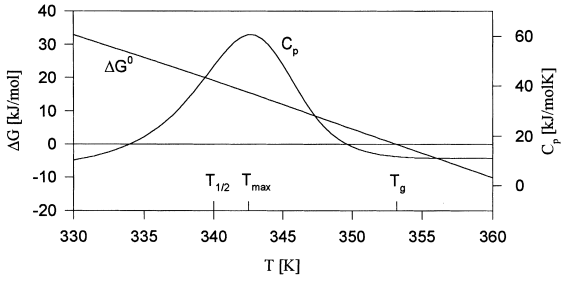

Fig. 4. Dependence on temperature of heat capacity C_p and the standard Gibbs energy change, ΔG^0, for an unfolding reaction following a 1:2 stoichiometry (same parameters as in Fig. 2). Different characteristic temperatures are indicated at the temperature axis: $T_{1/2}$ is the temperature at which populations of native and denatured proteins are equal. $T_{\max}$ is the temperature of maximal heat capacity and T_G is the temperature where the standard Gibbs energy change, ΔG^0, vanishes.

5. Coincidence of transition temperatures for large systems

The phenomena we have been describing are a clear manifestation of the small size of proteins. Small proteins are at the edge of the microscopic and macroscopic world. On the one hand they contain enough atoms to be defined as a macroscopic system [11] while on the other hand they are small enough to experience significant fluctuations around the mean energy value of the ensemble. What would happen if the single protein molecule could be made larger by increasing its mass with all specific intrinsic interaction parameters being held constant? Obviously the standard transition enthalpy, ΔH^0, being an extensive quantity, would also increase in proportion. That means we can use the magnitude of ΔH^0 as property that characterises the size of the protein in a thermodynamic sense. An increase in the transition enthalpy at $T_{1/2}$, $\Delta H^0(T_{1/2})$, is associated with an increase in the negative slope of the $\Delta G^0(T)$ curve at $T_{1/2}$ as delineated in the appendix [Eq. (A5)]. This in turn will reduce the difference between $T_{1/2}$ and T_G as an inspection of Fig. 4 shows. The quantitative relation is illustrated in Fig. 5 which shows the dependence of T_G on the magnitude of the transition enthalpy at $T_{1/2} = 340$ K. This temperature is the transition temperature of ROPwt protein. The abcisse shows $\Delta H^0(T_{1/2})$ in kJ/mol.

It is seen that for a small protein, such as ROP with 1:2 stoichiometry of unfolding and a transition enthalpy of $\Delta H^0(T_{1/2}) = 600$ kJ mol^{-1} one observes still a significant difference between T_G and $T_{1/2}$. However, for proteins ten times its size (similar specific transition enthalpies assumed) which would have transition enthalpies of 6000 kJ mol^{-1}, the difference $T_G - T_{1/2}$ becomes essentially minute. Experimentally, probably even smaller proteins with for example $\Delta H^0(T_{1/2}) =$ 2000 kJ mol^{-1} will no longer show differences in T_G and $T_{1/2}$ independent of whether they are analysed on the basis of the correct model II or the conventional model I, as the insert demonstrates. The dotted and solid heat capacity curves shown there are calculated for $T_{1/2} = 340$ K,

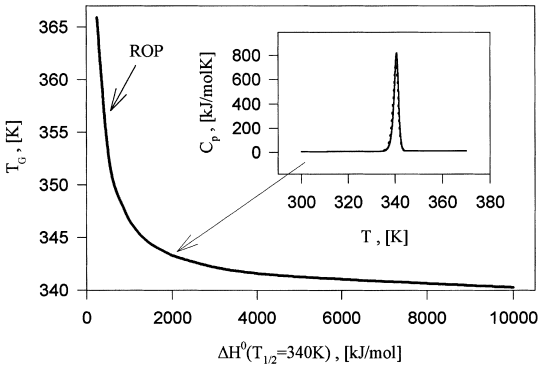

Fig. 5. Dependence of T_G on enthalpy (or equivalently mass). The same parameters as used in Fig. 1 were employed for the calculations with Eq. (A5) except for the variation in transition enthalpy, $\Delta H^0(T_{1/2})$. This parameter can be considered a thermodynamic measure of the size of the system. Already for $\Delta H^0(T_{1/2}) = 2000$ kJ/mol the experimental C_p curve can no longer be used for a discrimination of the two models as illustrated by the insert.

$\Delta H^0(T_{1/2}) = 2000$ J mol^{-1}, using model I and model II. It is evident that, unless extremely good data are obtained, the heat capacity curves cannot discriminate between the two models.

Calculating the mean protein energy by directly weighting ΔH^0 with the population sizes neglects the influence of stoichiometry. What is reflected in the heat capacity measurement is not only the mean energy and its fluctuation for a single protein, but also the stoichiometric contributions of the oligomerization equilibria. Therefore it is evident that the conventional way of enthalpy calculation is not adequate to predict the real behaviour of proteins, but only the proper approach which uses the partition function.

6. Summary

We have shown on the basis of two-state models that the isobaric heat capacity of proteins can be interpreted as a direct manifestation of the fluctuations in enthalpy that the single protein experiences. Though similar ideas have been proposed before [11] they have never illuminated the problem as detailed as done here. The reason why this aspect remained undetected for so long

is not clear. However, it may reside in the fact that for systems having 1:1 stoichiometry which were treated so far practically exclusively there is a coincidence of all thermodynamic parameters. Using statistical thermodynamics we have shown here rigorously that for systems having a stoichiometry other than 1:1 the conventional interpretations of the heat capacity of proteins lead to irreconcilable inconsistencies both with the experiment and the thermodynamic theory of protein folding.

Only at first glance does it appear paradoxical that conformation (population shifts) and enthalpy do not change in synchrony. This apparent paradox can readily be resolved, if the heat capacity is being viewed as the variance of enthalpy distribution that characterizes the equilibrium system of native and unfolded protein molecules at each temperature.

The model has passed its experimental test by providing an excellent description of the heat capacity curve of ROP protein which exhibits the unfolding equilibrium $N_2 = 2D$. This interpretation fits the data better than the model applied previously [8]. The deeper grounds for the extraordinary behaviour of proteins are to be found in the dualistic nature of proteins: these macromolecules can be described macroscopically as chemical entities participating in chemical reactions, such as association, dissociation and unfolding. However, microscopically the unfolding of a single protein involves — at least on the basis of the two state hypothesis — a discontinuity in the first derivative of the partition function, the enthalpy. This discontinuity fulfils the criterion of a first order phase transition [14,15].

Thus it is seen that the macroscopically observed temperature induced unfolding transitions of small proteins result from microscopically occurring fluctuations of single proteins that change conformation in a first order phase transition. In the thermodynamic limit of very large single proteins these fluctuations would become of minor importance. It may be speculated that the size of present day proteins has evolved to optimise the utilisation of these dynamic properties for function.

Acknowledgements

The authors gratefully acknowledge financial support by the German Science Foundation (DFG) within the 'Graduiertenkolleg' GRK 234/1-96.

Appendix A: Series representation of the Gibbs energy of denaturation

The calculation of the standard Gibbs energy change, ΔG^0, by a double Taylor expansion has been shown to be most useful for proteins [16]. For an $(n+1)$ times continuously differentiable function the series expansion yields

$$f(x) = \sum_{k=0}^{n} \frac{f^{(k)}(a)}{k}(x-a)^k + \frac{1}{n}\int_a^x (x-t)^n f^{(n+1)}(t)\mathrm{d}t \qquad \text{(A1)}$$

[17]. From Eq. (A1) one obtains with $n = 1$ and $f(x) = \Delta G^0(T)$

$$\Delta G^0(T) = \Delta G^0(T_{1/2}) - \Delta S^0(T_{1/2})\cdot(T - T_{1/2}) - \int_{T_{1/2}}^{T} \frac{T-t}{t}\Delta C_p(t)\,\mathrm{d}t \qquad \text{(A2)}$$

where a has been replaced by $T_{1/2}$, which is the midpoint temperature for which 50% of the native protein molecules have been converted to the unfolded state, or in other words where the fractional degree of unfolding α_D equals 0.5. Because of the relations

$$\Delta G^0(T_{1/2}) = -RT_{1/2}\,\ln\left[K(T_{1/2})\right] \quad \text{and}$$

$$\Delta G^0(T_{1/2}) = \Delta H^0(T_{1/2}) - T_{1/2}\Delta S^0(T_{1/2})$$

one obtains the following identity:

$$\Delta G^0(T_{1/2}) - \Delta S^0(T_{1/2})\cdot(T - T_{1/2}) = -RT\,\ln\left[K(T_{1/2})\right] - \Delta H^0(T_{1/2})\frac{T - T_{1/2}}{T_{1/2}} \qquad \text{(A3)}$$

Expanding also $\Delta C_p(T)$ into a series yields

$$\Delta C_p(T) = \sum_{k=0}^{\infty} \frac{\Delta C_p(T_{1/2})^{(k)}}{k!}(T - T_{1/2})^k.$$

In combination with Eq. (A2) and Eq. (A3) an expression for $\Delta G^0(T)$ can then be formulated. When using terms up to fourth order we obtain for the variation of the standard Gibbs energy with temperature the expression

$$\begin{aligned}\Delta G^0(T) = & -RT \ln\big(K(T_{1/2})\big) \\ & - \Delta H^0(T_{1/2}) \frac{T - T_{1/2}}{T_{1/2}} \\ & + \Delta C_p(T_{1/2}) \cdot \left(T - T_{1/2} - T \ln \frac{T}{T_{1/2}}\right) \\ & + \Delta C_p^{(1)}(T_{1/2}) \\ & \cdot \left(\frac{T_{1/2}^2 - T^2}{2} + TT_{1/2} \ln \frac{T}{T_{1/2}}\right) \\ & + \frac{\Delta C_p^{(2)}(T_{1/2})}{2} \\ & \cdot \left(\frac{T^3 - T_{1/2}^3}{3} + \frac{(T - T_{1/2})\big(TT_{1/2} - T^2\big)}{2} \right. \\ & \left. - T_{1/2}^2 T \ln \frac{T}{T_{1/2}}\right) \qquad \text{(A5)}\end{aligned}$$

For 1:1 stoichiometry one obtains $K(T_{1/2}) = 1$, for 1:2 stoichiometry $K(T_{1/2}) = c$. Only in the first case is $T_{1/2} = T_G$. In all other cases the steepness of the function $\Delta G^0(T)$ at $T_{1/2}$ determines how wide $T_{1/2}$ and T_G are apart. Eq. (A5) shows also that the variation of ΔG^0 with T is governed predominantly by the magnitude of $\Delta H^0(T_{1/2})$. But it is also influenced by the magnitude of $\Delta C_p(T_{1/2})$. This becomes evident when the term

$$\Delta C_p(T_{1/2}) \cdot \left(T - T_{1/2} - T \ln \frac{T}{T_{1/2}}\right) \qquad \text{(A6)}$$

is inspected. For positive $\Delta C_p(T_{1/2})$ the function is always negative except at $T = T_{1/2}$ where it vanishes.

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